intestinal vitamin D absorption was diminished in the elderly by as much as 40% compared to a younger population [27,28]. We have shown that an oral vitamin D supplementation with 800 IU or 20 μg given every day is safe and has proved its efficacy to maintain adequate 25OHD concentrations [29].

C. Medical Causes

Vitamin D insufficiency also may be due to malabsorption of vitamin D (Chapter 75) or to drugs known to alter the metabolism of vitamin D (Chapter 74). Vitamin D is a fat-soluble vitamin, and disorders that lead to steatorrhea are associated with vitamin D insufficiency. This is seen in subjects with hepatobiliary and gastrointestinal disorders, such as celiac disease, cystic fibrosis, chronic pancreatitis, and partial or total gastrectomy. In such subjects, the enteropatic circulation of 25OHD may be also disordered [30].

Vitamin D insufficiency may occur in patients taking anticonvulsant therapy, such as phenytoins, phenobarbital, and glutethimide (see Chapter 74). In these patients, the 25OHD concentrations are decreased; however, the mechanisms involved in this reduction are uncertain. One possibility is that anticonvulsants induce hepatic microsomal enzymes, which metabolize vitamin D to biologically inactive degradation products. Another possible mechanism may be drug interference in the synthesis of $1,25(OH)_2D$.

IV. CONSEQUENCES OF LOW VITAMIN D STATUS

A. Biochemical Consequences: Increased Serum PTH

As the synthesis of the active metabolite is substrate dependent, vitamin D insufficiency will lead to a small decrease in $1,25(OH)_2D$ levels. The lowest value of serum 25OHD necessary to maintain the normal synthesis of $1,25(OH)_2D$ is not known. In clinical studies, this threshold value was found to be lower than 12 ng/ml (30 nmol/liter) by Lips *et al*. [5] and 15 ng/ml (37.5 nmol/liter) by Bouillon *et al.* [16]. The subsequent subtle decrease in $1,25(OH)_2D$ levels leads to decreased serum calcium and induces an increase in serum PTH levels. This mechanism was suggested in 1983 by Riggs and Melton as a pathophysiological factor for so-called "type II osteoporosis" [31] (Fig. 2) (see Chapter 67). This significant increase in serum PTH with age in subjects living either at home or in an institution has been reported in many studies [32–35].

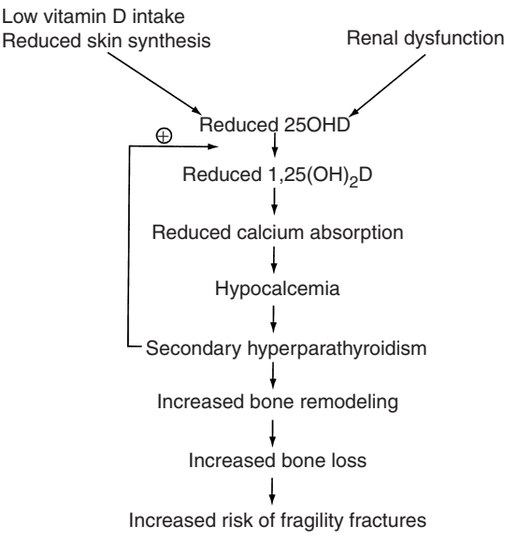

FIGURE 2 Pathophysiological mechanism of senile secondary hyperparathyroidism. Although increased PTH will stimulate $1,25(OH)_2D$ synthesis to correct hypocalcemia, this action is not efficient due to the decrease in the substrate (25OHD) concentration for 1α-hydroxylase activity.

It has been found to be partly related to renal dysfunction, age, and/or increasing years since menopause, and mainly to a fall in 25OHD levels [4,5,36] (Fig. 3) (see also Chapter 50). Renal insufficiency denoted by a declining glomerular filtration rate appears to be a cause [37], but not the main cause of the age-related rise in serum PTH [38]. Vitamin D insufficiency seems to be a significant determinant of senile secondary hyperparathyroidism.

We have found that the main determinants for PTH values in free-living elderly women were, at equal weight, age and 25OHD values and, to a lesser degree,

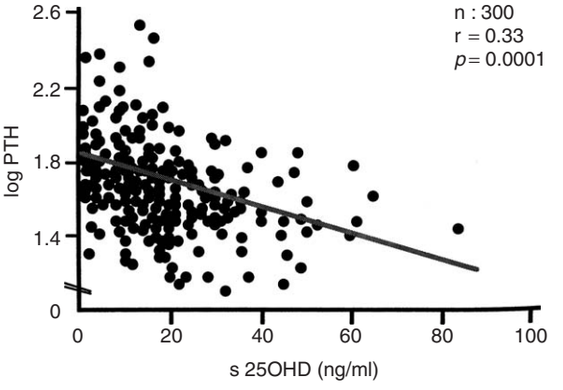

FIGURE 3 Inverse relationship between serum 25OHD and log PTH values in 300 elderly women living in institutions.

creatinine clearance [39]. When all these variables were adjusted in a multiple regression model, age and 25OHD were still significant predictors of PTH, but not creatinine clearance [39]. Ooms *et al.* [40] have found that only 25OHD values below a threshold of 10 ng/ml (25 nmol/liter) were related to PTH. Below this threshold, PTH increased 14.1% for every 4 ng/ml (10 nmol/liter) drop in serum 25OHD. Notwithstanding the fact that in several studies vitamin D only accounted for less than 10% of the variance in PTH, it must be emphasized that senile secondary hyperparathyroidism can be reversed by vitamin D supplements [4,5,29,41]. However, the association between 25OHD and PTH is likely to be influenced by the levels of calcium intake, as calcium supplementation was also demonstrated to be able to lower PTH levels in elderly subjects [42].

B. Impact on Bone

Severe and prolonged deficiency in vitamin D is associated with osteomalacia characterized by defective mineralized bone and reduced bone strength (Chapter 63). On the other hand, vitamin D insufficiency that is not severe enough to cause osteomalacia may nevertheless contribute to hip fracture risk in the elderly by decreasing calcium absorption and increasing PTH secretion, leading to increased bone turnover and bone loss, particularly in cortical bone (Fig. 2) [1,31,43,44]. This concept seems to be confirmed by the higher PTH levels found in patients with hip fractures than in matched controls [45,46]. It is also supported by histomorphometric studies that have shown an increase in resorption parameter values (i.e., reduced thickness of iliac cortices and increased number of osteoclasts per square millimeter of bone section) in patients with vertebral fractures [43,45]. These bone changes have been confirmed by Okano *et al.* [47]. On the other hand, overt osteomalacia, as determined by both increased thickness of osteoid seams and decrease in the calcification rate measured through tetracycline double labeling, is very rare and has been found in fewer than 10% of patients with hip fracture [43,44,48]. The histological picture of the first stage of vitamin D–deficient osteopathy (vitamin D insufficiency) is caused by secondary hyperparathyroidism and cannot be distinguished from hyperparathyroid bone disease on histological grounds [49] (also see Chapters 59 and 63).

Several studies have shown a relationship between femoral neck bone mineral density (BMD), vitamin D insufficiency, and secondary hyperparathyroidism not only in the elderly [40,50,51], but also in middle-aged women [52,53]. Martinez *et al.* found an association between low femoral BMD and low 25OHD levels in normal women older than 65 [54]. For Villareal *et al.*, there was a relationship between vertebral bone density assessed by quantitative computerized tomography and PTH values only in subjects with low 25OHD values (<15 ng/ ml or 38 nmol/liter) [55]. Ooms *et al.* [40] found similar results for the BMD of the hip, the best fit being obtained with a threshold value of 25OHD at 12 ng/ml (30 nmol/liter). The femoral BMD was 5% higher for every 4 ng/ml (10 nmol/liter) increase in 25OHD up to the threshold. In this study, serum PTH was negatively related to BMD at all measurements' sites, with the correlation coefficient ranging from –0.19 for the distal radius to -0.27 for the left femoral neck. In contrast, at a serum 25OHD level of 4 ng/ml (10 nmol/liter), the BMD of the femoral neck was reduced by 9.3%, which is 0.6 SD below the average BMD for an adequate vitamin D status (i.e., above 12 ng/ml or 30 nmol/liter). According to the data of Cummings *et al.* [56] and Ooms *et al.* [40], a 25OHD level of 4 ng/ml (10 nmol/liter) results in a relative risk of hip fracture of 1.8. In a German population, Scharla *et al.*, found a borderline significant positive correlation between femoral neck BMD and 25OHD values in men older than 70 years ($r = 0.34$, $p < 0.03$). In women between 50 and 70 years ($r = 0.36$, $p < 0.02$), there was no association between vertebral BMD and 25OHD values [57].

In a cross-sectional study in the United Kingdom, a positive relationship between serum 25OHD values and BMD of the lumbar spine, the femoral neck, and greater trochanter was observed in a group of 138 middle-aged women volunteers (45–65 years) [52]. In a prospective study, Dawson-Hughes *et al.* [41] have shown that vitamin D insufficiency noted in winter contributes to spinal bone loss in healthy postmenopausal women (mean age 61 years), which can be reduced by daily supplements with 10 μg of vitamin D.

Several studies have shown correlations between bone biochemical markers and the kinetic and histomorphometric evaluation of bone formation and resorption, and it has been possible to demonstrate the impact of 25OHD insufficiency on bone status using the biochemical markers of bone turnover. Brazier, Kamel, and coworkers [58,59] have reported a two- to three-fold elevation of resorption estimated by excretion of pyridinoline crosslinks in elderly subjects with vitamin D insufficiency and secondary hyperparathyroidism, as compared with vitamin D–sufficient elderly. In free-living healthy elderly women, we have found that the mean values of bone alkaline phosphatase, osteocalcin, and collagen C-telopeptide (crosslaps) were significantly increased as compared with the results obtained in young women [39] (Table I). For these

TABLE I Comparison of Biochemical Markers of Bone Remodeling in Elderly and Young Women during the Winter

Marker	Elderly women living at home (EPIDOS study, $n=405$)	Young women (OFELY study, $n=54$)
Bone alkaline phosphatase (μg/ml)	15.2±6.2 (a)	8.5±2.6
Osteocalcin (ng/ml)	24.9±9.6 (a)	14.9±4.4
Urinary calcium (mmol/nmol creatinine)	0.36±0.22 (b)	0.26±0.15
Urinary hydroxyproline (μmol/mmol creatinine)	29±12 (a)	19±7
Crosslaps* (μg/mmol creatinine)	311±168 (a)	186±108

Data are expressed as means ± SD. Lowercase letters show results of statistical analyses: (a), significantly different from young women $< p = 0.0001$; (b), significantly different from young women $p=0.001$. From Chapuy *et al.* [39].

* Collagen C-telopeptide.

markers, we found significant positive correlations with PTH values and negative correlations with hip BMD. In these healthy ambulatory women, we did not find a correlation between 25OHD and PTH with BMD values as reported in some studies [52,54,55], but other researchers have also been unable to show a significant correlation between BMD and PTH values [51,60].

Thus, if an increase in PTH levels secondary to vitamin D insufficiency is one certain cause of age-related bone loss and fragility, this suggests that other unidentified factors may play a major role in the increase in bone turnover. Ooms *et al.* [61] found that low serum 25OHD concentrations are associated with higher PTH and osteocalcin and lower BMD of the hip, but also that when high sex hormone-binding globulin (inverse measure of estrogen activity) is combined with vitamin D insufficiency, the secondary hyperparathyroidism is more severe. This suggests that low estrogen activity causes decreased sensitivity of the gut to $1,25(OH)_2D$ [62], leading to higher serum PTH levels and increasing the impact of vitamin D insufficiency. The observation, in several studies, of a seasonal variation in bone mineral density in normal subjects provides indirect evidence that relatively small changes in vitamin D status may have significant effects on bone mass. The demonstration that late wintertime bone loss could be prevented by small increases in vitamin D intake [41] provides a powerful argument in favor of the hypothesis that vitamin D status contributes to increased bone turnover and cortical bone loss (see also Chapter 63).

C. Other Effects: Muscle Weakness

Hypovitaminosis D with or without osteomalacia has been associated with muscle weakness, limb pain, and impaired muscle function. This might be explained by the fact that vitamin D receptors (VDR) exist in muscle, or it may be secondary to the mineral abnormality (see Chapters 55 and 102). Birge and Haddad have shown that 25OHD directly influences intracellular accumulation of phosphate by muscle and offered this as an important role in the maintenance of muscle metabolism and function [63]. A syndrome of hyperesthesia has been described in association with hypovitaminosis D in five homebound elderly subjects by Gloth *et al.* [64], but no systematic studies of pain and muscle strength have been performed in an older population before and after treatment of vitamin D insufficiency [65]. Pain and weakness can lead to functional disability that may prevent a person from venturing outdoors, and this, in turn, exacerbates a poor vitamin D status by decreasing exposure to sunlight. Corless *et al.,* in a multicenter study, were unable to demonstrate an effect of vitamin D supplements on the functional activity levels in elderly subjects [66]. On the other hand, Sorensen *et al.* have shown that treatment with lα-hydroxyvitamin D (1αOHD) was followed by a reduction in the time to dress [67]. This functional disability associated with vitamin D insufficiency may increase the incidence of falls and consequently the risk of hip fracture. Until relatively recently, studies on the prevention of senile osteoporosis and fractures with vitamin D supplements have failed to mention the incidence of falls [29,68,69], but a recent study run by Bischoff *et al.* [70] in 122 elderly institutionalized women has shown a reduction of 49% of the risk of falling in the group receiving 800 IU of vitamin D3 and 1200 mg/day of calcium compared to the group receiving only calcium.

V. PREVALENCE OF VITAMIN D INSUFFICIENCY

In analyzing the prevalence of vitamin D insufficiency in populations, it is necessary to divide the

subjects according to age, country of residence, and season in which the study was performed (see also Chapters 47 and 62). To facilitate the analysis of vitamin D status, countries were grouped according to geographic regions: North America, Scandinavia, Europe, and others. Elderly subjects were subdivided into healthy elderly persons living at home and institutionalized subjects. In addition, the seasonal variations in serum 25OHD levels were taken into account. To analyze the values reported in the literature, we have largely relied on the review by McKenna [24] in which 117 studies of vitamin D status from 27 regions published between 1971 and 1990 were analyzed. In some groups of subjects, and especially in healthy elderly, the number of studies is very small.

A. Vitamin D Insufficiency in Adults (30–70 years)

In North America where dairy products are fortified with vitamin D, vitamin D insufficiency in young adults is rare [71–73]. In the study by Sherman *et al.* [35], fewer than 1% of men and 4% of women had 25OHD values lower than 14 ng/ml (35 nmol/liter), and these low values were found in May–June, when mean values are at their nadir. In Scandinavian countries, where vitamin D intake is high, vitamin D insufficiency was noted in 4 to 9% of young adults during winter and in up to 5% during summer [74]. In Ireland [75], Italy [76,77], and England [24], 15 to 40% of young adults have hypovitaminosis D during the winter (Fig. 4). In a cross-sectional survey in a large Swiss population (3276 subjects aged 25–74 years) between November 1988 and June 1989, 6% of subjects had 25OHD values lower than 8 ng/ml (20 nmol/liter), and 34% had values lower than 15 ng/ml (37.5 nmol/liter) [78]. In subjects younger than 65 years, there was a small but clear cyclical seasonal variation with a nadir in February (median 41 nmol/liter i.e., 16.4 ng/ml) and a zenith in June (median 55 nmol/liter i.e., 22 ng/ml). In contrast with previous studies which reported more important differences between young and elderly adults, in this population-based cohort, there was only a small and nonsignificant decrease in 25OHD values between the ages of 24 and 75 years. This may be because the upper age limit was only 75 years.

In a study that we performed on a large adult French population from the SUVIMAX project (Supplementation with Vitamins Minerals and Antioxidants), 25OHD and PTH status were measured in 1584 healthy adults living in 16 large towns between November 1994 and April 1995 (772 men aged 45–60 years and 812 women aged 35–60 years) [79] (Table II).

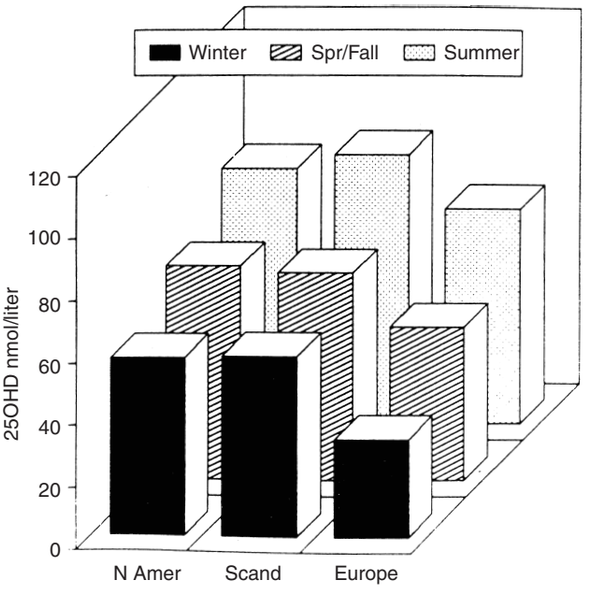

FIGURE 4 Mean of average serum 25OHD levels in young adults reported during the winter, spring and fall combined, and summer from studies collated according to geographic region. From McKenna [24] with permission.

The SUVIMAX project is a large international epidemiologic study to assess the effects of vitamins, minerals, and antioxidants in the prevention of global and specific mortality and morbidity due to cardiovascular diseases, cancers, and cataracts. In this adult population, 14% of subjects had 25OHD lower than 12 ng/ml (30 nmol/liter). There was no age effect, but a sex difference between 25OHD levels was apparent (vitamin D insufficiency was present in 15.5% of women and only in 12.4% of men; $p < 0.04$). Major differences were observed between different regions within the country. In addition, there was a significant negative correlation between 25OHD and PTH values for the whole population ($r = 0.20$, $p < 0.001$). The mean PTH levels were 48.2 ± 22.0 pg/ml for subjects with 25OHD concentrations at or below 30 ng/ml and 37.9 ± 14.2 pg/ml for the others ($p < 0.01$). This study demonstrates that vitamin D insufficiency is present in a substantial percentage of the general adult urban population in France, a finding that has been confirmed by two other studies [80,81].

In low latitude countries, hypovitaminosis D is also present in adults. For example, in Saudi Arabia up to 40% of native inhabitants and immigrants from Africa have hypovitaminosis D in winter [82]. In healthy young adult Japanese, the frequency of hypovitaminosis D reaches 5% in males and 25% in females [83]. Actually, it appears that the vitamin D status of the young adult

TABLE II Prevalence of Vitamin D Insufficiency in the General Adult Population Living in 16 Large French Towns[a]

Region	*n*	Mean serum 25OHD (nmol/liter)[b]	Vitamin D insufficiency[c] (% hypovitaminosis D)	Mean serum PTH (pg/ml)[d]
North	200	43±21	29	42±15
Center	99	49±25	27	40±15
Northeast	199	52±26	18	42±16
Northwest	300	58±29	14	38±17
Paris	98	59+25	13	46±24
Rhone-Alpes	200	62±27	9	40+15
Mediterranean coat	299	68±27	7	35±13
South	89	81±27	6	40±11
Southwest	100	94±38	0	37±11

[a]A total of 1584 subjects were studied: 772 males from 45 to 60 years old and 812 females from 35 to 60 years old. From Chapuy *et al.* [79].
[b]To convert nmol/liter to ng/ml 25OHD, divide by 2.5.
[c]Vitamin D insufficiency is deemed as a serum 25OHD level below 30 nmol/liter.
[d]PTH normal range is 55 pg/ml.

population needs to receive more attention, especially in winter, in countries where foods are not fortified. In the studies referenced by the McKenna analysis [24], the time of the year at which the measurements was performed is generally recorded, so as to permit a comparison of results with reference to the season of study [72,84,85]. In the studies performed earlier, the season and therefore the individual exposure to sunlight are not always reported.

B. Vitamin D Insufficiency in the Elderly (70–90 years)

Many studies, primarily those from Europe, have indicated that low levels of vitamin D may be more prevalent in older persons even if they do not appear related to the aging process per se (Fig. 5). In the healthy elderly population in North America and Scandinavia, nearly 25% of subjects had low values in winter but less than 5% had low levels throughout the year [25,86–90]. So, in these countries, even if the vitamin D intake is adequate and equal or greater than the recommended dietary allowance, the fraction of the vitamin D pool due to sunlight exposure is very important. As was found in normal adults, in elderly subjects there is a marked seasonal variation in 25OHD levels. In Europe, the frequency of vitamin D insufficiency in winter ranges from 8 to 60% [16,24,84,91–94].

The Euronut Seneca study has evaluated the 25OHD concentrations in 824 free-living elderly people from 16 towns (latitudes between 35° and 61° N) in 11 European countries between December 1988 and March 1989 [95]. Town-specific mean 25OHD concentrations range from 10 to 24 ng/ml (25–59 nmol/liter) for men and from 8 to 19 ng/ml (21–48 nmol/liter) for women. Overall 36% of men and 47% of women had 25OHD concentrations below 12 ng/ml (30 nmol/liter), the lowest concentrations being found surprisingly in southern European towns in France, Spain, and Italy. The low

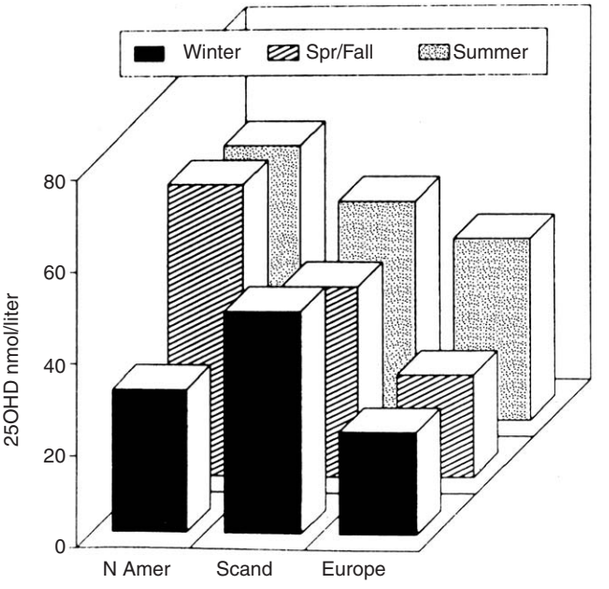

FIGURE 5 Mean of average serum 25OHD levels in healthy elderly reported during winter, spring and fall combined, and summer from studies collated according to geographic region. From McKenna [24] with permission.

TABLE III Comparison of Biochemical Values in Elderly and Young Women during the Winter*

	Elderly women living at home	Young (EPIDOS)	Elderly (OFELY)
Number of subjects	440	59	54
Age (years)	80±3	87±6 (a)	34±5 (a)
Calcium intake (mg/day)	569±338	–	810±280 (b)
Serum calcium	2.35±0.07	2.22±0.09 (a)	2.35±0.07 (mmol/liter)
25OHD	42.5±25.0	15.5±6.5 (c)	62.0±40.0 (b) (nmol/liter)
PTH (pg/ml)	63±28	76±49 (d)	43±15 (c)

*Data are expressed as means ± SD. Lowercase letters show results of statistical analyses: (a), $p < 0.0001$ compared to EPIDOS and OFELY studies; (b), $p < 0.0001$ compared to EPIDOS study; (c), $p < 0.001$ compared to EPIDOS study; (d), $p < 0.05$ compared to EPIDOS study. From Chapuy *et al.* [39].

25OHD values in this study were largely explained by attitudes toward sunlight exposure. We have studied [39] the vitamin D status of 440 healthy, free-living elderly women aged 75–90 years during winter and living in five French cities whose latitude varies from 49°9′ to 43°6′ N. The mean 25OHD level was not different among the five cities. However, in all of the cities, the mean 25OHD level was significantly lower than the mean level found in young healthy women from the OFELY cohort study that were recruited during the same winter period (42.5±25 versus 62.0±40.0 nmol/liter). (OFELY is a prospective study of the bone-loss determinants in women aged 30–95 years randomly selected from a large insurance company in Lyon, France.) Nevertheless, the mean values of the free-living elderly women were significantly higher ($p = 0.03$) than the winter values obtained in 59 institutionalized elderly women (42.5 ± 25 versus 15.5 ± 6.5 nmol/liter) (Table III). Among the institutionalized elderly women, 39% exhibited vitamin D insufficiency (25OHD values < 12 ng/ml, i.e., 30 nmol/liter) and only 16% had normal 25OHD values greater than or equal to 25 ng/ml (62.5 nmol/liter), the mean value of young women (Table II). Vitamin D insufficiency was associated in these healthy women with biochemical indices of secondary hyperparathyroidism and increased bone turnover.

In comparison to the few studies of free-living people, the vitamin D status of homebound subjects has been extensively studied. In the review by McKenna [24], the prevalence of vitamin D insufficiency in the institutionalized elderly population varies from 3 to 28% in North America. This prevalence reaches 54% in the study by Gloth *et al.* [96], who compared homebound elderly in community versus nursing homes. Despite apparently adequate vitamin D intake (200 to 400 IU/day or 5 to 10 μg/day), several studies have shown that between 30 and 50% of older homebound subjects have low vitamin D status, with 25OHD levels at or below 10 ng/ml (25 nmol/liter) [97–99]. In Europe, vitamin D insufficiency in elderly homebound subjects ranged from 70 to 100% [24]. The European 25OHD values in institutionalized subjects are significantly lower than both Scandinavian and North American values (Fig. 6). Only a few studies were conducted during specific seasons, but one would not expect much seasonal change as these elderly subjects have little or no exposure to sunlight [100]. The mean European values, based on year-round determinations of 25OHD and calculated from values reported in 16 European studies [24], is lower than 8 ng/ml (20 nmol/liter).

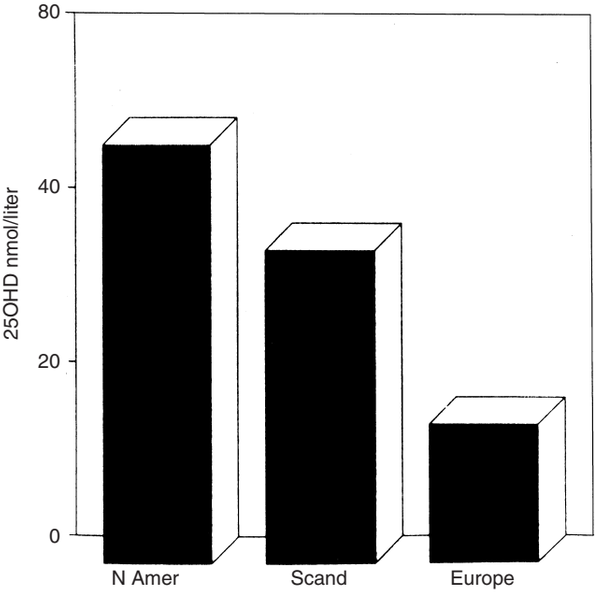

FIGURE 6 Mean of average serum 25OHD levels in institutionalized elderly reported throughout the year from studies collated according to geographic region. From McKenna [24] with permission.

In our DECALYOS I study, we have measured the 25OHD values at baseline in 280 very elderly women (84 ± 6 years) who were ambulatory but lived in nursing homes. (The DECALYOS study was the prospective study undertaken to determine the effects of vitamin D and calcium supplements on hip fracture incidence [29] in Lyon, France.) The mean 25OHD values were 14 ± 10 ng/ml (35 ± 25 nmol/liter), and 44% of subjects had 25OHD values lower than or equal to 12 ng/ml (30 nmol/liter). In another sample of elderly institutionalized women recruited for the recent DECALYOS II study [101], we found 66% of women with both an inadequate calcium intake (< 800 mg/day) and low vitamin D status (serum 25OHD <12 ng/ml). This suggests a valid rationale for combined calcium and vitamin D supplementation. Similarly, in the Dutch study of Ooms *et al.* [61], 65% of the 330 healthy women, residents of home apartments for the elderly, had 25OHD values below 12 ng/ml (30 nmol/liter), and in 34% the 25OHD level was below 8 ng/ml (20 nmol/liter). In winter 83% and in summer 50% of the subjects had 25OHD levels that were below 12 ng/ml (30 nmol/liter). The median levels of 25OHD were significantly higher for inhabitants of apartments for the elderly than for residents of homes for the elderly (11.6 and 8.8 pg/ml, respectively, i.e., 29 and 22 nmol/liter; $p < 0.0001$).

VI. PREVENTIVE MEASURES: CORRECTION OF LOW VITAMIN D STATUS

The effects on bone turnover of vitamin D insufficiency and secondary hyperparathyroidism have suggested that correction of subclinical hypovitaminosis D in the elderly may have a beneficial effect on the secretion of PTH, bone loss, and consequently the risk of fracture in elderly subjects.

A. Effects on Secondary Hyperparathyroidism

In the late 1980s, in a six-month trial of supplementation with vitamin D_2 (800 IU or 20 μg/day) and calcium (1 g/day) given to elderly subjects with vitamin D insufficiency living in an institution, we were able to reduce serum PTH concentrations by more than 30% with a parallel normalization of 25OHD concentrations [4]. With only 400 IU/day (10 μg) of vitamin D_3, Ooms *et al.* restored to normal 25OHD concentrations and decreased the mean PTH level by about 6 to 15% in an elderly Dutch population [40]. These two studies demonstrated that supplementation with low doses of vitamin D (400 to 800 IU/day or 10 to 20 μg/day) leads to adequate improvement of vitamin D status and parathyroid function with a greater effect of the 800 IU daily dose. There does not appear to be a need for a higher dose, as has been suggested by others [1]. In contrast, vitamin D supplementation with 10 μg of vitamin D of nursing home residents in the United States having 25OHD levels of 16 ng/ml or greater (>40 nmol/liter) [102], or of about 50 nmol/liter [103] did not significantly decrease PTH levels.

B. Effects on Bone Mass

By increasing the vitamin D intake from 100 to 500 IU daily, Dawson-Hughes *et al.* [41] were able to significantly reduce the late wintertime bone loss and to improve the net bone density of the spine. Among normal, middle-aged women, Khaw *et al.* found a significant direct relationship between serum 25OHD and bone mass of the spine and femoral neck, with an inverse correlation between bone density and PTH concentration, which is consistent with effects of small changes in vitamin D status on bone mass [52]. The use of 15,000 IU or 375 μg of oral vitamin D_2 weekly has also been shown to reduce metacarpal bone loss in normal women aged 65 to 74 years living in the community [104].

In our DECALYOS I study [29], the elderly women treated daily for 18 months with 800 IU or 20 μg of vitamin D_3 and 1.2 g of calcium showed an increase of 2.7% in the BMD of the proximal femur. During the same period, the women in the placebo group showed a decrease of femoral BMD equal to 4.6%. In DECALYOS II study, femoral neck BMD decreased in the placebo group (–2.4% per year) while remaining unchanged in women treated with 800 IU of vitamin D_3 and 1200 mg/day of calcium (+0.3% per year). No significant difference between groups was found for changes in distal radius BMD [101]. In the Dutch study of Ooms *et al.,* a daily treatment of elderly women (mean calcium intake 859 mg/daily) with 400 IU or 10 μg of vitamin D_3 over a two-year period was associated with an increase in femoral neck BMD equal to 2.3% in comparison with the placebo group. In these women at baseline, hip BMD was positively correlated with the serum 25OHD concentration below a threshold level (serum 25OHD < 12 ng/ml or 30 mmol/liter [40]).

C. Effects on Fracture Rate

The effects of vitamin D supplements on bone mass suggested that correction of vitamin D insufficiency in the elderly with low doses of vitamin D may have

beneficial effects on fracture incidence [105]. This was the aim of several studies. In our DECALYOS I study, 3270 healthy ambulatory women (mean age 84 ± 6 years) living in a nursing home received daily either 800 IU or 20 µg of vitamin D_3 plus 1.2 g of elemental calcium or a double placebo. After 18 months of follow-up, analysis showed that there was a 43% reduction in hip fractures ($p < 0.05$) and a 32% reduction in all nonvertebral fractures ($p = 0.015$) in the treated group. The results of the intention-to-treat analysis were also similar: 80 hip fractures in the vitamin D_3–calcium group and 110 in the placebo group (27% reduction; $p < 0.01$). At the same time, serum PTH decreased by 46% ($p < 0.001$) and the serum 25OHD level increased by 160% ($p < 0.001$) without change in the $1,25(OH)_2D$ levels. After a further 18 months of treatment for 1404 women, the beneficial effects of the treatment on nonvertebral fractures was confirmed. At the end of 36 months of follow-up in the intention-to-treat analysis, there were 17.2% fewer nonvertebral fractures (255 versus 308, $p < 0.02$) and 23% fewer hip fractures (137 versus 178, $p < 0.02$) in the vitamin D_3–calcium group (Fig. 7). The probability of hip fractures was decreased (odds ratio 0.73, CI 0.62 to 0.84), as was that of all nonvertebral fractures (odds ratio 0.72, CI 0.60 to 0.84) [29,68] (Fig. 8). This study has pointed out the importance of vitamin D insufficiency as a major determinant of senile secondary hyperparathyroidism and bone loss (see also Chapter 78). However, the study did not permit the elucidation of the relative importance of calcium versus vitamin D. Also, the possible effect of vitamin D supplements on the incidence of falls was not studied. In the DECALYOS II study performed in 583 ambulatory institutionalized women (mean age 85.2 years), the relative risk (RR) of hip fracture in the placebo group compared with the active treatment (800 UI vitamin D_3 and 1200 mg calcium/day) was 1.69 (CI 0.96 to 3.0), which is similar to that found in DECALYOS I (RR = 1.7; CI 1.0 to 2.8) [101].

In the study of Heikinheimo *et al.* [106], 799 elderly men and women, living either in residential care or in their own home, were followed for 2 to 5 years. In those treated with an annual injection of 150,000 to 300,000 IU of vitamin D_2, there was a significant reduction in the number of fractures in the upper limbs and ribs but no significant reduction in hip fractures. The reduction in hip fracture incidence reached 22% (9.4% in the control group and 7.3% in the intervention group), which is similar to the reduction found in our DECALYOS I study (23%). One reason for the lack of significance was possibly the smaller sample size. The prevention of fracture with vitamin D alone found by Heikinheimo might suggest that the results obtained with the association of calcium and vitamin D would be primarily due to vitamin D [106,107]. In a prospective double blind trial of 2578 men and women (mean age 80 ± 6 years) living either at home or in an institution, Lips *et al.* studied the effect of a daily supplement with 400 IU of vitamin D_3 [108]. After one year of vitamin D supplementation in a subgroup of

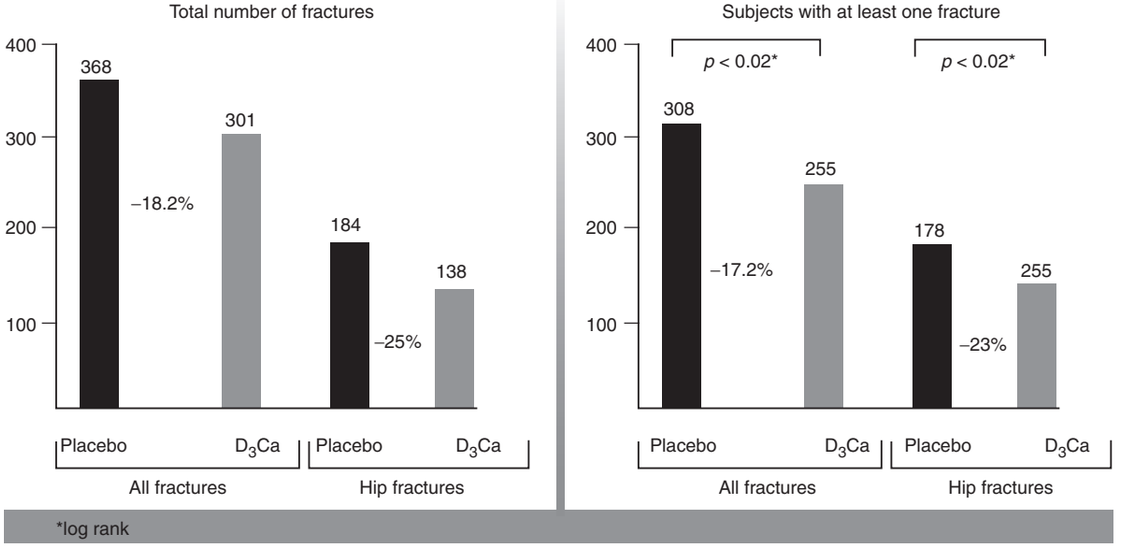

FIGURE 7 Effects of vitamin D_3 and calcium supplementation during three years on numbers of fractures in elderly women (DECALYOS I study). One group received placebo, and the other group (D_3Ca) received 800 IU of vitamin D_3 plus 1.2 g elemental calcium [29].

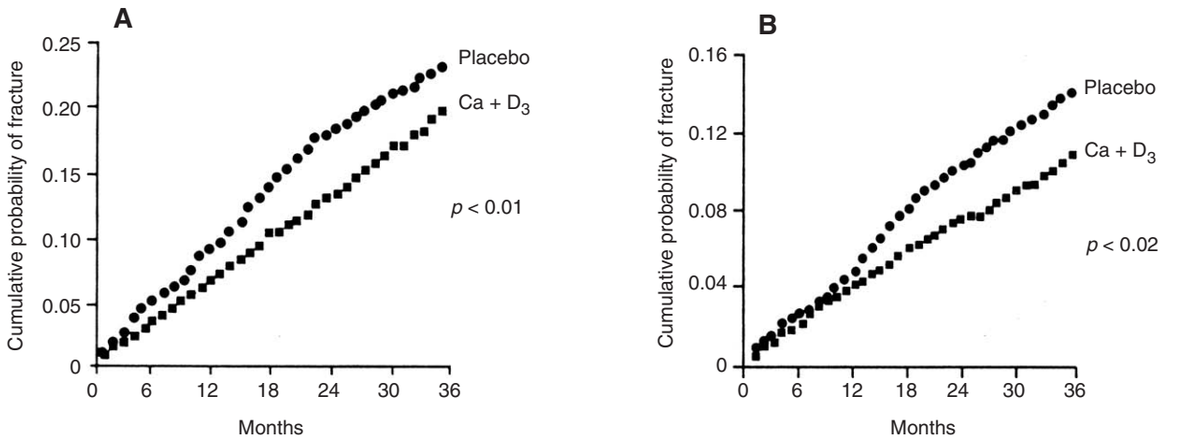

FIGURE 8 Cumulative probability of hip fractures (**A**) and all fractures (**B**) in the placebo and vitamin D_3–calcium groups (DECALYOS I study). From Meunier *et al.* [50] with permission.

women [40], the PTH level was reduced by only 6% from baseline, and after two years the BMD of the femoral neck had increased 2.2% in the vitamin D group compared to the placebo group. Nevertheless, the vitamin D supplementation did not significantly decrease the incidence of hip fracture after a maximum treatment period of 3.5 years. Similarly, in the recent study reported by Meyer *et al*. [103] and performed in residents of 51 Norwegian nursing homes (mean age 85.0 years), an intervention with 400 IU of vitamin D_3 per day over two years had no effect on the incidence of hip fracture or all nonvertebral fractures.

The differences observed in the results of the French and the Dutch studies may be explained by the lower dietary calcium intake in France than in the Netherlands and the use of calcium in the French study. In addition, the participants in the French study were four years older, less active at baseline, and were all residents of nursing homes. Hip fracture incidence was lower in the Dutch study (29 per 1000 versus 40 per 1000), so its power may have been insufficient to demonstrate significant differences in the fracture rates. Nevertheless, the increase in 25OHD levels and more importantly the reduction in PTH secretion were much lower in the Dutch and the Norwegian studies, raising the possibility that the daily dose of 400 IU of vitamin D_3 was suboptimal (minus 39% in DECALYOS I study—confirmed in DECALYOS II study—versus minus 6% in the Dutch study and no change in the Norwegian study after one year of treatment). These results indicate that a larger treatment effect was obtained in the French studies [68,101] where 800 IU/day of vitamin D had been given.

In addition to these four prospective studies on the preventive effect of vitamin D on hip fracture incidence, Banstam and Kanis in a preliminary retrospective report from the MEDOS (MEDOS: Mediterranean Osteoporosis Study) study did not find that the use of vitamin D was associated with a significant decrease in the risk of hip fracture, as was the use of calcium, estrogen, or calcitonins [109]. When the data were reanalyzed including not all the hip fractures but only the low energy fractures, the use of vitamin D supplement was associated with a 26% (but nonsignificant) decrease in the risk of hip fracture [93]. In addition, the risk reduction was influenced by age and body mass index (BMI). For women 80 years or older, the reduction in hip fracture risk for vitamin D users was 37% ($p = 0.04$), and for these women with a BMI less than 20 kg/m^2, the use of vitamin D was associated with a marked and significant reduction in hip fracture risk of 55% ($p = 0.01$). In this study, vitamin D was taken for a time ranging from 1 to 20 years, and the doses used were not known.

From all these studies, it appears that vitamin D supplements are undoubtedly useful in the prevention of hip fracture, but there are still two critical questions that need to be answered. What is the optimal dose, and who should receive supplements?

VII. CONCLUSIONS

There is now convincing evidence that there are several forms of low vitamin D states that induce different forms of bone disease. Severe and prolonged "deficiency" of vitamin D, which is no longer very common, is associated with osteomalacia. In contrast, less severe vitamin D depletion, which might be called vitamin D "insufficiency," is very common in elderly subjects

living in institutions or at home but also appears not to be rare in healthy adults, particularly in winter. A progressive state of vitamin D insufficiency per se does not imply that bone disease is present but does identify a high risk status [1]. Vitamin D insufficiency will ultimately lead to a state of vitamin D deficiency. However, before overt clinical symptoms of osteomalacia become apparent, vitamin D insufficiency may give rise to problems at the bone level as a consequence of secondary hyperparathyroidism, resulting in increased bone turnover, bone loss, and risk of fractures. The observation of seasonal variation in bone mineral density in normal subjects provides evidence that relatively small changes in vitamin D status may have significant effects on bone mass.

In agreement with several authors, we propose that vitamin D insufficiency might be redefined by a 25OHD level equal to or under 30 ng/ml (75 nmol/liter), this value being required for maximal PTH suppression. In elderly subjects it has been shown that 800 IU of vitamin D_3 are usually needed to reach this optimal level of serum 25OHD. In many countries, natural dietary sources do not readily permit an intake of the recommended amounts of vitamin D; thus, the available mode of prevention of vitamin D insufficiency might be increased exposure to sunlight, fortification of foodstuffs, and oral or injectable vitamin D supplementation. Because the prevalence of vitamin D insufficiency is very high among elderly subjects, an increase in vitamin D intake becomes essential, especially for those living in an institution. Promoting sun exposure is difficult to realize, and placing ultraviolet lamps in the living room would probably not be efficient enough. Supplementation by vitamin D preparations seems to be the best solution. This supplementation should be higher than 400 lU/day in the healthy elderly to increase 25OHD levels, because this dose was not sufficient for prevention of hip fractures in elderly subjects [103,108]. However, 800 IU of vitamin D_3 with 1.2 g of calcium was able to decrease by 25% the hip fractures' incidence of institutionalized elderly women in three years and to maintain normal 25OHD and PTH concentrations. As it appears that vitamin D supplementation is effective and safe in preventing vitamin D insufficiency, daily low dose treatment (800 IU/daily) may be the best regimen. However, an intermittent high dose (100,000 IU) given orally or by injection every six months may be an effective alternative [94]. In contrast, very high single doses, like 600,000 IU twice a year, should be used cautiously because of the risk of inducing transient hypervitaminosis D and side effects due to hypercalcemia, including increased calcification rate of vascular atheromatous plaques.

References

1. Parfitt MA, Gallagher JC, Heaney RP, Johnston CC, Neer R, Whedon GD 1982 Vitamin D and bone health in the elderly. Am J Clin Nutr **36**:1014–1031.
2. Walters MR, Kollenkirchen U, Fox J 1992 What is vitamin D deficiency? Proc Soc Exp Biol Med **199**:385–393.
3. Peacock M, Selby PL, Francis RM, Brown WB, Hordon L 1985 Vitamin D deficiency, insufficiency, sufficiency and intoxication. What do they mean? In: Norman A, Schaefer K, Grigolett MG, Herrath DV (eds) Sixth Workshop on Vitamin D. De Gruyter, Berlin and New York, pp. 569–570.
4. Chapuy MC, Chapuy P, Meunier PJ 1987 Effect of calcium and vitamin D supplements on calcium metabolism in the elderly. Am J Clin Nutr **46**:324–328.
5. Lips P, Wierzinga A, Van Ginkel FC, Jongen MJM, Netelenbos JC 1988 The effect of vitamin D supplementation on vitamin D status and parathyroid function in elderly subjects. J Clin Endocrinol Metab **67**:644–650.
6. Krall EA, Sahyoun N, Tannebaum S, Dallal GE, Dawson-Hughes B 1989 Effect of vitamin D intake on seasonal variations in parathyroid hormone secretion in postmenopausal women. N Engl J Med **321**:1777.
7. Gloth FM, Gundberg CM, Hollis BW, Haddad JG, Tobin JD 1995 Vitamin D deficiency in homebound elderly persons. JAMA **274**:1683–1686.
8. Gloth FM, Tobin JD 1995 Vitamin D deficiency in older people. J Am Geriatr Soc **43**:822–828.
9. Webb AR, Pilbeam C, Hanafin N, Holick MF 1990 An evaluation of the relative contributions of exposure to sunlight and of diet to the circulating concentrations of 25-hydroxyvitamin D in an elderly nursing home population in Boston. Am J Clin Nutr **51**:1075–1081.
10. Chapuy MC, Preziosi P, Maamer M, Arnaud S, Galan P, Herckberg S, Meunier PJ 1997 Prevalence of vitamin D insufficiency in an adult normal population. Osteoporos Int **7**:439–443.
11. Thomas MK, Lloyd-Jones DM, Thadhani RI, Shaw AC, Deraska DJ, Kitch BT, Vamvakas EC, Dick IM, Prince RL, Finkelstein JS 1998 Hypovitaminosis D in medical inpatients. N Engl J Med **338**:777–783.
12. Malabanan AO, Veronikis IE, Holick MF 1998 Redefining vitamin D insufficiency. Lancet **351**:805–806.
13. Dawson-Hughes B, Harris SS, Dallal GE 1997 Plasma calcidiol, season and serum parathyroid hormone concentrations in healthy elderly men and women. Am J Clin Nutr **65**:67–71.
14. Tangpricha V, Pearce EN, Chen TC, Holick MF 2002 Vitamin D insufficiency among free-living healthy young adults. Am J Med **112**:659–6620.
15. Vieth R, Ladak Y, Walfish PG 2003 Age-related changes in the 25-hydroxyvitamin D versus parathyroid hormone relationship suggest a different reason why older adults require more vitamin D. J Clin Endocrinol Metab **88**:185–191.
16. Bouillon RA, Auwerx JD, Lissens WD, Pelemans WK 1987 Vitamin D status in the elderly, seasonal substrate deficiency causes 1,25-dihydroxycholecalciferol deficiency. Am J Clin Nutr **45**:755–763.
17. Lips P, Chapuy MC, Dawson-Hughes B, Pols HAP, Holick MF 1999 An international comparison of serum 25-hydroxyvitamin D measurements. Osteoporos Int **9**:394–397.
18. Hollis B 1996 Assessment of vitamin D nutritional and hormonal status: What to measure and how to do it. Calcif Tissue Int **58**:4–5.

19. Holick MF, McCollum Award lecture 1994 Vitamin D—New horizons for the 21st century. Am J Clin Nutr **60**:619–630.
20. Gannagé-Yared MH, Chemali R, Yaacoub N, Halaby G 2000 Hypovitaminosis D in a sunny country: relation to lifestyle and bone markers. J Bone Miner Res **15**:1856–1862.
21. Holick MF, Matsuoka LY, Wortsman J 1989 Age, vitamin D, and solar ultraviolet. Lancet **1**:1104–1105.
22. Need AG, Morris HA, Horowitz M, Nordin BEC 1993 Effects of skin thickness, age, body fat, and sunlight on serum 25-hydroxyvitamin D. Am J Clin Nutr **58**:882–885.
23. Chel VGM, Ooms ME, Popp-Snijders C, Pavel S, Schothorst AA, Meulemans CCE, Lips P 1998 Ultraviolet irradiation corrects vitamin D deficiency and suppresses secondary hyperparathyroidism in the elderly. J Bone Miner Res **13**:1238–1242.
24. McKenna MJ 1992 Differences in vitamin D status between countries in young adults and in elderly. Am J Med **93**:69–77.
25. Clemens TL, Zhou X, Myles M, Endres D, Lindsay R 1986 Serum vitamin D_2 and vitamin D_3 metabolite concentrations and absorption of vitamin D_2 in elderly subjects. J Clin Endocrinol Metab **63**:656–660.
26. Holick MF 1986 Vitamin D requirements for the elderly. Clin Nutr **5**:121–129.
27. Weisman Y, Schen RJ, Einsenberg Z, Edelstein S, Harell A 1981 Inadequate status and impaired metabolism of vitamin D in elderly. J Med Sci **17**:19–21.
28. Baragry JM, France MW, Corles D, Gupta SP, Switala S, Boucher BJ, Cohen RD 1978 Intestinal cholecalciferol absorption in the elderly and in younger adults. Clin Sci Mol Med **55**:213–220.
29. Chapuy MC, Arlot ME, Duboeuf F, Brun J, Crouzet B, Arnaud S, Delmas PD, Meunier PJ 1992 Vitamin D_3 and calcium to prevent hip fractures in elderly women. N Engl J Med **237**:1637–1642.
30. Mundy GR 1989 In: Calcium Homeostasis: Hypercalcemia and Hypocalcemia. Martin Dunitz, London, pp. 185–186.
31. Riggs BL, Melton LJ 1983 Evidence of two distinct syndromes of involutional osteoporosis. Am J Med **75**:899–901.
32. Chapuy MC, Durr F, Chapuy P 1983 Age-related changes in parathyroid hormone and 25-hydroxycholecalciferol levels. J Gerontol **38**:19–22.
33. Forero MS, Klein RF, Nissenson RA, Nelson K, Heath H, Arnaud CD, Riggs BL 1987 Effect of age on circulating immunoreactive and bioactive parathyroid hormone levels in women. J Bone Miner Res **2**:363–366.
34. Epstein S, Bryce G, Hinman JW, Miller ON, Riggs BL, Hui SL, Johnston CC 1986 The influence of age on bone mineral regulating hormones. Bone **7**:421–425.
35. Sherman SS, Hollis BW, Tobin JD 1990 Vitamin D status and related parameters in a healthy population: The effects of age, sex, and season. J Clin Endocrinol Metab **71**:405–413.
36. Prince RL, Dick I, Devine A, Price RI, Gutteridge DH, Kerr D, Criddle A, Garcia-Webb P, St John A 1995 The effects of menopause and age on calciotropic hormones: A cross sectional study on healthy women aged 35 to 90. J Bone Miner Res **10**:835–842.
37. Freaney R, McBrinn Y, Me Kenna MJ 1993 Secondary hyperparathyroidism in elderly people: Combined effect of renal insufficiency and vitamin D deficiency. Am J Clin Nutr **58**:187–191.
38. Young G, Marcus R, Minkoff JR, Kirn LY, Segre GV 1987 Age-related rise in parathyroid hormone in men: The use of intact and midmolecule antisera to distinguish hormone secretion from retention. J Bone Miner Res **2**:367–374.
39. Chapuy MC, Schott AM, Garnero P, Hans D, Delmas PD, Meunier PJ 1996 Healthy elderly French women living at home have secondary hyperparathyroidism and high bone turnover in winter. J Clin Endocrinol Metab **81**: 1129–1133.
40. Ooms ME, Roos JC, Bezemer PD, Van Der Vijch WJF, Bouter LM, Lips P 1995 Prevention of bone loss by vitamin D supplementation in elderly women: A randomized double blind trial. J Clin Endocrinol Metab **80**:1052–1058.
41. Dawson-Hughes B, Dallal GE, Krall EA, Harris S, Sokoll LJ, Falconer G 1991 Effect of vitamin D supplementation on overall bone loss in healthy postmenopausal women. Ann Intern Med **115**:505–512.
42. Kochersberger G, Westlund R, Lyles KW 1990 Calcium supplementation lowers serum parathyroid hormone levels in elderly subjects. J Gerontol **45**:M159–M162.
43. Lips P, Netelendos C, Jongen MJM, Van Ginkel FC, Altuis AL, Vanschaik CL, Vandervijch WJF, Vermeiden JPW, Van Der Meer C 1982 Histomorphometric profile and vitamin D status in patients with femoral neck fracture. Metab Bone Dis Related Res **4**:85–93.
44. Lips P 2001 Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. Endocrine Reviews **22**:477–501.
45. Johnston CC, Norton J, Khairi MRA, Kernek C, Edouard C, Meunier PJ 1985 Heterogeneity of fracture syndromes in postmenopausal women. J Clin Endocrinol Metab **61**:551–556.
46. Compston JE, Silver AC, Croucher PI, Brown RC, Woodhead JS 1989 Elevated serum intact parathyroid hormone levels in elderly patients with hip fracture. Clin Endocrinol **31**:667–672.
47. Okano T, Yamamoto K, Hagino H, Hishimoto H 1992 Iliac bone histomorphometry in Japanese women with hip fracture. In: Bone Morphometry, Sixth International Congress, Lexington, 4–9 October, Abstract 99.
48. Benhamou CL, Chappard D, Gauvain JB 1991 Hyperparathyroidism in proximal femur fractures: Biological and histomorphometric study in 21 patients over 75 years old. Clin Rheumatol **10**:144–160.
49. Rao DS, Villanueva A, Mathews SM 1983 Histologic evaluation of vitamin D depletion in patients with intestinal malabsorption or dietary deficiency. In: Frame B, Potts J Jr (eds) Clinical Disorders of Bone and Mineral Metabolism. Excerpta Medica, Amsterdam, pp. 224–226.
50. Meunier PJ, Chapuy MC, Arlot ME, Delmas PD, Duboeuf F 1994 Can we stop bone loss and prevent hip fractures in the elderly? Osteoporosis Int **4**(Suppl 1):S71–S76.
51. Rosen CJ, Morrison A, Zhou H, Storm D, Hunter SJ, Musgrave K, Chen T, Wei W, Holick MF 1994 Elderly women in northern New England exhibit seasonal changes in bone mineral density and calciotropic hormones. Bone Miner **2**:83–92.
52. Khaw KT, Sheyd MJ, Compston J 1992 Bone density, parathyroid hormone, and 25-hydroxyvitamin D concentrations in middle-aged women. Br Med J **305**:273–277.
53. Lukert B, Higgins J, Stoskopf M 1992 Menopausal bone loss is partially regulated by dietary intake of vitamin D. Calcif Tissue Int **51**:173–179.
54. Martinez ME, Delcampo MJ, Sanchez-Cabezudo MJ, Garcia JA, Sanchez-Calvin MT, Torrijos A, Coya J 1994 Relations between calcidiol serum levels and bone mineral density in postmenopausal women with low bone density. Calcif Tissue Int **55**:253–256.

55. Villareal DT, Civitelli R, Chines A, Avioli LV 1991 Subclinical vitamin D deficiency in postmenopausal women with low vertebral bone mass. J Clin Endocrinol Metab **72**:628–634.
56. Cummings SR, Black DM, Nevitt MC, Browner W, Cauley J, Ensrud K, Genant HK, Palermo L, Scott J, Vogt TM 1993 Bone density at various sites for prediction of hip fractures. Lancet **341**:72–75.
57. Scharla SH, Scheidt-Nave C, Leidig G, Seibel M, Ziegler R 1994 Association between serum 25-hydroxyvitamin D and bone mineral density in a normal population sample in Germany. In: Norman AW, Bouillon R, Thomasset M (eds) Vitamin D: A Pluripotent Steroid Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications. de Gruyter, Berlin, p. 863.
58. Brazier M, Kamel S, Maamer M, Agbomson F, Elseper I, Garabedian M, Desmet G, Sebert JL 1995 Markers of bone remodeling in the elderly subjects: Effects of vitamin D insufficiency and its correction. J Bone Miner Res **10**:1753–1761.
59. Kamel S, Brazier M, Picar C, Boitte F, Samson L, Desmet G, Sebert JL 1994 Urinary excretion of pyridinoline crosslinks measured by immunoassay and HPLC techniques in normal subjects and elderly patients with vitamin D deficiency. Bone Miner **26**:197–208.
60. Dawson Hughes B, Harris SS, Krall EA, Dallal GE, Falconer G, Green CL 1995 Roles of bone loss in postmenopausal women randomly assigned to one or two dosages of vitamin D. Am J Clin Nutr **61**:1140–1145.
61. Ooms ME, Lips P, Roos JC, Vandervijch WJF, Popp-Snijdeers C, Bezemer PD, Bouter LM 1995 Vitamin D status and sex hormone binding globulin: Determinants of bone turnover and bone mineral density in elderly women. J Bone Miner Res **10**:1177–1184.
62. Gennari C, Agnusdei D, Nardi P, Civitelli R 1990 Estrogen preserves a normal intestinal response to 1,25-dihydroxyvitamin D_3 in oophorectomized women. J Clin Endocrinol Metab **71**:1288–1293.
63. Birge SJ, Haddad JC 1975 25-Hydroxycholecalciferol stimulation of muscle metabolism. J Clin Invest **56**:1100–1107.
64. Gloth FM, Lindsay JM, Zelesnick LB, Greenough WB 1991 Can vitamin D deficiency produce an unusual pain syndrome. Arch Intern Med **151**:1662–1664.
65. Gloth FM, Smith CE, Hollis BW, Tobin JD 1995 Functional improvement with vitamin D replenishment in a cohort of frail vitamin D–deficient older people. J Am Geriatr **43**:1269–1271.
66. Corless D, Dawson D, Fraser F, Ellis M, Evans SJ, Perry JD, Reisner C, Silver CP, Beer M, Boucher BJ 1985 Do vitamin D supplements improve the physical capabilities of elderly hospital patients. Age Aging **14**:76–84.
67. Sorensen OH, Lund BI, Saltin B 1979 Myopathy in bone loss ageing: Improvement by treatment with 1α-hydroxycholecalciferol and calcium. Clin Sci **56**:157–161.
68. Chapuy MC, Arlot ME, Delmas PD, Meunier PJ 1994 Effect of calcium and cholecalciferol treatment for three years on hip fractures in elderly women. Br Med J **308**:1081–1082.
69. Tilyard MW, Spears GFS, Thomson J, Dovey S 1992 Treatment of postmenopausal osteoporosis with calcitriol or calcium. N Engl J Med **326**:357–362.
70. Bischoff HA, Stahelin HB, Dick W, Akos R, Knecht M, Salis C, Nebiker M, Theiler R, Pfeifer M, Begerow B, Lew RA, Conzelmann M 2003 Effects of Vitamin D and calcium supplements on falls: a randomized controlled trial. J Bone Miner Res **18**:343–351.
71. Haddad JG, Hahn TJ 1973 Natural and synthetic sources of circulating 25-hydroxyvitamin D in man. Nature **244**:515–517.
72. Styrd RP, Gilbertson TJ, Brunden MN 1979 A seasonal variation study of 25-hydroxyvitamin D_3 serum levels in normal humans. J Clin Endocrinol Metab **48**:771–775.
73. Chesney RW, Rosen JF, Hamstra AJ, Smith C, Mahaffey K, DeLuca HF 1981 Absence of seasonal variation in serum concentrations of 1,25-dihydroxyvitamin D despite a rise in 25-hydroxyvitamin D in summer. J Clin Endocrinol Metab **53**:139–142.
74. Lund B, Sorenson OH 1979 Measurement of 25-hydroxyvitamin D in serum and its relation to sunshine, age, and vitamin D intake in the Danish population. Scand J Clin Lab Invest **39**:23–30.
75. Vik T, Try K, Stromme JH 1980 The vitamin D of man at 70° north. Scand J Clin Lab Invest **40**:227–232.
76. Benucci A, Tommasi M, Fantappie B, Scardigli S, Ottanelli S, Pratesi E, Romano S 1993 Serum 25-hydroxyvitamin D levels in normal subjects: Seasonal variation and relationship with parathyroid hormone and osteocalcin. J Nucl Biol Med **37**:77–82.
77. Carnevale V, Modoni S, Pileri S, Di Giorgio A, Chiodini I, Minisola S, Vieth R, Scillitani A 2001 Longitudinal evaluation of vitamin D status in healthy subjects from Southern Italy: seasonal and gender differences Osteoporos Int **12**:1026–1030.
78. Burnand B, Slovtskis D, Gianoli F, Cornuz J, Rickenback M, Paccaud F, Burckhardt P 1992 Serum 25-hydroxyvitamin D: distribution and determinants in the Swiss population. Am J Clin Nutr **56**:537–542.
79. Chapuy MC, Preciozi P, Maamer M, Arnaud S, Galan P, Hercberg S, Meunier PJ 1997 Prevalence of hypovitaminosis D in the femoral adult French population. Osteoporos Int **7**:439–443.
80. Ribot C, Pouilles JM, Tremollieres F 1996 Vitamin D status in a large cohort of early postmenopausal French women. Osteoporosis Int **6**(Suppl. l):abstract PSU218.
81. Baudoin C, Aquino D, Charlier C, Cohen Solal M, Gueris J, Devernejoul MC 1996 Vitamin D deficiency in postmenopausal women in region of Paris. Osteoporosis Int **6**(Suppl. l):abstract PSU281.
82. Sedrani SH, Elidrisy AWTH, El Arabi KM 1983 Sunlight and vitamin D status in normal Saudi subjects. Am J Clin Nutr **38**:129–132.
83. Takeuchi A, Okano T, Ishida Y, Kovayashi T 1995 Dietary vitamin D intake and vitamin D nutritional status in the healthy young and institutionalized elderly people. In: Burckhardt P Heaney RP (eds) Nutritional Aspects of Osteoporosis. Ares Serono Symposia Publications, Rome, pp. 351–355.
84. Stamp TCB, Round JM 1974 Seasonal changes in human plasma levels of 25-hydroxyvitamin. Nature **247**:563–565.
85. Poskitt EME, Cole TJ, Lawson DEM 1979 Diet, sunlight, and 25-hydroxyvitamin D in healthy children and adults. Br Med J **1**:221–223.
86. Delvin EE, Imbach A, Copti M 1988 Vitamin D nutritional status and related biochemical indices in an autonomous elderly population. Am J Clin Nutr **48**:373–378.
87. Von Knorring J, Slatis P, Weber TH, Helenius T 1982 Serum levels of 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D and parathyroid hormone in patients with femoral neck fracture in southern Finland. Clin Endocrinol **17**:189–194.
88. Omdahl JL, Garry PJ, Hunsaker LA, Hunt WC, Goodwin JS 1982 Nutritional status in a healthy elderly population: Vitamin D. Am J Clin Nutr **36**:1125–1133.

89. Lukert BP, Carey M, McCarty B, Tiemann S, Goodnight L, Helm M, Hassanein R, Stevenson S, Stoskopf M, Doolan L 1987 Influence of nutritional factors on calcium regulating hormone and bone mass. Calcif Tissue Int **40**:119–125.
90. Schmidt-Gayk H, Goosen J, Lendle F, Seidel D 1997 Serum 25-hydroxycholecalciferol in myocardial infarction. Atherosclerosis **26**:55–58.
91. Rapin CH, Lagier R, Boivin G, Jung A, McGee W 1982 Biochemical findings in blood of aged patients with femoral neck fractures: A contribution to the detection of occult osteomalacia. Calcif Tissue Int **34**:465–469.
92. Newton HMV, Sheltawy M, Hay AWM, Morgan B 1985 The relations between vitamin D_2 and D_3 in elderly women in Great Britain. Am J Clin Nutr **41**:760–764.
93. Ranstam J, Kanis JA 1995 Influence of age and body mass on the effects of vitamin D on hip fractures risk. Osteoporosis Int **5**:450–454.
94. Byrne PM, Preatney R, McKenna MJ 1995 Vitamin D supplementation in the elderly: Review of safety and effectiveness of different regimes. Calcif Tissue Int **56**:518–520.
95. Van Der Wielen RP, Lowik MRH, Van Der Berg H, Degroot L, Haller J, Moreras O, Vanstaveren WA 1995 Serum 25OHD concentrations among elderly people in Europe. Lancet **346**:207–210.
96. Gloth MD, Tobin JD, Smith CE, Hollis BW, Gunberg CM 1993 The prevalence of vitamin D deficiency in homebound elderly: Community vs nursing home. J Am Geriatr Soc **41**(Suppl.):17–22.
97. Gloth FM, Tobin JD, Shermann SS, Hollis BW 1991 Is the recommended daily allowance for vitamin D too low in the homebound elderly? J Am Geriatr Soc **39**:137–141.
98. McMurtry CT, Yound SE, Downs RW, Adler RA 1992 Mild vitamin D deficiency and secondary hyperparathyroidism in nursing home patients receiving adequate dietary vitamin D. J Am Geriatr Soc **40**:343–347.
99. O'Dowd KJ, Clemens TL, Kelsey JL, Lindsay R 1993 Exogenous calciferol (vitamin D) and vitamin D endocrine status among elderly nursing home residents in the New York City area. J Am Geriatr Soc **41**:414–421.
100. Davies M, Mawer EB, Hann JT, Taylor JL 1986 Seasonal changes in the biochemical indices of vitamin D deficiency in the elderly: A comparison of people in residential homes, long stay ward, and attending a day hospital. Age Aging **15**:77–83.
101. Chapuy MC, Pamphile R, Paris E, Kempf C, Schlichting M, Arnaud S, Garnero P, Meunier PJ 2002 Combined calcium and vitamin D_3 supplementation in elderly women: confirmation of reversal of secondary hyperparathyroidism and hip fracture risk: the Decalyos II study. Osteoporos Int **13**:257–264.
102. Himmelstein S, Clemens TL, Rubin A, Lindsay R 1990 Vitamin D supplementation in elderly nursing home residents increases 25OHD but not $1,25(OH)_2D$. Am J Clin Nutr **52**:701–706.
103. Meyer HE, Smedshaug GB, Kvaavik E, Falch JA, Tverdal A, Pedersen JI 2002 Can vitamin D supplementation reduce the risk of fracture in the elderly? A randomized controlled trial. J Bone Min Res **17**:709–715.
104. Nordin BEC, Baker MR, Horsman A, Peacock M 1985 A prospective trial on the effect of vitamin D supplementation on metacarpal bone loss in elderly women. Am J Clin Nutr **42**:470–747.
105. Compston JE 1995 The role of vitamin D and calcium supplementation in the prevention of osteoporotic fractures in the elderly. Clin Endocrinol **43**:393–405.
106. Heikinheimo RJ, Inkovaara JA, Harjv EJ, Haavisto MV, Kaarela RH, Kajata JM, Kokko AML, Kokko LA, Rajala SA 1992 Annual injections of vitamin D and fractures of aged bone. Calcif Tissue Int **51**:105–110.
107. Torgeson D, Campbell M 1994 Vitamin D alone may be helpful. Br Med J **309**:193 (letter).
108. Lips P, Graafmans WC, Ooms ME, Bezemer PD, Bouter LM 1996 Vitamin D supplementation and fracture incidence in elderly persons. Ann Intern Med **124**:400–406.
109. Kanis JA, Johnell O, Gullberg B, Allander E, Dilsen G, Gennari C, Lopez-Vaz AA, Lyritis JP, Mazuoli G, Miravet L, Passeri M, Cano RP, Rapado A, Ribot C 1992 Evidence for the efficacy of bone active drugs in the prevention of hip fracture. Br Med J **305**:1124–1128.

CHAPTER 67

Vitamin D and Osteoporosis

RICHARD EASTELL AND B. LAWRENCE RIGGS
Bone Metabolism Group, Division of Clinical Sciences (North), University of Sheffield, Sheffield, England and the Department of Endocrinology and Metabolism, Mayo Clinic and Mayo Foundation, Rochester, Minnesota, USA

I. INTRODUCTION

Involutional osteoporosis begins in middle life and becomes progressively more common with advancing age. It is clinically manifest as fractures. It is likely that changes in calcium homeostasis contribute substantially to the pathogenesis of osteoporosis. A proposed model for these interactions on bone loss with aging is shown in Figure 3.

II. EFFECT OF AGE ON LEVELS OF 25OHD

Most studies have shown that plasma levels of 25OHD decrease with age by about 50% in both men and women [1–14] (Fig. 1). The importance of this decline in 25OHD to the pathogenesis of osteoporosis has been a major topic of study in the osteoporosis field over the past few years [15].

Lips [16] has proposed that the lower limit of reference range for 25OHD in the summer is taken as 30 nmol/l (see Chapter 62), based on the following arguments:

1. This is the lower limit of the reference range established for the summer based on blood donors.
2. This is the level of 25 OHD above which $1,25(OH)_2D$ is not substrate-dependent.
3. This is the level of 25OHD above which BMD does not correlate with 25OHD [17].
4. This is the level of 25OHD above which further treatment with vitamin D will not suppress PTH any further.

Based on this figure, 25 to 50% of the elderly are vitamin D–deficient in either Europe [18,19] or the United States [13]. This figure is as high as 75% for the housebound elderly [18]. In Europe, latitude is not a major determinant of 25OHD status. Indeed, people living in southern Europe have lower levels of 25OHD mainly as a result of their attitudes to sunshine exposure, such as the use of long-sleeved clothes [19]. This threshold figure of 30 nmol/L may be too conservative (see Section IX) and thus vitamin D deficiency may be more prevalent than these figures suggest (see Chapter 61).

A. Mechanisms for the Age-related Decrease in 25OHD

There are several possible mechanisms for the age-related decrease in plasma 25OHD, and these will be considered in turn.

1. DIETARY INTAKE OF VITAMIN D

Most elderly subjects do not consume the Recommended Daily Allowance for vitamin D, but this is also true for younger subjects [20]. McKenna [20] found no evidence for differences in vitamin D intake between young adults and the elderly in any geographical region, although average intake varied greatly between countries.

2. VITAMIN D ABSORPTION AND C25 HYDROXYLATION

Evidence relating to the effect of age on vitamin D absorption is conflicting. Barraguy *et al.* [3] administered ^{3}H-vitamin D to young and elderly subjects and found that absorption was greater in young subjects (13.2% vs. 7.6% in 6 hr). However, administration of vitamin D_2 results in similar increments in plasma 25OHD in young and elderly subjects [21,22]. One explanation for these apparently conflicting results is

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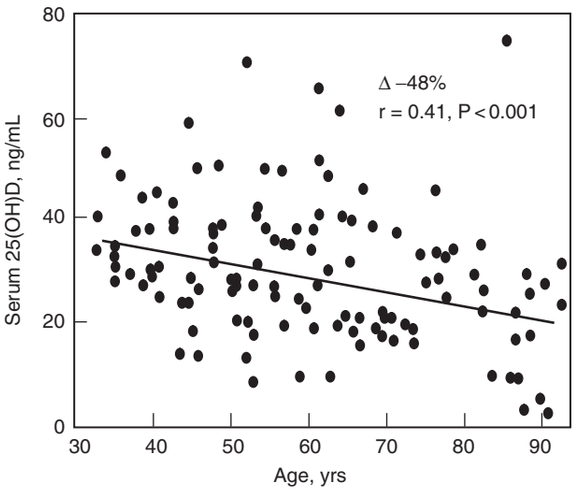

FIGURE 1 Influence of age on the serum concentration of 25OHD in a sample of healthy women [from Tsai *et al.* [13], with permission].

that vitamin D absorption might be decreased in the elderly, but that C25 hydroxylase activity could be increased (possibly as a result of low levels of $1,25(OH)_2D$ in the elderly [23]).

3. Effect of Age on Production of Vitamin D in Skin

Exposure to sunshine is often less in the elderly than in the young [2,6]. It has been suggested that widespread use of sunscreens to prevent skin cancer may make the elderly more susceptible to vitamin D deficiency [24], although it is not clear whether sunscreen use increases with age.

Evidence regarding the effect of age on the capacity of human skin to produce vitamin D remains controversial (see Chapter 50). MacLaughlin and Holick reported a decrease in 7-dehydrocholesterol (provitamin D) in skin biopsy specimens with aging [25]. Furthermore, conversion of 7-dehydrocholesterol by ultraviolet light was decreased in skin from elderly subjects. Exposure of subjects to ultraviolet light results in an increase in circulating levels of 25OHD, and this response has been reported to be the same in young and old subjects [26] or fourfold greater in young subjects [27]. It has been suggested that the age-related decrease in skinfold thickness might be related to declining levels of 25OHD [28].

4. Effect of Age on Clearance of 25OHD

The effect of age on the metabolic clearance rate of 25OHD has not been studied directly, but the half-life of plasma 25OHD after administration of ^{3}H-labeled vitamin D was reported as 21 to 27 days [3] and did not increase with age. Clemens *et al*. reported similar findings [21].

III. EFFECT OF AGE ON LEVELS OF $1,25(OH)_2D$

Plasma levels of $1,25(OH)_2D$ probably decrease with age in most populations, especially after age 65 [5,7,12, 21,29–33]. However, many studies have reported unchanged or increasing levels over a wide age range in studies of both men and women [9,11,34–39], while others have reported increasing levels from age 35 up to age 65, followed by a decrease [37,38,40,41]. Aksnes *et al.* found that $1,25(OH)_2D$ showed no age-related decrease in active healthy subjects living at home, but decreased significantly in hospitalized geriatric patients not receiving Vitamin D [1].

These conflicting findings suggest that more than one mechanism could account for the age-related changes in $1,25(OH)_2D$, and that the importance of these mechanisms could vary in different populations.

A. Mechanisms for Age-related Changes in $1,25(OH)_2D$

The age-related decline in levels of $1,25(OH)_2D$ could be due to a change in the level of vitamin D–binding protein (DBP), 25OHD substrate deficiency, estrogen deficiency, decreased renal 1α-hydroxylase activity, or increased metabolic clearance of $1,25(OH)_2D$. These possible mechanisms will be considered in turn.

1. Changes in Vitamin D–binding Protein

Interpretation of $1,25(OH)_2D$ levels is complicated by the effect of vitamin D–binding protein (DBP), which may also modulate its action. Less than 1% of circulating $1,25(OH)_2D$ is free, and the non DBP-bound fraction may correspond better to its biological action under certain circumstances [42]. Changes in vitamin D–binding protein (DBP) can cause profound changes in plasma $1,25(OH)_2D$ levels, but recent experiments in DBP knockout mice have questioned whether levels of DBP can affect the action of $1,25(OH)_2D$, even though the levels of total vitamin D metabolites are decreased [43] (see Chapter 9). The increases found in pregnancy appear to represent, at least in part, increases in serum free serum $1,25(OH)_2D$ [44]. Moreover, a change in DBP concentration is unlikely to account for a decrease in $1,25(OH)_2D$ with aging, because DBP concentrations have been reported to show no significant change with age [1,30], or a minimal

decrease [12], or even an increase, and when a free calcitriol index is calculated this clearly decreases with age in postmenopausal women [45].

2. 25OHD Substrate Deficiency

A marked reduction in the level of 25OHD is likely to be a rate-limiting factor for $1,25(OH)_2D$ production [16]. However, in most elderly populations, the level of 25OHD is probably not rate limiting. This is likely to account for the different effects of vitamin D supplementation in the elderly, with increased levels of $1,25(OH)_2D$ reported in some elderly populations [18,46], but not in others [47,48]. Lips *et al.* [18] found that the increase in $1,25(OH)_2D$ after vitamin D supplementation was inversely related to the initial 25OHD concentration. Some studies have shown a decrease in $1,25(OH)_2D$ with aging, even though the level of 25OHD did not decline [5,33].

3. Estrogen Deficiency and $1,25(OH)_2D$

The contribution of the menopause and estrogen deficiency to the age-related decline in $1,25(OH)_2D$ is uncertain. Sowers *et al.* [32] found that the age-related decrease in $1,25(OH)_2D$ was largely accounted for by menopausal status, but other studies have found that the menopause has no effect on calculated free $1,25(OH)_2D$ [49,50]. Similarly, some studies have shown that postmenopausal estrogen administration increases total $1,25(OH)_2D$ [51,52], as well as calculated free $1,25(OH)_2D$ [51] while others have found that estrogen administration results in a transient increase in $1,25(OH)_2D$ and DBP, with no change in calculated free $1,25(OH)_2D$ [53].

4. Renal 1α-hydroxylase Activity

In subjects without marked 25OHD substrate deficiency, the most likely cause for the decrease in plasma $1,25(OH)_2D$ levels with aging is decreased renal 1α-hydroxylase activity. Renal 1α-hydroxylase activity is increased by PTH, growth hormone, hypocalcemia and hypophosphatemia, and decreased by $1,25(OH)_2D$ [54–56]. lα-hydroxylase activity is also affected by the amount of functioning renal tissue [57]. In early chronic renal failure, the glomerular filtration rate (*GFR*) determined by radioisotopic DTPA (diethylene triamine penta-acetic acid) clearance is positively correlated with $1,25(OH)_2D$ [57], and these changes are first detectable when renal function is only slightly impaired (GFR < 70 ml/min).

There are at least three possible reasons for reduced renal lα-hydroxylase activity with aging. First, aging is associated with a decline in renal mass, and an association between impaired renal function and $1,25(OH)_2D$ levels with normal aging have been reported in some studies [33,58], but not in others [37,59,60].

Secondly, the rise in $1,25(OH)_2D$ in response to PTH infusion is blunted with aging [13,33,61]. This refractoriness to PTH with aging does not appear to be due to estrogen deprivation [50,52]. Studies of $1,25(OH)_2D$ production in renal slices from rats of different ages have indicated that the refractoriness to PTH may be a specific defect, and that the $1,25(OH)_2D$ response to calcitonin is preserved [62].

Finally, growth hormone stimulates renal $1,25(OH)_2D$ production [56,63,64], and growth hormone secretion decreases with age [65,66]. In one cross-sectional study [12], serum IGF-I was found to be the most important determinant of the fall in $1,25(OH)_2D$ with age. Although lα-hydroxylase activity is decreased by rising phosphate concentrations [54], this is not likely to account for an age-related decline in $1,25(OH)_2D$ because serum phosphate tends to decline with age [30,37,67–69].

5. Clearance of $1,25(OH)_2D$

Studies in rats indicate that the age-related decrease in $1,25(OH)_2D$ may be due to increased metabolic clearance of $1,25(OH)_2D$ [70,71]. However, Eastell *et al.* found that the clearance of infused $[^3H]1,25(OH)_2D$ tended to decrease with age in healthy women (age range 26 to 88 years), and that this was associated with an age-related increase in serum levels of $1,25(OH)_2D$ [40].

In another study, Halloran *et al.* found that clearance of $1,25(OH)_2D$ did not vary with age in men, but in this study subjects were selected so as not to show the usual age-related decrease in creatinine clearance [72].

IV. THE EFFECT OF AGE ON CALCIUM INTAKE AND ABSORPTION

Net calcium absorption is determined both by dietary calcium intake and the efficiency of calcium absorption. Cross-sectional studies in the United States have shown that calcium intake declines by about 10% in men and women between 35 and 75 years of age [73]. This decrease is due to a reduction in overall caloric intake as the calcium density of the diet does not decline with age [73].

Calcium is absorbed from the intestine by an active $1,25(OH)_2D$ dependent process, and by passive vitamin D–independent mechanisms [74]. The vitamin D–dependent mechanisms are saturated at low intake, and differences in fractional calcium absorption due to variations in the level of $1,25(OH)_2D$ may only be evident when calcium intake is low [74].

Several studies have shown that the efficiency of intestinal calcium absorption decreases with age. This decrease is found by calcium balance studies [75,76],

by jejunal perfusion studies [77], and by radiocalcium absorption tests [5,75,78–80]. Nevertheless, there is some evidence that the actual amount of calcium absorbed from the habitual diet might not vary markedly with age. Eastell *et al.* [40] found that when true fractional calcium absorption (TFCA) was measured by tracing the habitual diet over 24 hours, true calcium absorption (TFCA multiplied by dietary calcium) did not decline with age. Ebeling *et al.* [36] reported similar findings using the same technique employing stable isotopes of calcium. In a longitudinal study, Heaney *et al.* [75] measured fractional calcium absorption by balance and double-tracer methods from a diet constructed to match each subject's current dietary calcium intake. Although fractional calcium absorption clearly decreased in women who went through the menopause between their first and second study, fractional absorption decreased only slightly (about 0.2% per year) in women who experienced no change in estrogen status. Furthermore, in this study, calcium intake increased slightly with aging, and therefore the decline in net calcium absorption is likely to have been minimal.

These differences may relate to methodological factors. The interpretation and relevance of these tests depend on the test calcium load and the length of time over which measurements are made. Calcium absorption tests that use small fixed amounts of calcium carrier measure predominantly active calcium absorption. It is likely that active, but not passive calcium absorption, decreases with age, and in general the lower the amount of fixed calcium carrier, the greater the apparent age-related decrease is in calcium absorption. Using the small intestine perfusion technique, Ireland and Fordtran [77] found that calcium absorption in elderly subjects on a high calcium diet (2000 mg/day) was not different from that in younger subjects, but it was lower in elderly subjects when both groups were studied on a low calcium diet (300 mg/day). Restriction of measurements to the early time period after radiotracer ingestion (<6 hours) may also overestimate the effect of age on calcium absorption. At this time, part of the ingested dose is still in the large intestine, and isotopic equilibrium is not achieved until about 24 hours [81]. Colonic absorption probably accounts for about 5% calcium absorption in healthy subjects, and this proportion is likely to increase in subjects in whom absorptive efficiency is low [81]. Calcium absorption tests which limit blood sampling to within one hour of tracer ingestion [34,82] may also overestimate the effect of aging because gastric emptying may be considerably delayed in the elderly [83]. Calcium absorption tests in which calcium tracer is administered without food are also likely to exaggerate the effect of age on calcium absorption. Gastric acidity is markedly reduced in a substantial proportion of elderly subjects [84]. In subjects with achlorhydria Recker *et al.* found that calcium absorption was very low when administered while fasting, but was normal in the presence of food [85].

In summary, there is good evidence to suggest that calcium absorption is somewhat less efficient in the elderly. Elderly subjects show poor adaptation to variations in calcium intake and calcium balance in the elderly is likely to be more sensitive to calcium intake than in young adults. However, the apparent effect of age may be exaggerated when samples are collected in the early time-period after tracer ingestion, when the carrier dose is low, and when calcium is administered without food.

A. Mechanisms for Decreased Calcium Absorption with Aging

1. Decreased $1,25(OH)_2D$

When subjects with widely varying levels of $1,25(OH)_2D$ are studied, calcium absorption and $1,25(OH)_2D$ are highly correlated. This correlation is greatest when subjects are fed low calcium meals [74]. However, in population studies, a decrease in the level of $1,25(OH)_2D$ with age is not a universal finding (see earlier), and several studies have not found a relationship between calcium absorption and $1,25(OH)_2D$ [34,40,86], unless they have included a wide range of vitamin D status (see below).

2. Intestinal $1,25(OH)_2D$ Resistance

Another factor that could be related to a decrease in calcium absorption with age is a decrease in intestinal responsiveness to $1,25(OH)_2D$. In rat studies, calcium uptake by isolated duodenal cells, the number of intestinal receptors for $1,25(OH)_2D$, the calbindin response to $1,25(OH)_2D$, and the effect of $1,25(OH)_2D$ therapy on calcium absorption have been reported to decline with age, but there is disagreement about the relative importance of low serum $1,25(OH)_2D$, and $1,25(OH)_2D$ resistance in this model [87–90].

In human studies, there are three main lines of evidence supporting a possible role of intestinal $1,25(OH)_2D$ resistance. First, some studies have shown an age-related increase in the level of $1,25(OH)_2D$ in association with unchanged calcium absorption [34,91].

Second, direct measurements of tissue $1,25(OH)_2D$ receptor (VDR) content in the duodenal mucosa have shown an age-related decrease of about 30% between age 20 and age 80 [34], although this has not been found by all investigators [92].

Third, the positive relationship between fractional calcium absorption and an index of free $1,25(OH)_2D$

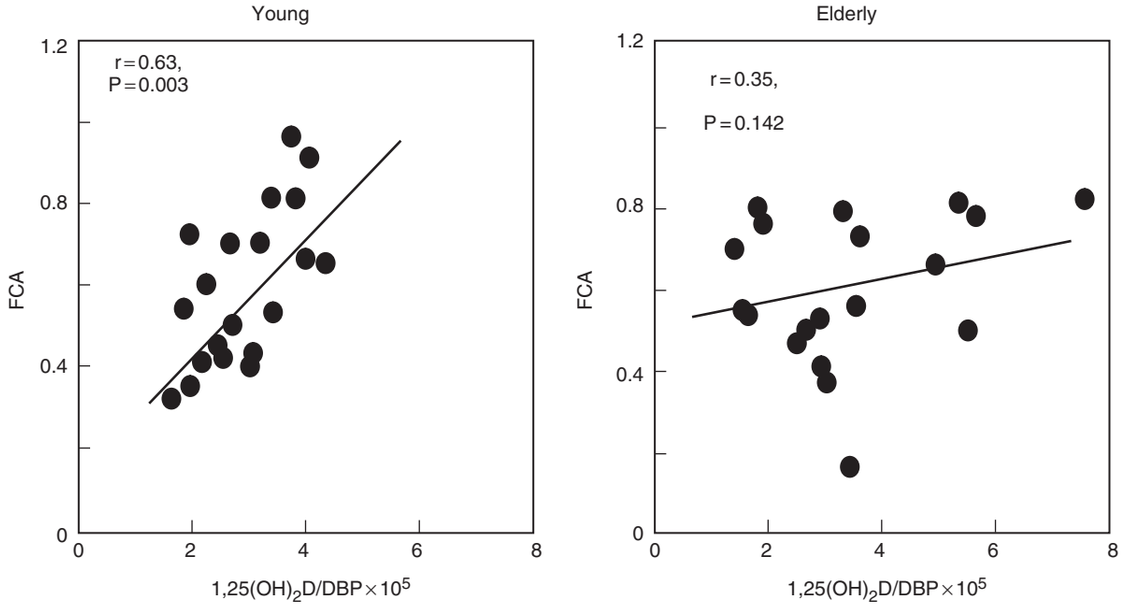

FIGURE 2 Relationship between fractional calcium absorption (assessed by double isotope method with 100 mg calcium carrier) and the serum free $1,25(OH)_2D$ index ($1,25(OH)_2D$/Vitamin D binding protein) in 20 young adult women and in 19 elderly normal women. Experimental protocol induced levels of serum free $1,25(OH)_2D$ index that ranged from below to above normal. Note that the significant correlations present in the young adult women were lost in the elderly women. From Pattanaungkul *et al.* [39], with permission.

levels (total $1,25(OH)_2D$/DBP) found in young women is not found in the elderly [39,93]. This was studied over a wide range of vitamin D levels, induced by altering the dietary calcium or by administering oral $1,25(OH)_2D$ (Fig. 2).

However, the response to an increase in endogenous $1,25(OH)_2D$ induced by a low calcium diet was not affected by age [36]. This study measured the important variable, true fractional calcium absorption, however, this measures both active and passive absorption. It is possible that had a lower calcium carrier been used, so as to trace active transport, that a blunted response to $1,25(OH)_2D$ might have been observed.

An important recent observation is that common allelic variants in the gene encoding the vitamin D receptor might predict up to 75% of the genetic effect on bone mineral density in healthy adults, although this effect is likely to be much smaller as evaluated by a meta-analysis [94] (see Chapter 68). Women with the low bone mineral density genotype (BB) also appear to have higher bone turnover than women with the BB genotype. These differences could relate to decreased VDR expression in the BB genotype, with consequent effects on intestinal calcium absorption, PTH secretion, and bone mineralization. However, no changes in VDR expression have been found with the common VDR genotype variations studied so far.

3. Estrogen Deficiency and Calcium Absorption

The menopause is likely to account for a substantial proportion of the age-related decrease in calcium absorption in women. In a longitudinal study, Heaney *et al.* [75] found that the age-related decline in fractional absorption was considerably larger in women who went through the menopause between studies, compared to subjects who had no change in estrogen status. Estrogen administration increases intestinal calcium absorption [95] in postmenopausal osteoporotic women and maintains normal calcium absorption and responsiveness to $1,25(OH)_2D$ in premenopausal women undergoing elective ovariectomy [96]. Also, estrogen may have a direct effect on uptake of calcium by duodenal cells [97].

4. Calcium Absorption and the Aging Stomach

Atrophic gastritis and reduced gastric acid production is common among the elderly, with an estimated prevalence of 20 to 50% in the sixth and seventh decades of life [84]. Calcium bioavailability is probably not affected by high gastric pH *per se*, although this is controversial [85,98–100]. Although atrophic

gastritis in the elderly is rarely severe enough to cause pernicious anemia, patients with pernicious anemia do appear to have reduced bone mineral density [101] and are at increased risk of osteoporotic fracture [102], even though calcium absorption from food is usually unimpaired [85,101]. It has been suggested that bone loss in pernicious anemia could be due to decreased secretion of a bone-stimulating factor produced by the gastric mucosa [102]. These findings raise the intriguing possibility that changes in the aging stomach could be associated with bone loss independently of any effect on calcium absorption. Partial gastrectomy is also associated with bone mineral loss, and it has been suggested that this could be related to reduced postprandial calcitonin secretion [103].

B. Which Form of Vitamin D Is the Major Determinant of Calcium Absorption?

The most potent natural metabolite of vitamin D is $1,25(OH)_2D$, and its potency ratio to 25OHD is approximately 2000:1. However, the circulating level of 25OHD is almost 1000 times higher than $1,25(OH)_2D$. It has been calculated by Barger-Lux *et al.* [104] that 25OHD contributes about 25% of the circulating vitamin D activity. Colodro *et al.* [105] tested the effect on calcium absorption in renal failure of different metabolites of vitamin D and calculated that the potency ratio in humans may be closer to 400:1. This would indicate that 25OHD contributes up to 90% of the circulating vitamin D activity. This would explain why several groups have found stronger relationships between 25OHD and calcium absorption fraction than between $1,25(OH)_2D$ and calcium absorption fraction [104,106]. However, these studies were done before it was recognized that 1α-hydroxylase is present in many target tissues, including intestine, and that local conversion of 25OHD to $1,25(OH)_2D$ may confound these calculations.

V. AGE AND PARATHYROID HORMONE

A. Effect of Age on Parathyroid Function

The principal immunoreactive form of PTH in the plasma is heterogeneous C-terminal fragments, especially when renal function is impaired. Several cross-sectional studies using fragment-detecting assays showed increases in the concentration of PTH with age [11,12,30,68,107–112]. These studies are difficult to interpret, because renal function declines with age, resulting in increased retention of C-terminal fragments. However, many more recent studies have confirmed an age-related increase in intact PTH in women, which is probably progressive after the menopause [12,37,82,91,111,113–117], although a few studies have not found this to be the case [118,119]. Serum intact PTH also increases with age in men [120,121].

In concurrent studies, the age-related increase in carboxyl-terminal PTH is about fourfold higher than the increase in intact PTH [12]. The effects of age on the level of intact PTH are modest, with average reported increases of about 10% per decade after the menopause. The rise in intact PTH is probably similar in men [37].

Bioactive PTH measured using an adenylate cyclase bioassay also appears to increase with age [122]. Moreover, there is evidence of increased PTH activity with age, based on excretion of cyclic AMP [11,109–111], and a decrease in the theoretical renal phosphate threshold, TmP/GFR [107,109,111], between the second and the ninth decade of life. This decrease in TmP/GFR is the likely cause of the decreased serum phosphorus discussed previously.

It may be important to consider the pattern of PTH release when considering the effect of age on PTH secretion. PTH secretion shows a marked circadian rhythm, and the form of this rhythm may alter with aging [123].

Pulsatility of PTH secretion has also been shown in several studies using frequent sampling techniques [124–126], and it has been postulated that the pattern of pulsatility may govern the biological action of PTH. The pulse amplitude of PTH secretion may be markedly reduced in osteoporosis [124]. The effect of aging on the pattern of PTH release has not been studied, but this is clearly of some interest.

B. Mechanisms for Increased Intact PTH with Aging

Theoretically, increased PTH levels could be due to hypocalcemia, a greater parathyroid cell mass (parathyroid hyperplasia), a shift in the set-point of calcium stimulated PTH secretion, or decreased PTH clearance. $1,25(OH)_2D$ can alter PTH secretion by altering the set-point [127–130], as well as the maximum secretory capacity [131].

It is possible that subtle hypocalcemia is an important cause of age-related changes in PTH secretion. Total and ionized calcium in serum decrease slightly with age in most studies (see earlier). Dawson-Hughes *et al.* showed that healthy postmenopausal women with levels of serum ionized calcium in the lowest quintile had significantly higher levels of intact PTH than subjects with ionized calcium in the highest quintile [132].

A similar inverse relationship between ionized calcium and intact PTH has been shown by other investigators [12,133]. In rats, however, the age-related increase in PTH does not appear to be related to low plasma calcium [71,134].

Aging could also be associated with an increase in the set-point for suppression of PTH secretion by calcium (implying higher PTH secretion at any given concentration of ionized calcium). A shift in set-point could be a primary effect of aging on the sensitivity of the parathyroid gland to calcium, or could be secondary to other factors, such as low $1,25(OH)_2D$ or decreased parathyroid responsiveness to $1,25(OH)_2D$. However, Landin-Wilhelmsen *et al.* [7] reported a positive correlation between $1,25(OH)_2D$ and PTH, and this would argue against a decrease in $1,25(OH)_2D$ as a cause of the increase in PTH. They did report a negative correlation between PTH and 25OHD (as did Hegarty *et al.* [135]), and this again raises the question of the relative contribution of 25OHD to the biologicial activity of vitamin D (see above). Studies in rats have shown that PTH secretion is higher at any given level of calcium in aged animals *in vivo* [136,137] and *in vitro* [138].

However, in elderly human subjects, the set-point does not appear to change with aging, but basal secretion and maximum secretory capacity are increased [131]. This suggests that the rise in PTH with aging is due to parathyroid hyperplasia, possibly secondary to chronic hypocalcemia, rather than an altered set-point. Furthermore, in this study the magnitude of the decrease in basal and maximal PTH secretion after $1,25(OH)_2D$ therapy was similar in young and elderly subjects, suggesting that parathyroid responsiveness to $1,25(OH)_2D$ is preserved in the elderly. A number of studies have shown a significant inverse relationship between levels of intact PTH and $1,25(OH)_2D$ in elderly subjects [12,48], but this may be secondary to impaired calcium absorption rather than an effect of low $1,25(OH)_2D$ itself. Treatment of elderly subjects with vitamin D results in a reduction in the level of PTH [18,139,140].

A possible relationship between declining renal function and increase in intact PTH has been explored in several studies. Some studies have shown that the level of intact PTH is independently related to declining renal function [60,116]. In contrast, other studies in rats [71,116,134] and humans [12,59] have shown that declining renal function is not independently associated with the age-related increase in intact PTH.

There is no evidence for a change in the clearance of intact PTH with age in man, and studies in rats have suggest that a decline in PTH clearance cannot account for high levels of PTH with advancing age [134].

VI. INTERRELATIONSHIPS BETWEEN AGE-RELATED BONE LOSS, ESTROGEN, PTH, AND VITAMIN D METABOLISM

The age-related increase in PTH may play a pivotal role in age-related bone loss [141]. In this model, decreased net intestinal calcium absorption (resulting from dietary calcium deficiency and reduced levels of $1,25(OH)_2D$) results in mild hypocalcemia and secondary hyperparathyroidism. PTH excess results in increased bone resorption and net bone loss (see Figure 3).

Although it is clear that PTH and vitamin D metabolism alter with aging, the relationship between these changes and bone loss is not certain. Inappropriately elevated PTH in primary hyperparathyroidism is associated with reduced bone density [142,143] but it is uncertain whether mild asymptomatic hyperparathyroidism is associated with an increased risk of fracture or progressive bone loss [144–146].

There are conflicting data on whether the age-related increase in PTH or decrease in 25OHD and $1,25(OH)_2D$ are causally related to the age-related increases in bone turnover and decreases in BMD. Population-based studies from Rochester, MN have shown that serum PTH is positively correlated with bone turnover markers and negatively correlated with BMD at several sites [120,147]. Moreover, when young adult premenopausal women and elderly postmenopausal women were compared, equalization of serum PTH levels by calcium infusion lead to an equalization of bone turnover markers, suggesting a causal relationship [148]. Others have shown a relationship between BMD and the level of $1,25(OH)_2D$ [32,149], PTH and 25OHD [149]. Ooms *et al.* [17] reported that 25OHD levels did correlate with hip BMD below the threshold of 30 nmol/l [17]. In one study, PTH did relate to rates of bone loss from the forearm in premenopausal women [150]. Others have failed to find significant correlations between serum PTH or vitamin D metabolites and age-related changes in bone turnover [117], bone mineral density [13,37,59,114], or bone loss [151]. These differences may be explained in part by the use of samples that were not population-based, the selection of healthy volunteers, or the lack of statistical power.

In contrast to the above findings, some studies have shown a relationship between bone mineral density and the level of $1,25(OH)_2D$. It is likely that differences in net calcium absorption account for only a small proportion (possibly about 10%) of the variance in bone density in the elderly population [152].

Pharmacological doses of calcium may reduce the rate of bone loss in elderly subjects [153,154], especially in subjects with a low habitual calcium intake. However, most of the variance in the rate of bone loss is likely to be independent of intestinal calcium supply. Orwoll *et al.* found that healthy men (30 to 87 years old) show substantial bone loss in the radius (1.0%/year) and spine (2.3%/year), which is not altered by calcium supplementation (1000 mg/day) or vitamin D (25 mg/day) [155].

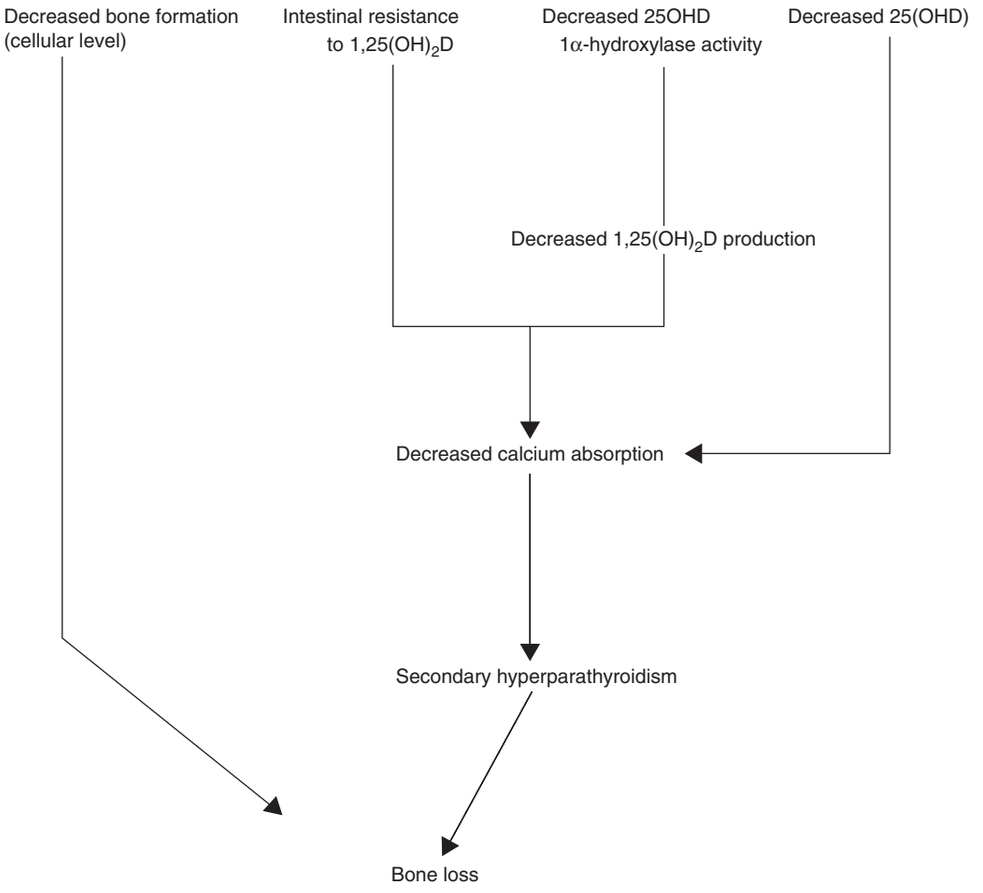

FIGURE 3 A model for the proposed changes in calcium homeostasis and bone turnover with age.

Devine *et al.* reported that calcium absorption measured using the single-tracer stable strontium method did not correlate with bone density in women more than 10 years past the menopause [86].

Riggs *et al.* [156,157] have proposed that the increase in PTH with aging may be a consequence of estrogen deficiency. The strongest evidence for this is the effect of estrogen on PTH and bone resorption in older women—both are decreased [158] (Table I). This could be explained by an effect of estrogen on the intestine and kidney. The intestine has been shown to contain functional estrogen receptors [159] and estrogen increases intestinal calcium absorption both in rodents [159] and in humans [96,160]. This effect is a consequence of upregulation of the epithelial calcium channel (CaT1) in the duodenum, and the mechanism is independent of vitamin D [161].

In women who had been ovariectomized six months earlier, the responsiveness of calcium absorption to short-term treatment with $1,25(OH)_2D$ was blunted, and this was restored by estrogen replacement [96].

TABLE I Comparison of 30 Young Premenopausal Women (Pre M), 30 Untreated Postmenopausal Women (Post M), and 30 (Post M) Women Receiving Long-Term Estrogen Therapy (after McKane *et al.* [158])

Variables	Pre M	Post M	Post M + estrogen
Number	30	30	30
Age, years	32	74	74
Estrogen status	Replete	Untreated	ERT
Serum PTH, pmol/L	2.7	3.6**	2.5
Urinary NTX, nmol/mmol creatinine	29	43***	25

**, $p<0.01$, ***, $p<0.001$, comparison of Pre M and Post M. There were no differences between Pre M and Post M + estrogen.

ERT, estrogen replacement therapy.

Estrogen treatment in elderly postmenopausal women increases renal tubular reabsorption of calcium [162].

VII. SUMMARY OF CHANGES IN VITAMIN D WITH AGING

Thus, with age there are a number of changes in vitamin D and its actions.

1. There is a decrease in 25OHD that probably results from decreased UV light exposure, decreased effect of UV light on skin synthesis of vitamin D, and decreased absorption of vitamin D from the diet.
2. There is a decrease in $1,25(OH)_2D$ after the age of 65 years that is partly a result of the decrease in the substrate, 25OHD, and partly a result of the decrease of renal lα-hydroxylase activity.
3. There is a decrease in active calcium absorption that results from the decrease in vitamin D metabolites (25OHD and $1,25(OH)_2D$), from intestinal resistance to the action of vitamin D, and to estrogen deficiency.
4. There is an increase in PTH secretion.

There are further changes in patients with osteoporosis-related fractures, and these indicate different pathogenetic factors for these fracture syndromes, supporting the concept of type I and type II osteoporosis. In type I osteoporosis, the major fractures are in the forearm and vertebra, and postmenopausal women under the age of 75 years are affected. In type II osteoporosis the major fractures are of the proximal forearm and vertebra, and both men and women over the age of 70 years are affected.

1. In type I osteoporosis, there is a decrease in both calcium absorption and $1,25(OH)_2D$, but the PTH levels are lower than expected, indicating that the primary defect is likely to be an increase in net bone resorption (see Fig. 4).
2. In type II osteoporosis, there is an increase in PTH and a decrease in 25OHD that is greater than these changes that occur in all subjects with aging. This supports the importance of borderline vitamin D deficiency as an important cause of hip fracture.

VIII. TREATMENT OF ESTABLISHED OSTEOPOROSIS WITH VITAMIN D

A. Rationale and General Principles

A distinction must be made between the use of physiological replacement dosages of vitamin D to treat nutritional deficiency and the use of therapeutic dosages of vitamin D or natural vitamin D metabolites to treat osteoporosis when vitamin D stores are normal. Physiologic replacement of vitamin D can be achieved with small dosages of 1000 IU per day or less. Because vitamin D plays a key role in the regulation of calcium metabolism and in the maintenance of bone mass,

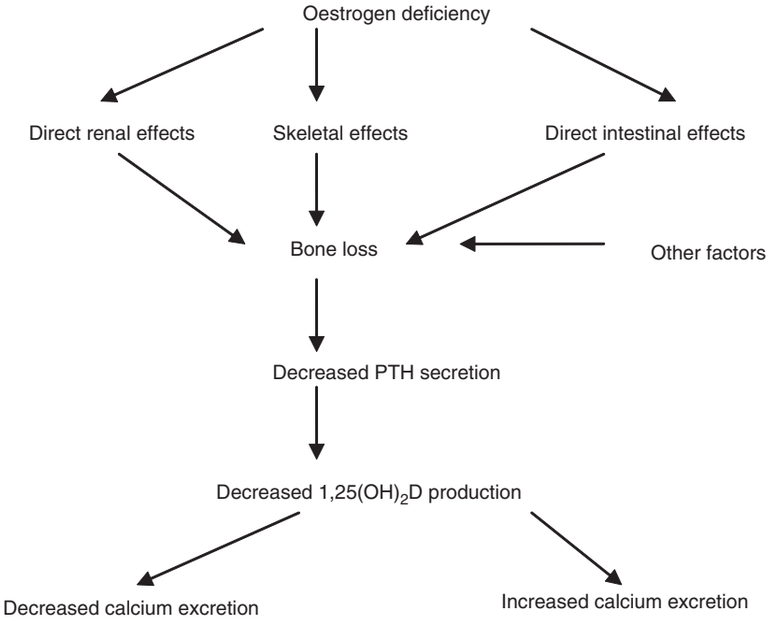

FIGURE 4 A model for the proposed changes in calcium homeostasis and bone turnover in type I osteoporosis

deficiency states should always be searched for and, when present, corrected with relatively small dosages of vitamin D.

As reviewed earlier in this chapter, there is substantial evidence that many patients with osteoporosis have an impairment in the metabolism of vitamin D to its physiologically active metabolite, $1,25(OH)_2D$, which contributes to their negative calcium metabolism and bone loss. This impairment can be overcome by using large dosages of vitamin D (10,000 IU to 25,000 IU per day) or by small dosages of $1,25(OH)_2D_3$. Both vitamin D and its main circulating metabolite, 25OHD are stored in muscle and fat and over time large amounts can be retained. Because of these large stores, severe hypercalcemia and hypercalciuria due to vitamin D intoxication can occur relatively suddenly when pharmacologic dosages of vitamin D are used long-term and, when it does occur, it may last for weeks or even months. In contrast, $1,25(OH)_2D$ does not have significant long-term storage and if hypercalcemia or hypercalciuria occur, they rapidly resolve over a few days. Because of this and because physiological or near physiological dosages are effective in increasing calcium absorption [163], $1,25(OH)_2D_3$ is preferred over pharmacologic dosages of vitamin D in the treatment of osteoporosis.

Calcium absorption is impaired in both postmenopausal and age-related osteoporosis, and it can be corrected by therapy with $1,25(OH)_2D_3$. If increasing calcium absorption were the only effect of $1,25(OH)_2D_3$ in the treatment of osteoporosis, large dosages of oral calcium might effectively substitute for it. However, as reviewed elsewhere in this book (Section III), $1,25(OH)_2D_3$ also enhances renal calcium conservation, increases the differentiation of bone cells, acts directly on muscle, and has many other beneficial effects.

Finally, from a therapeutic standpoint, it should be recognized that the effects of $1,25(OH)_2D_3$ on calcium metabolism and bone turnover are triphasic. In dosages below 0.5 μg per day, the drug fails to increase calcium absorption in many subjects [163], and thus, these dosages should be considered to be incompletely effective. In dosages above 0.75 μg per day, some subjects will have increases in urine and serum calcium levels, particularly if the intake of calcium is not restricted, and there also may be evidence of increased bone resorption mediated by stimulation of osteoclasts by $1,25(OH)_2D_3$. In dosages above 1.0 μg per day, these adverse effects are much more common. The most favorable results from $1,25(OH)_2D_3$ treatment of established osteoporosis have been achieved using dosages in the intermediate range of 0.5 μg per day to 0.75 μg per day. Thus, the optimal therapeutic range appears to be quite narrow and may vary among patients. This restricted therapeutic range constitutes one of the major limitations in the use of this drug for treatment. Differences in dosages employed in different clinical trials may explain some of the inconsistent and conflicting therapeutic results reported in the scientific literature.

1. CORRECTION OF NUTRITIONAL VITAMIN D DEFICIENCY

Although vitamin D deficiency classically is associated with osteomalacia, milder deficiency states can lead to osteoporosis [164]. As reviewed elsewhere in this book (Chapters 61 and 62), states of mild vitamin D deficiency may be more common than has been believed previously, particularly among the elderly who often have poorer diets and have decreased solar exposure. This is particularly likely to occur in those counties that do not supplement dairy products with vitamin D, in countries with more northerly latitudes, and during the winter months.

There is considerable uncertainty about the level of 25OHD that merits vitamin D replacement. Malabanan *et al.* [165] treated 35 patients for 8 weeks with 50,000 IU vitamin D orally per week. They stratified the baseline 25OHD to find out if there is some threshold above which there is no benefit, as assessed by suppression of PTH. They identified 50 nmol/L as such a threshold. Jesudason *et al.* [166] identified a similar threshold (60 nmol/L) by examining the level of 25OHD below which bone turnover markers (and PTH) increased in older women attending a metabolic bone center. Supplementation with vitamin D resulted in increases in calcium absorption up to a level of 25OHD of 80 nmol/L [167]. Thus, the threshold of 30 nmol/L mentioned earlier in the chapter may need to be revised upwards [15].

Chapuy *et al.* [140] randomly treated 3,270 elderly French women who were confined to either nursing homes or apartments for the elderly with either 800 IU of vitamin D and 500 mg of elemental calcium per day or double placebo. After 18 months, the numbers of hip fractures were 43 percent lower and nonvertebral fractures were 32% lower and serum parathyroid hormone was 44% lower, all statistically significant changes (see Chapter 66). Heinkinheimo *et al.* [168] treated or followed without treatment a group of 621 elderly (>85 yrs of age), free living or institutionalized men and women with annual intramuscular injections of ergocalciferol over a four-year period. The treatment group had a slightly, but significantly lower (16%) fracture rate than did the control group (22%). Ooms *et al.* [169] treated 348 elderly Dutch women with 400 IU per day of vitamin D or placebo and found that there were small, but significant decreases in serum PTH and increases in femoral neck density. Lips *et al.* [170] treated 2,578 elderly (>80 yrs of age) women and men

who were living independently or in nursing homes with 400 IU of placebo. Over 3.5 years of observation, there were no differences in the number of hip or total fractures. The major difference between this study and that of the French study was that the subjects in Chapuy's study had lower levels of serum 25(OH)D and received a higher dosage of vitamin D and a calcium supplement. Trivedi *et al.* [171] recently reported that oral vitamin D given 100,000 IU orally every four months to men and women over 65 years for 5 years reduced the risk of fractures.

Dawson-Hughes *et al.* [172] treated men and women over the age of 65 years with calcium (500 mg/day) and vitamin D (700 IU/day) for 3 years. There was prevention of bone loss and a reduction in the risk of nonvertebral fractures.

From these and other studies, it appears that many elderly persons have varying degrees of vitamin D deficiency and that correction of this with small replacement dosages is beneficial. Lips *et al.*[173] found that an average of 300 IU per day of vitamin D was sufficient to correct vitamin D deficiency in elderly women. However, because of evidence that the elderly may absorb vitamin D less well than the young and because low dosages are safe and inexpensive, it would seem prudent to ensure that elderly individuals take 800 IU per day of vitamin D and have a calcium intake of 1,000 mg per day or more.

2. TREATMENT WITH $1,25(OH)_2D_3$

Despite more than 15 years of clinical trials, $1,25(OH)_2D_3$ still has not been approved for the treatment of established osteoporosis in the United States. It is approved, however, in the United Kingdom, Australia, Italy, Japan, New Zealand, as well as in 16 other countries. However, $1,25(OH)_2D_3$ has been approved in most countries for treatment of hypocalcemia and renal osteodystrophy, and thus is available to physicians for "off label" usage.

B. Efficacy in Postmenopausal Osteoporosis

Only randomized, prospective clinical trials with objective endpoints will be reviewed here. The earliest evaluation of $1,25(OH)_2D_3$ in postmenopausal osteoporotic women was reported by Gallagher *et al.* [174] who compared the effect of six months treatment with 0.5 μg per day of $1,25(OH)_2D_3$ or placebo. As compared with either baseline measurements or with changes in the placebo group, they found that $1,25(OH)_2D_3$ significantly increased calcium absorption, decreased bone resorption rate, and improved calcium balance. The most systematic study reported thus far was a large three-center study comparing the effects of $1,25(OH)_2D_3$ on the rate of change in bone density in postmenopausal osteoporotic women. All patients had their calcium intake adjusted to 1000 mg per day. Unfortunately, the protocol required that the dosage of $1,25(OH)_2D_3$ be increased until toxicity occurred and then reduced to a dosage that did not produce hypercalcemia or hypercalciuria. In the centers of Aloia *et al.* [175] and Gallagher *et al.* [176], there were significant increases in bone density, whereas in the center of Ott and Chesnut [177], there were no significant differences from the placebo group. However, Ott and Chesnut enrolled patients with milder disease (the mean number of vertebral fractures in their study was 1.2 versus 2.9 and 4.1 in the other two studies), and they had reduced the mean daily dosage of $1,25(OH)_2D_3$ to a much lower dosage (0.43 μg versus 0.80 μg and 0.67 μg in the other two centers). Subsequently, Ott and Chesnut [177] reanalyzed their data and found that those subjects who received 0.5 μg per day or more responded by increases in bone density, whereas those with lower dosages did not.

Orimo *et al.* [178] found in 80 postmenopausal osteoporotic Japanese women that one year of treatment with 1α-hydroxyvitamin D [1α(OH)D] increased bone density by 1.8% at the lumbar spine and by 4.6% at the femoral trochanter as compared with the placebo group, whereas there was no significant change at the femoral neck. Christiansen *et al.* [179] failed to demonstrate a significant effect of a small dosage of 0.25 μg per day of $1,25(OH)_2D_3$ on retarding bone loss in perimenopausal normal women. However, Need *et al.* [180] found that treatment of postmenopausal osteoporosis for 15 months with a dosage of 0.25 μg per day combined with a calcium supplement of 1000 mg per day reduced urine hydroxyproline excretion and increased bone density as compared with a control group.

Some studies have reported that $1,25(OH)_2D_3$ treatment decreases the occurrence of vertebral fractures. In a two-center double-blind study over three years, $1,25(OH)_2D_3$ resulted in a 65% reduction in vertebral fractures, although only the first year was placebo-controlled [181]. Tilyard *et al.* [182] compared the effect of $1,25(OH)_2D_3$ treatment in postmenopausal osteoporotic women with a control group receiving only calcium supplementation. At the end of three years, they found a significant 63 percent decrease in vertebral fractures compared to the control group. However, the data were only significant when the results of the first year and subjects having more than five fractures were excluded. Also, inexplicably, the vertebral fracture rate increased in the group receiving calcium supplementation. Finally, Orimo *et al.* [178] found that one year's treatment of 80 Japanese women

with $1\alpha(OH)D_3$ reduced significantly the vertebral fracture rate by 83% as compared with placebo.

Thus far, there has been no controlled study of the effect of $1,25(OH)_2D_3$ in the treatment of elderly women with age-related (type II) osteoporosis.

Gallagher *et al.* [160] recently have reported the results of a three-year double-blind prospective controlled trial in 489 elderly women randomized in a factorial design to treatment with placebo, $1,25(OH)_2D_3$ (0.5 μg/day), estrogen replacement therapy (ERT) or ERT, plus $1,25(OH)_2D_3$. In compliant subjects, they found that the $1,25(OH)_2D_3$ treatment group had a greater increase in BMD (~2%, $P<0.005$) at various scanning sites than the placebo group, although the group receiving ERT had a substantially greater increase in BMD. However, the group receiving both $1,25(OH)_2D_3$ and ERT had the greatest increase in BMD. Interestingly, the two groups receiving $1,25(OH)_2D_3$ had 50% fewer fractures than the two groups who did not receive $1,25(OH)_2D_3$. Because of the factorial nature of the design, the relative effects of $1,25(OH)_2D_3$ and estrogen on BMD could be calculated. Using this approach, the estimated proportion of the bone effect of estrogen that was mediated by $1,25(OH)_2D_3$ was 34.5% for the mean of four scanning sites, whereas the proportion of the bone effect of $1,25(OH)_2D_3$ that was independent of estrogen was estimated to be 18.3%. Although this study provides useful data, a major caveat is that subjects were excluded who had BMD values below the age-adjusted normal range. In previous studies, we have found that osteopenic and osteoporotic patients have the greatest abnormalities of calcium absorption and vitamin D metabolism [5,183]. Thus, these must be considered to be minimal estimates, and the true impact of primary abnormalities of vitamin D metabolism and action is likely to be larger.

There have been few studies comparing vitamin D preparations with bisphosphonates for the treatment of osteoporosis. Shiraki *et al.* [184] studied 210 patients with osteoporosis and compared alendronate (5 mg/day) with alfacalcidol (1 μg/day) for 48 weeks and found significantly greater increases in BMD with alendronate than with alfacalcidol.

C. Meta-analysis of the Clinical Trials of Vitamin D

Papadimitropoulos *et al.* [185] performed a meta-analysis of standard vitamin D (vitamin D2 and D3, and 25OHD) and "active" vitamin D [$1,25(OH)_2D_3$ and 1-alpha(OH)D] for the period 1966 to 1999 using predefined criteria. They found eight suitable studies for vertebral fracture and six suitable studies for nonvertebral fracture, and the results are shown in Table II. These show a decrease in the risk of vertebral fracture and possibly nonvertebral fracture, although most of the studies of vertebral fractures were with "active" vitamin D, and most of those of nonvertebral fracture were with standard vitamin D. They reported more consistent effects of "active" vitamin D on bone mineral density than vitamin D. However, not all the studies quoted above were captured by this meta-analysis [171] and so we look forward to an update. They found that patients were more likely to discontinue vitamin D than placebo as a result of adverse effects or abnormal laboratory tests (presumably raised serum calcium).

D. Safety

This remains a concern because of the relatively narrow therapeutic range for this drug. With dosages higher than 0.75 μg per day, hypercalciuria and mild hypercalcemia may occur in a minority of patients, particularly in those with unrestricted calcium intake. Thus far, significant deterioration of renal function has not been reported in subjects receiving recommended dosages, and the occurrence of kidney stones is rare. With the recommended dosages of 0.5 to 0.75 μg per day and with calcium intake restricted to 1000 mg per day, the drug appears to be quite safe. Thus, Tilyard [182] found no evidence of hypercalcemia, hypercalciuria, or nephrocalcinosis after 528 patient-years of observation. Nonetheless, monitoring of serum and urine calcium values no less than yearly is recommended.

1. Calcium and Vitamin D as Supplements to Other Treatments

Almost all of the Phase III clinical trials for the treatment of postmenopausal osteoporosis included a calcium supplement and most included vitamin D [186]. However, when these treatments were approved by the regulatory authorities, the only recommendation made was that the diet should contain sufficient calcium and vitamin D. There is thus an important question as to whether the calcium and vitamin D is simply a placebo in these studies, and whether women with osteoporosis commonly attain adequate intakes of calcium and vitamin D without supplementation.

There have been many clinical trials of drugs for postmenopausal osteoporosis. A summary of the major clinical trials is included in Table III. It can be seen that the usual dose of calcium supplementation was between 500 and 1000 mg/day and that of vitamin D between 250 and 600 IU/day. These amounts of vitamin D would

TABLE II Meta-analysis of Fracture Trials with Vitamin D [185]

Reference	Vertebral fracture, relative risk (95% confidence interval)	Nonvertebral fracture, relative risk (95% confidence interval)	n
a. Standard Vitamin D (vitamin D or 25-hydroxyvitamin D)			
Baeksgaard [197]	0.33 (0.01 to 8.05)		180
Chapuy [140]		0.75 (0.61 to 0.91)	3270
Lips [170]		1.04 (0.77 to 1.41)	1916
Dawson-Hughes [172]		0.45 (0.22 to 0.91)	213
Pooled estimate		0.78 (0.55 to 1.09)	5399
b. "Active" Vitamin D ($1,25(OH)_2D_3$ and 1-α-OHD_3)			
Gallagher [198]	0.90 (0.42 to 1.89)		50
Orimo [178]	0.37 (0.09 to 1.44)	1.10 (0.02 to 2.0)	80
Ott [177]	1.4 (0.59 to 3.62)	2.20 (0.52 to 9.24)	86
Tilyard [182]	0.43 (0.31 to 0.61)	0.50 (0.25 to 1.00)	622
Guesens [199]	0.88 (0.43 to 1.80)		32
Orimo [200]	0.46 (0.31 to 0.69)		86
Caniggia [201]	0.20 (0.01 to 3.54)		14
Pooled	0.64 (0.44 to 0.92)	0.87 (0.29 to 2.59)	970

be expected to increase serum 25-hydroxyvitamin D (25-OHD) by between 6 and 15 nmol/L [187].

The recommendation in the Fracture Intervention Trial (FIT) study was that a food frequency questionnaire be used to evaluate dietary calcium [188]. If the dietary calcium was below 1000 mg/day, then the patient should be given 500 mg/day calcium per day. About 82% of women with osteoporosis failed to achieve this level.

In the Vertebral Efficacy with Risedronate Therapy (VERT) studies, the recommendation was to measure 25OHD and if the value was below 40 nmol/L to supplement with vitamin D (up to 500 IU/day). About 36% of patients in the VERT multinational study required supplementation [189]. This threshold is quite low—it is becoming accepted that a threshold of 80 nmol/L is desirable to prevent the biochemical changes of subclinical osteomalacia (high parathyroid hormone and bone turnover markers) [187]. Thus, it is likely that most people receiving treatment for osteoporosis are in need of supplementation with calcium and vitamin D if the conditions under which these trials were conducted are to be reproduced.

E. Rationale for Supplementation

There is a positive calcium balance after starting therapy with antiresorptive therapy. The total body bone mineral content increases by 1% at one year after treatment with alendronate plus calcium (500 mg/day) [190].

TABLE III The Level of Calcium and Vitamin D Supplementation in Several Large Clinical Trials of Postmenopausal Osteoporosis

Name of trial	Antiresorptive Drug	Calcium, mg/day	Vitamin D, IU/day
FIT 1 and 2 [188,202]	Alendronate	500*	250
VERT MN and NA [189,203]	Risedronate	1000	Up to 500**
PROOF [204]	Calcitonin	1000	400
MORE [205]	Raloxifene	500	Up to 600

*If dietary calcium less than 1000 mg/day (in 82% of subjects in FIT 1)
** If 25OHD less than 40 nmol/L (in 36% of subjects in VERT-MN)

This is equivalent to a positive balance of about 20 mg/day. In women given 500 mg/day of calcium alone, there was 0.5% decrease in total body mineral content, and this would be equivalent to a negative calcium balance of about 10 mg/day, so the extra calcium requirement resulting from alendronate 10 mg/day administration would be 30 mg/day. This could be met by an adaptation of the body by an increase in the fraction of dietary calcium absorbed [191]. However, this mechanism is less effective in the elderly. It could be met by the calcium supplement—if about 10% were absorbed, then an extra 50 mg/day of calcium would be available to support the positive calcium balance.

Vitamin D deficiency prevents the antiresorptive effect of bisphosphonates and impairs muscle strength. Koster *et al.* [192] recruited 28 osteopenic patients with vitamin D deficiency (25OHD < 40 nmol/L) and compared their BMD response to one year of cyclical etidronate with a control group with normal 25OHD. The control group showed an increase in BMD of 2 to 6% in response to cyclic etidronate, depending on the site of measurement. The vitamin D–deficient group had little or no improvement in the BMD, and further deterioration of the vitamin D deficiency, as indicated by a decrease in serum calcium and an increase in parathyroid hormone and osteocalcin. It is likely that the vitamin D deficiency prevented the increase in calcitriol and hence calcium absorption in response to the etidronate therapy.

There is some evidence that vitamin D therapy might reduce the risk of falls in postmenopausal women (see Chapter 102). Pfeifer *et al.* [193] reported that 148 women with low 25OHD (<50 nmol/L) were given a calcium supplement (1000 mg/day) and randomized to vitamin D (800 IU/day) or placebo for one year. The vitamin D resulted in decreased serum PTH, a decrease in body sway, and in the average number of falls per subject. Bischoff *et al.* [194] reported that 122 elderly women were given a calcium supplement (1200 mg/day) and randomized to vitamin D (800 IU/day) or placebo for 3 months. The vitamin D resulted in a decrease in the average number of falls per subject (by 49%), and an improvement in musculoskeletal function. These studies both assessed the number of falls and not the number of subjects who fell and this is a limitation. Nonetheless, this reduction in the risk of falling could translate into a decrease in the risk of fracture.

Thus, almost all of the clinical trials of treatments for osteoporosis included calcium and/or vitamin D as supplements. These supplements are more effective than placebo and act in their own right to reduce the risk of fracture. It is likely, although not proven, that they add to the antifracture effects of the antiresorptive drugs with which they are given. It would appear that the dose that would help reduce fractures and strengthen muscle would be 1000 mg of calcium and 800 IU of vitamin D per day. This type of supplementation is now commonly included in guidelines for the management of osteoporosis [195].

IX. CONCLUSIONS

Although data in the literature are conflicting, the large majority of studies indicate that $1,25(OH)_2D_3$ is moderately effective in reducing bone loss and possibly also in reducing vertebral fracture occurrence in women with type I osteoporosis. Efficacy seems to be dose-related with the best results occurring in patients treated with 0.5 to 0.75 μg per day. The drug appears to be quite safe in this dosage range, although restriction of calcium intake and monitoring serum and urine values for calcium is recommended. The osteoporotic women who are most likely to benefit from $1,25(OH)_2D_3$ are those with impaired calcium absorption. Unfortunately, it is not practical to measure calcium absorption directly. However, Riggs and Nelson [183] correlated calcium absorption and urinary calcium excretion and found that most osteoporotic women who had intestinal calcium malabsorption on a normal calcium diet had values for urine calcium excretion below 100 mg per day. Calcium and vitamin D should be considered standard adjuncts to treatment with antiresorptive therapy, such as bisphosphonates.

Many of the elderly patients with osteoporosis are vitamin D–deficient and should be treated with small dosages of vitamin D. For the remainder, treatment with small dosages of calcitriol, 0.25 μg per day combined with 1000 mg per day of elemental calcium seems rational because of the documented decrease in serum $1,25(OH)_2D$ levels in many patients. However, the value and safety of this drug in these elderly patients clearly needs better documentation.

The other area that requires further development is the use of synthetic metabolites of vitamin D. Shevde *et al.* [196] have described the effects of 2-methylene-19-nor-(205)-$1,25(OH)_2D_3$ in rats. There is a potent effect on osteoblasts and a large increase in bone mineral density with little change in calcium absorption (see Chapter 87). These selective vitamin D receptor modulators may prove to be useful therapies for the treatment of osteoporosis.

References

1. Aksnes L, Rodland O, Odegaard OR, Bakke KJ, Aarskog D 1989 Serum levels of vitamin D metabolites in the elderly. Acta Endocrinol **127**:27–33.

2. Baker MR, Peacock M, Nordin BEC 1980 The decline in vitamin D status with age. Age & Aging **9**:249–252.
3. Barraguy JM, France MW, Corless D, Gupta SP, Switala S, Boucher BJ *et al.* 1978 Intestinal cholecalciferol absorption in the elderly and in younger adults. Clin Sci Mol Biol **55**: 213–220.
4. Chapuy MC, Durr F, Chapuy P 1983 Age-related changes in parathyroid hormone and 25 hydroxycholecalciferol levels. J Gerontol **38**:19–22.
5. Gallagher JC, Riggs BL, Eisman J, Hamstra A, Arnaud SB, DeLuca HF 1979 Intestinal calcium absorption and serum vitamin D metabolites in normal subjects and osteoporotic patients. J Clin Invest **64**:729–736.
6. Guggenheim K, Kravitz M, Tal R, Kaufmann NA 1979 Biochemical parameters of vitamin D nutrition in old people in Jerusalem. Nutr Metab **23**:172–178.
7. Landin-Wilhelmsen K, Wilhalmsen L, Wilske J, Lappas G, Rosen T, Lindstedt G *et al.* 1995 Sunlight increases serum 24(OH) vitamin D concentration whereas $1,25(OH)_2D_3$ is unaffected. Eur J Clin Nutr **49**:400–407.
8. Lore F, Di Cairano G, Signorini AM, Caniggia A 1981 Serum levels of 25-hydroxyvitamin D in postmenopausal osteoporosis. Calcif Tiss Int **33**:467–470.
9. Lund B, Sorensen OH 1979 Measurement of 25-hydroxyvitamin D in serum and its relation to sunshine, age, and vitamin D. Scand J Clin Lab Invest **39**:23–30.
10. Omdahl JL, Garry PJ, Hunsaker LA, Hunt WC, Goodwin JS 1982 Nutritional status in a healthy elderly population: vitamin D. Am J Clin Nutr **36**:1225–1233.
11. Orwoll ES, Meier DE 1986 Alterations in calcium, vitamin D, and parathyroid hormone physiology in normal men with aging: relationship to the development of senile osteopenia. J Clin Endocrinol Metab **63**:1262–1269.
12. Quesada JM, Coopmans W, Ruiz B, Aljama P, Jans I, Bouillon R 1992 Influence of vitamin D on parathyroid function in the elderly. J Clin Endocrinol Metab **75**: 494–501.
13. Tsai KS, Wahner HW, Offord KP, Melton LJI, Kumar R, Riggs BL 1987 Effect of aging on vitamin D stores and bone density in women. Calcif Tissue Int **40**:241–243.
14. Weisman Y, Schen RJ, Eisenberg Z, Edelstein S, Harell A 1981 Inadequate status and impaired metabolism of vitamin D in the elderly. Isr J Med Sci **17**:19–21.
15. Heaney RP 2003 Vitamin D, nutritional deficiency, and the medical paradigm. J Clin Endocrinol Metab **88**(11):5107–5108.
16. Lips P 1996 Vitamin D deficiency and osteoporosis: the role of vitamin D deficiency and treatment with vitamin D and analogues in the prevention of osteoporosis-related fractures. Eur J Clin Invest **26**:436–442.
17. Ooms MEW, Lips P, Roos JC, van der Vijgh WJF, Popp-Snijders C, Bezemer PD *et al.* 1995 Vitamin D status and sex hormone binding globulin: determinants of bone turnover and bone mineral density in elderly women. J Bone Miner Res **10**:1177–1184.
18. Lips P, Wiersinga A, Vanginkel FC, Jongen MJM, Netelenbos JC, Hackeng WHL *et al.* 1988 The effect of vitamin D supplementation on vitamin D status and parathyroid function in elderly subjects. J Clin Endocrinol Metab **67**:644–650.
19. Van Der Wielaen RPJ, Lowik MRH, Van Der Berg H, de Groot LCPGM, Haller J, Moreiral O *et al.* 1995 Serum vitamin D concentrations among elderly people in Europe. Lancet **346**:207–210.
20. McKenna MJ 1992 Differences in vitamin D status between countries in young adults and the elderly. Am J Med **93**:69–77.
21. Clemens TL, Zhou X-Y, Myles M, Endres D, Lindsay R 1986 Serum vitamin D_2 and vitamin D_3 metabolite concentrations and absorption of vitamin D_2 in elderly subjects. J Clin Endocrinol Metab **63**:656–660.
22. Somerville PJ, Lien JWK, Kaye M 1977 The calcium and vitamin D status in an elderly female population and their response to administered supplemental vitamin D_3. J Gerontol **32**:659–663.
23. Bell NH 1985 Vitamin D endocrine system. J Clin Invest **76**:1–6.
24. Mutsuoka LY, Wortsman J, Hanifan N, Holick MF 1988 Chronic sunscreen use decreases circulating concentrations of 25-hydroxyvitamin D. Arch Dertmatol **124**:1802–1804.
25. Maclaughlin J, Holick MF 1985 Aging decreases the capacity of human skin to produce vitamin D_3. J Clin Invest **76**: 1536–1538.
26. Davie M, Lawson, DEM 1980 Assessment of plasma 25-hydroxyvitamin D response to ultraviolet irradiation over a controlled area in young and elderly subjects. Clin Sci **58**: 235–242.
27. Holick MF, Matsuoka LY, Wortsman J 1989 Age, vitamin D and solar ultraviolet. Lancet **2**:1104–1105.
28. Need AG, Morris HA, Horowitz M, Nordin BEC 1992 Skinfold thickness, age, and vitamin D. J Bone Miner Res **7**(Supplement):165.
29. Dandona P, Menon RK, Shenoy R, Houlder S, Thomas M, Mallinson, WJW 1986 Low 1,25-dihydroxyvitamin D, secondary hyperparathyroidism, and normal osteocalcin in elderly subjects. J Clin Endocrinol Metab **63**:459–462.
30. Fujisawa Y, Kida K, Matsuda H 1984 Role of change in vitamin D metabolism with age in calcium and phosphorus metabolism in normal human subjects. J Clin Endocrinol Metab **59**:719–726.
31. Manolagas SC, Howard J, Culler F, Catherwood BD, Deftos LJ 1982 Cytoreceptor assay for $1,25(OH)_2D$: a simple, rapid, and reliable test for clinical application. Clin Res **30**:527A.
32. Sowers MR, Wallace RB, Hollis BW 1990 The relationship of 1,25-dihydroxyvitamin D and radial bone mass. Bone & Mineral **10**:139–148.
33. Tsai K-S, Heath HI, Kumar R, Riggs BL 1984 Impaired vitamin D metabolism with aging in women. Possible role in pathogenesis of senile osteoporosis. J Clin Invest **73**: 1668–1672.
34. Ebeling PR, Sandgren ME, Dimagno EP, Lane AW, DeLuca HF, Riggs BL 1992 Evidence of an age-related decrease in intestinal responsiveness to vitamin D: relationship between serum 1,25 dihydroxyvitamin D_3 and intestinal vitamin D receptor concentrations in normal women. J Clin Endocrinol Metab **75**:176–182.
35. Dokoh S, Morita R, Fukunaga M, Yamamoto I, Torizuka K 1978 Competitive protein binding assay for 1,25-dihydroxyvitamin D in human plasma. Endocrinol Japan **25**:431–436.
36. Ebeling PR, Yergey AL, Vieira NE, Burritt MF, O'Fallon WM, Kumar R *et al.* 1994 Influence of age on effects of endogenous 1,25-dihydroxyvitamin D on calcium absorption in normal women. Calcif Tiss Int **55**:330–334.
37. Sherman SS, Hollis BW, Tobin JD 1990 Vitamin D status and related parameters in a healthy population: the effects of age, sex, and season. J Clin Endocrinol Metab **71** No **2**:405–413.
38. Kinyamu HK, Gallagher JC, Balhorn KE, Petranick KM, Rafferty KA 1997 Serum vitamin D metabolites and calcium absorption in normal young and elderly free-living women and in women living in nursing homes. Am J Clin Nutr **65**(3):790–797.

39. Pattanaungkul S, Riggs BL, Yergey AL, Vieira NE, O'Fallon WM, Khosla S 2000 Relationship of intestinal calcium absorption to 1,25-dihydroxyvitamin D [1,25(OH)2D] levels in young versus elderly women: evidence for age-related intestinal resistance to 1,25(OH)2D action. J Clin Endocrinol Metab **85**(11):4023–4027.
40. Eastell R, Yergey AL, Vieira NE, Cedel SL, Kumar R, Riggs BL 1991 Interrelationship among vitamin D metabolism, true calcium absorption, parathyroid function, and age in women: evidence of an age-related intestinal resistance to 1,25-dihydroxyvitamin D action. J Bone Miner Res **6**(2):125–132.
41. Epstein S, Bryce G, Hinman JW, Miller ON, Riggs BL, Hui SL *et al.* 1986 The influence of age on bone mineral regulating hormones. Bone **7**:421–425.
42. Vargas S, Bouillon R, Van Baelen H, Raisz LG 1990 Effects of vitamin D-binding protein on bone resorption stimulated by 1,25-dihydroxyvitamin D_3. Calcif Tissue Int **47**:164–168.
43. Safadi FF, Thornton P, Magiera H, Hollis BW, Gentile M, Haddad JG *et al.* 1999 Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D–binding protein. J Clin Invest **103**(2):239–251.
44. Bikle DD, Gee E, Halloran B, Haddad JG 1984 Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. J Clin Invest **74**:1966–1971.
45. Prince RL, Dick I, Devine A, Price RI, Gutteridge DH, Kerr D *et al.* 1995 The effects of menopause and age on calciotropic hormones: a cross-sectional study of 655 healthy women aged 35 to 90. J Bone Miner Res **10**:835–842.
46. Bouillon R, Auwerx J, Dekeyser L, Fevery J, Lissens W, DeMoor P 1984 Serum vitamin D metabolites and their binding protein in patients with liver cirrhosis. J Clin Endocrinol Metab **59**:86–89.
47. Fraher LJ, Caveney AN, Hodsman AB 1990 Vitamin D status of elderly institutionalized Canadians: effect of season and oral vitamin D2 on circulating 1,2(OH)2D. J Bone Miner Res **5**(Supplement 2):133.
48. Himmelstein S, Clemens TL, Rubin A, Lindsay R 1990 Vitamin D supplementation in elderly nursing home residents increases 25(OH)D but not 1,25(OH)2D. Am J Clin Nutr **52**:701–706.
49. Falch JA, Oftebro H, Hang E 1987 Early postmenopausal bone loss is not associated with a decrease in circulating levels of 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, or vitamin D–binding protein. J Clin Endocrinol Metab **64**:836–841.
50. Prince RL, Dick I, Garcia-Webb P, Retallack RW 1990 The effects of the menopause on calcitriol and parthyroid hormone: responses to a low dietary calcium stress test. J Clin Endocrinol Metab **70**:1119–1123.
51. Cheema C, Grant BF, Marcus R 1989 Effects of estrogen on circulating "free" and total 1,25-dihydroxyvitamin D and on the parathyroid vitamin D axis in postmenopausal women. J Clin Invest **83**:537–542.
52. Marcus R, Villa ML, Cheema M, Cheema C, Newhall K, Holloway L 1992 Effects of conjugated estrogen on the calcitriol response to parathyroid hormone in postmenopausal women. J Clin Endocrinol Metab **74**:413–418.
53. Hartwell D, Hassager C, Overgaard K, Riis BJ, Podenphant J, Christiansen C 1990 Vitamin D metabolism in osteoporotic women during treatment with estrogen, an anabolic steroid, or calcitonin. Acta Endocrinol **122**:715–721.
54. Kumar R, Cahan DH, Madias NE, Harrington JT, Kurtin P, Dawsonhughes BF 1991 Vitamin D and calcium transport. Kidney Int **40**:1177–1189.
55. Reichel H, Koeffler P, Norman AW 1989 The role of the vitamin D endocrine system in health and disease. New Eng J Med **320**:980–991.
56. Spencer EM, Halloran B 1992 The dependence of PTH's action on the renal 1-hydroxylase on insulin-like growth factor-1. J Bone Miner Res **7**(Supplement 1):172.
57. St John A, Thomas MB, Davies CP, Mullan B, Dick I, Hutchison B *et al.* 1992 Determinants of intact parathyroid hormone and free 1,25-dihydroxyvitamin D levels in mild and moderate renal failure. Nephron **61**:422–427.
58. Francis RM, Peacock M, Barkworth SA 1984 Renal impairment and its effects on calcium metabolism in elderly women. Age & Aging **13**:14–20.
59. Sherman SS, Tobin JD, Hollis BW, Gundberg CM, Roy TA, Plato CC 1992 Biochemical parameters associated with low bone density in healthy men and women. J Bone Miner Res **7**:1123–1130.
60. Webb AR, Pilbeam C, Hanafin N, Holick MF 1990 An evaluation of the relative contributions of exposure to sunlight and of diet to the circulating concentrations of 25-hydroxyvitamin D in an elderly nursing home population in Boston. Am J Clin Nutr **51**:1075–1081.
61. Slovik DM, Adams JS, Neer RM, Holick MF, Potts JT 1981 Deficient production of 1,25-dihydroxyvitamin D in elderly osteoporotic patients. New Eng J Med **305**:372–374.
62. Armbrecht HJ, Wongsurawat N, Paschal R 1987 Effect of age on renal responsiveness to parathyroid hormone and calcitonin in rats. J Endocrinol **114**:173–178.
63. Bouillon R. Growth hormone and bone. Horm Res **36**:49–55.
64. Spencer EM, Tobiassen O 1981 The mechanism of the action of growth hormone on vitamin D metabolism in the rat. Endocrinol **108**:1064–1070.
65. Rudman D, Rao UMP 1991 The hypothalamic-growth hormone-Somatomedin C Axis: the effect of aging. In: Morley JE, editor. Endocrinology and metabolism in the elderly. Blackwell USA, 1991:35–55.
66. Zadik Z, Chalew SA, McCarter RJ, Meistas M, Kowarski AA 1985 The influence of age on the 24-hour integrated concentration of growth hormone in normal individuals. J Clin Endocrinol Metab **60**:513–516.
67. Tietz NW, Shuey DF, Wekstein DR 1992 Laboratory values in fit aging individuals—sexagenarians through centenarians. Clin Chem 38(6):1167–1185.
68. Wiske PS, Epstein S, Bell NH, Queener SF, Edmondson J, Johnston CC 1979 Increases in immunoreactive parathyroid hormone with age. New Eng J Med **300**:1419–1421.
69. Yendt ER, Cohanim M, Rosenberg GM 1986 Reduced serum calcium and inorganic phosphate levels in normal elderly women. J Gerontol **41**:325–330.
70. Johnson JA, Goff JP, Beitz RL, Horst RL, Reinhardt TA 1992 Expression of 1,25 hydroxyvitamin D-24-hydroxylase changes with aging. J Bone Miner Res **7**(Supplement 1): 150.
71. Wada L, Daly R, Kern D, Halloran B 1992 Kinetics of 1,25-Dihydroxyvitamin D metabolism in the aging rat. Am J Physiol **262**:E906–E910.
72. Halloran BP, Portale AA, Lonergan ET, Morris RC Jr 1990 Production and metabolic clearance of 1,25-dihydroxyvitamin D in men: effect of advancing age. J Clin Endocrinol Metab **70** No 2:318–323.
73. Carroll MD, Abraham S, Dresser CM 1983 Dietary intake source data: United States, 1976–80. Vital and health statistics. DHHS Publ **231**(Series 11 (PHS)):83–1681.
74. Sheikh MS, Schiller LR, Fordtran JS 1990 In vivo intestinal absorption of calcium in humans. Miner Electrolyte Metab **16**:130–146.

75. Heaney RP, Recker RR, Stegman MR, Moy AJ 1989 Calcium absorption in women: Relationships to calcium intake, estrogen status, and age. J Bone Miner Res **4**:469–475.
76. Nordin BEC, Wilkinson R, Marshall DH, Gallagher JC, Williams A, Peacock M 1976 Calcium absorption in the elderly. Calcif Tiss Int **21**:442–451.
77. Ireland P, Fordtran JS 1973 Effect of dietary calcium and age on jejunal calcium absorption in humans studied by intestinal perfusion. J Clin Invest **52**:2672–2681.
78. Alevizaki CC, Ikkos DG, Singhelakis P 1973 Progressive decrease of true intestinal calcium absorption with age in normal man. J Nucl Med **14**:760–762.
79. Avioli LV, McDonald JE, Lee SW 1965 The influence of age on the intestinal absorption of 47-Ca in women and its relation to 47-Ca absorption in postmenopausal osteoporosis. J Clin Invest **44**:1960–1967.
80. Bullamore JR, Gallagher JC, Wilkinson R, Nordin BEC, Marshall DH 1970 Effect of age on calcium absorption. Lancet **2**:535–537.
81. Barger-Lux MJ, Heaney RP, Recker RR 1989 Time course of calcium absorption in humans: Evidence for a colonic component. Calcif Tissue Int **44**:308–311.
82. Chan ELP, Lau E, Shek CC, MacDonald D, Woo J, Leung PC *et al.* 1992 Age-related changes in bone density, serum parathyroid hormone, calcium absorption and other indices of bone metabolism in Chinese women. Clin Endocrinol **36**: 375–381.
83. Evans MA, Triggs EJ, Cheung M, Creasey H, Paull PD 1981 Gastric emptying rate in the elderly: implications for drug therapy. J Am Geriatr Soc **29**:201–205.
84. Russell RM 1992 Changes in gastrointestinal function attributed to aging. Am J Clin Nutr **55**:1203S–1207S.
85. Recker RR 1985 Calcium absorption and achlorhydria. N Engl J Med **313**:70–73.
86. Devine A, Prince RL, Kerr DA, Dick IM, Criddle A, Kent GN *et al.* 1993 Correlates of intestinal calcium absorption in women 10 years past the menopause. Calcif Tissue Int **52**:358–360.
87. Armbrecht HJ, Bolz M, Strong R, Richardson A, Bruns MEH, Christakos S 1989 Expression of calbindin D decreases with age in intestine and kidney. Endocrinol **125**:2950–2956.
88. Horst RL, Goff JP, Reinhardt TA 1990 Advancing age results in reduction of intestinal and bone 1,25 dihydroxyvitamin D receptor. Endocrinol **126**:1053–1057.
89. Liang CT, Barnes J, Takamato S, Sacktor B 1989 Effect of age on calcium uptake in isolated duodenum cells: role of 1,25-dihydroxyvitamin D_3. Endocrinol **124**:2830–2836.
90. Takamoto S, Seino Y, Sacktor B, Liang CT 1990 Effect of age on duodenal 1,25 dihydroxyvitamin D_3 receptors in Wistar rats. Biochem Biophys Acta **1034**:22–28.
91. Lissner L, Bengtsson C, Hansson T 1991 Bone mineral content in relation to lactation history in pre- and postmenopausal women. Calcif Tiss Int **48**:319–325.
92. Kinyamu HK, Gallagher JC, Prahl JM, DeLuca HF, Petranick KM, Lanspa SJ 1997 Association between intestinal vitamin D receptor, calcium absorption, and serum 1,25 dihydroxyvitamin D in normal young and elderly women. J Bone Miner Res **12**(6):922–928.
93. Barger-Lux MJ, Heaney RP, Hayes J, DeLuca HF, Johnson ML, Gong G 1995 Vitamin D receptor gene polymorphism, bone mass, body size, and vitamin D receptor density. Calcif Tissue Int **57**(2):161–162.
94. Cooper GS, Umbach DM 1996 Are vitamin D receptor polymorphisms associated with bone mineral density? A meta-analysis. J Bone Miner Res **11**(12):1841–1849.
95. Gallagher JC, Riggs BL, DeLuca HF 1980 Effect of estrogen on calcium absorption and serum vitamin D metabolism in postmenopausal osteoporosis. J Clin Endocrinol Metab **51**:1359–1364.
96. Gennari C, Agnusdei D, Nardi P, Civitelli R 1990 Estrogen preserves a normal intestinal responsiveness to 1,25-dihydroxyvitamin D_3 in oophorectomized women. J Clin Endocrinol Metab **71**:1288–1293.
97. Arjmandi BH, Salih MA, Herbert DC, Sims SH, Kalu DN 1993 Evidence for estrogen receptor-linked calcium transport in the intestine. Bone Miner **21**:63–74.
98. Bo-Linn GW, Davis GR, Baddrus DJ, Morawski SG, Ana CS, Fordtran JS 1984 An evaluation of the importance of gastric acid secretion in the absorption of dietary calcium. J Clin Invest **73**:640–647.
99. Eastell R, Vieira NE, Yergey AL, Wahner HW, Silverstein MN, Kumar R *et al.* 1992 Pernicious anaemia as a risk factor for osteoporosis. Clin Sci **82**:681–685.
100. Knox TA, Kassarjian Z, Dawsonhughes B, Golner BB, Dallal GE, Arora S *et al.* 1991 Calcium absorption in elderly subjects on high-fiber and low-fiber diets—effect of gastric acidity. Am J Clin Nutr **53**:1480–1486.
101. Eastell R, Vieira NE, Yergey AL, Wahner HW, Silverstein MN, Kumar R *et al.* 1992 Pernicious anemia as a risk factor for osteoporosis. Clin Sci (Colch) **82**(6):681–685.
102. Goerss JB, Kim CH, Atkinson EJ, Eastell R, O'Fallon WM, Melton LJ3 1992 Risk of fractures in patients with pernicious anemia. J Bone Miner Res **7**(5):573–579.
103. Filipponi P, Gregorio F, Cristallini S, Mannarelli C, Blass A, Scarponi AM *et al.* 1990 Partial gastrectomy and mineral metabolism: effects on gastrin-calcitonin release. Bone Miner **11**(2):199–208.
104. Barger-Lux MJ, Heaney RP, Lanspa SJ, Healy JC, DeLuca HF 1995 An investigation of sources of variation in calcium absorption efficiency. J Clin Endocrinol Metab **80**: 406–411.
105. Colodro IH, Brickman AS, Coburn JW, Osborn TW, Norman AW 1978 Effect of 25-hydroxyvitamin D_3 on intestinal absorption of calcium in normal man and patients with renal failure. Metabolism **27**:745–753.
106. Francis RM, Peacock M, Storer JH, Davies AEJ, Brown WB, Nordin BEC 1983 Calcium malabsorption in the elderly: the effect of treatment with oral 25-hydroxyvitamin D_3. Eur J Clin Invest **13**:391–396.
107. Berlyne GM, Ben-Ari J, Kushelevsky A, Idelman A, Galinsky D, Hirsch M *et al.* 1975 The aetiology of senile osteoporosis: secondary hyperparathyroidism due to renal failure. Quart J Med **64**:505–521.
108. Delmas P, Stenner D, Wahner HW, Mann KG, Riggs BL 1983 Increase in serum bone gamma-carboxyglutamic acid protein with aging in women: implications for the mechanism of age-related bone loss. J Clin Invest **71**:1316–1321.
109. Insogna KL, Lewis AM, Lipinski BA, Byrant C, Baran DT 1981 Effect of age on serum immunoreactive parathyroid hormone and its biological effects. J Clin Endocrinol Metab **53**:1072–1075.
110. Kotowicz MA, Melton LJ, Cedel SL, O'Fallon WM, Riggs BL 1990 Effect of age on variables relating to calcium and phosphorus metabolism in women. J Bone Miner Res **5**:345–352.
111. Marcus R, Madvig P, Young G 1984 Age-related changes in parathyroid hormone and parathyroid hormone action in normal humans. J Clin Endocrinol Metab **58**:223–230.
112. Van Breukelen FJM, Bijvoet OLM, Van Oosterom AT 1979 Inhibition of osteolytic bone lesions by (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate (APD). Lancet **I**:803–805.

113. Eastell R, Peel NFA, Hannon RA, Blumsohn A, Price A, Colwell A *et al.* 1993 The effect of age on bone collagen turnover as assessed by pyridinium crosslinks and procollagen I c-terminal peptide. Osteoporosis International **3**(Suppl 1):S100–S101.
114. Flicker L, Lichtenstein M, Colman P, Buirski G, Kaymakei B, Hopper JL *et al.* 1992 The effect of aging on intact PTH and bone density in women. J Am Geriatr Soc **40**:1135–1138.
115. Klee GG, Preissner CM, Schryver PG, Taylor RL, Kao PC 1992 Multisite immunochemiluminometric assay for simultaneously measuring whole-molecule and amino-terminal fragments of human parathyrin. Clin Chem **38**:628–635.
116. Yendt ER, Cohanim M, Jarzylo S, Jones G, Rosenberg G 1991 Bone mass is related to creatinine clearance in normal elderly women. J Bone Miner Res **6**:1043–1050.
117. Young G, Marcus R, Minkoff JR, Kim LY, Sergre GV 1987 Age-related rise in parathyroid hormone in man—the use of intact and midmolecule antisera to distinguish hormone secretion from retention. Bone Mineral Res **2**:367–374.
118. Bouillon R, Coopmans W, Degroote DEH, Radoux D, Ellard PH 1990 Immunoradiometric assay of parathyrin with polyclonal and monoclonal region-specific antibodies. Clin Chem **36**:271–276.
119. Sokoll LJ, Morrow FD, Quirbach DM, Dawson-Hughes B 1988 Intact parathyrin in postmenopausal women. Clin Chem **34**:407–410.
120. Khosla S, Melton LJ, III, Atkinson EJ, O'Fallon WM, Klee GG, Riggs BL 1998 Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen. J Clin Endocrinol Metab **83**(7):2266–2274.
121. Fatayerji D, Eastell R 1999 Age-related changes in bone turnover in men. J Bone Mineral Res **14**(7):1203–1210.
122. Forero MS, Klein RF, Nissenson RA, Nelson K, Heath HI, Arnaud CD *et al.* 1987 Effect of age on circulating immunoreactive and bioactive parathyroid hormone levels in women. J Bone Miner Res **2**:363–366.
123. Eastell R, Simmons PS, Colwell A, Assiri AM, Burritt MF, Russell RG *et al.* 1992 Nyctohemeral changes in bone turnover assessed by serum bone gla-protein concentration and urinary deoxypyridinoline excretion: effects of growth and aging. Clin Sci (Colch) **83**(3):375–382.
124. Harms HM, Prank K, Brosa U, Schlinke E, Neubauer O, Brabant G *et al.* 1992 Classification of dynamical diseases by new mathematical tools: Application of multidimensional phase space analyses to the pulsatile secretion of parathyroid hormone. Eur J Clin Invest **22**:371–377.
125. Kitamura N, Shigeno C, Shiomi K 1990 Episodic fluctuation in serum intact parathyroid hormone concentration in men. J Clin Endocrinol Metab **70**:252–263.
126. Samuels MH, Veldhuis J, Cawley C, Urban RJ, Luther M, Bauer R *et al.* 1993 Pulsatile secretion of parathyroid hormone in normal young subjects—assessment by deconvolution analysis. J Clin Endocrinol Metab **77**:399–403.
127. Cantley LK, Russell J, Lettieri D, Sherwood LM 1985 1,25-dihydroxyvitamin D_3 suppresses parathyroid hormone secretion from bovine parathyroid cells in tissue culture. Endocrinol **117**:2114–2119.
128. Gruson M, Demignon J, Del Pinto Montes J, Miravet L 1982 Comparative effects of some hydroxylated vitamin D metabolites on parathyrin secretion by dispersed rat parathyroid cells in vitro. Steroids **40**:275–285.
129. Russell J, Lettieri D, Sherwood LM 1986 Suppression by $1,25(OH)_2D_3$ of transcription of the preproparathyroid hormone gene. Endocrinol **119**:2864–2866.
130. Silver J, Naveh-Many T, Mayer H, Schmelzer HJ, Popoutzer MM 1986 Regulation by vitamin D metabolites of parathyroid hormone gene transcription in vivo in the rat. J Clin Invest **78**:1296–1301.
131. Ledger GA, Burritt MF, Kao PC, O'Fallon WM, Riggs BL, Khosla S 1994 Abnormalities of parathyroid hormone secretion in elderly women that are reversible by short-term therapy with 1,25-dihydroxyvitamin D_3. J Clin Endocrinol Metab **79**:211–216.
132. Dawson-Hughes B, Harris S, Dallal GE 1991 Serum ionized calcium, as well as phosphorus and parathyroid hormone, is associated with the plasma 1,25-dihydroxyvitamin D_3 concentration in normal postmenopausal women. J Bone Miner Res **6**:461–468.
133. Sorva A, Risteli J, Risteli L, Valimaki M, Tilvis R 1991 Effects of vitamin-D and calcium on markers of bone metabolism in geriatric patients with low serum 25-Hydroxyvitamin-D levels. Calcif Tissue Int **49**:S88–S89.
134. Fox J, Mathew MB 1991 Heterogeneous response to PTH in aging rats—evidence for skeletal PTH resistance. Am J Physiol **260**:E933–E937.
135. Hegarty V, Woodhouse P, Khaw KT 1994 Seasonal variation in 25-hydroxyvitamin D and parathyroid hormone concentrations in healthy elderly women. Age Aging **23**:478–482.
136. Fox J 1991 Regulation of parathyroid-hormone secretion by plasma calcium in aging rats. Am J Physiol **260**:E220–E225.
137. Uden P, Halloran B, Daly R, Duh QY, Clark O 1992 Set-point for parathyroid hormone release increases with postmaturational aging in the rat. Endocrinology **131**:2251–2256.
138. Wongsurawat N, Armbrecht HJ 1987 Comparison of calcium effect on in vitro calcitonin and parathyroid hormone release by young and aged thyroparathyroid glands. Exp Gerontol **22**:263–269.
139. Chapuy MC, Chapuy P, Meunier PJ 1987 Calcium and vitamin D supplements: effects on calcium-metabolism in elderly people. Am J Clin Nutr **46**:324–328.
140. Chapuy MC, Arlot ME, Duboeuf F, Brun J, Crouzet B, Arnaud S *et al.* 1992 Vitamin D_3 and calcium to prevent hip fractures in elderly women. New Eng J Med **327**:1637–1642.
141. Gallagher JC 1992 Pathophysiology of osteoporosis. Semin Nephrol **12**:109–115.
142. Eastell R, Kennedy NSJ, Smith MA, Tothill P, Edwards CRW 1986 Changes in total body calcium following surgery for primary hyperparathyroidism. Bone **7**:269–272.
143. Martin P, Bergmann P, Gillet C, Fuss M, Kinnaert P, Corvilain J *et al.* 1986 Partially reversible osteopenia after surgery for primary hyperparathyroidism. Arch Int Med **146**:689–691.
144. Melton LJ, Atkinson EJ, Ofallon WM, Heath H 1992 Risk of age-related fractures in patients with primary hyperparathyroidism. Arch Intern Med **152**:2269–2273.
145. Heath HI 1991 Clinical spectrum of primary hyperparathyroidism: evolution with changes in medical practice and technology. J Bone Miner Res **6**(Supplement 2):63–70.
146. Parfitt MA, Rao DS, Kleerekoper M 1991 Asymptomatic primary hyperparathyroidism discovered by multichannel biochemical screening: clinical course and considerations bearing on the need for surgical intervention. J Bone Miner Res **6**(Supplement 2):97–101.
147. Delmas PD, Stenner D, Wahner H, Mann KG, Riggs BL 1983 Increase in serum bone gamma-carboxyglutamic acid protein with aging in women. J Clin Invest **71**:1316–1321.
148. Ledger GA, Burritt MF, Kao PC, O'Fallon WM, Riggs BL, Khosla S 1995 Role of parathyroid hormone in mediating nocturnal and age-related increases in bone resorption. J Clin Endocrinol Metab **80**(11):3304–3310.

149. Martinez ME, Del Campo MT, Sanchez-Cabezudo MJ 1994 Relations between calcidiol serum levels and bone mineral density in postmenopausal women with low bone density. Calcif Tissue Int **55**:253–256.
150. Lukert B, Higgins J, Stoskopf M 1992 Menopausal bone loss is partially regulated by dietary intake of vitamin D. Calcif Tissue Int **51**:173–179.
151. Kleerekoper M, Wilson P, Peterson E, Nelson DA 1992 Acute parathyroid hormone response to intramuscular calcitonin may predict femoral neck bone loss in older postmenopausal women. Journal of Bone and Mineral Research **7**[Suppl 1], S312. Ref Type: Abstract.
152. Avioli LV, Heaney RP 1991 Calcium intake and bone health. Calcif Tissue Int **48**:221–223.
153. Cumming RC 1990 Calcium intake and bone mass: A quantitative review of the evidence. Calcif Tissue Int **47**: 194–201.
154. Dawson-Hughes B, Dallal GE, Drall EA, Sadowski L, Sahyoun N, Tannerbaum S 1990 A controlled trial of the effect of calcium supplementation on bone density in postmenopausal women. New Eng J Med **323**(13):878–883.
155. Orwoll EW, Oviatt SK, McClung MR, Deftos LJ, Sexton G 1990 The rate of bone mineral loss in normal men and the effects of calcium and cholecalciferol supplementation. Ann Int Med **112**:29–34.
156. Riggs BL, Khosla S, Melton LJ III 1998 A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men [see comments]. J Bone Miner Res **13**(5):763–773.
157. Riggs BL, Khosla S, Melton LJ, III 2002 Sex steroids and the construction and conservation of the adult skeleton. Endocr Rev **23**(3):279–302.
158. McKane WR, Khosla S, Risteli J, Robins SP, Muhs JM, Riggs BL 1997 Role of estrogen deficiency in pathogenesis of secondary hyperparathyroidism and increased bone resorption in elderly women. Proc Assoc Am Physicians **109**(2):174–180.
159. Thomas ML, Xu X, Norfleet AM, Watson CS 1993 The presence of functional estrogen receptors in intestinal epithelial cells. Endocrinol **132**(1):426–430.
160. Gallagher JC, Fowler SE, Detter JR, Sherman SS 2001 Combination treatment with estrogen and calcitriol in the prevention of age-related bone loss. J Clin Endocrinol Metab **86**(8):3618–3628.
161. Van Cromphaut SJ, Rummens K, Stockmans I, Van Herck E, Dijcks FA, Ederveen AG *et al.* 2003 Intestinal calcium transporter genes are upregulated by estrogens and the reproductive cycle through vitamin D receptor-independent mechanisms. J Bone Miner Res **18**(10):1725–1736.
162. McKane WR, Khosla S, Burritt MF, Kao PC, Wilson DM, Ory SJ *et al.* 1995 Mechanism of renal calcium conservation with estrogen replacement therapy in women in early postmenopause—a clinical research center study. J Clin Endocrinol Metab **80**(12):3458–3464.
163. Klein RG, Arnaud SB, Gallagher JC, DeLuca HF, Riggs BL 1977 Intestinal calcium absorption in exogenous hypercortisonism. J Clin Invest **60**:253–259.
164. Parfitt AM, Gallagher JC, Heaney RP, Johnston CC, Neer R, Whedon GD 1992 Vitamin D and bone health in the elderly. Am J Clin Nutr **36**:1014–1031.
165. Malabanan A, Veronikis IE, Holick MF 1995 Redefining vitamin D insufficiency. Lancet **351**(9105):805–806.
166. Jesudason D, Need AG, Horowitz M, O'Loughlin PD, Morris HA, Nordin BE 2002 Relationship between serum 25-hydroxyvitamin D and bone resorption markers in vitamin D insufficiency. Bone **31**(5):626–630.
167. Heaney RP, Dowell MS, Hale CA, Bendich A 2003 Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D. J Am Coll Nutr **22**(2):142–146.
168. Heikinheimo RJ, Inkovaara JA, Harju EJ, Haavisto MV, Kaarela RH, Kataja JM *et al.* 1992 Annual injection of vitamin D and fractures of aged bones. Calcif Tissue Int **51**(2):105–110.
169. Ooms ME, Roos JC, Bezemer PD, van der Vijgh WJF, Bouter LM, Lips P 1995 Prevention of bone loss by vitamin D supplementation in elderly women: A randomized double-blind trial. J Clin Endocrinol Metab **80**:1052–1058.
170. Lips P, Graafmans WC, Ooms ME, Bezemer PD, Bouter LM 1996 Vitamin D supplementation and fracture incidence in elderly persons. A randomised, placebo-controlled clinical trial. Ann Int Med **124**:400–406.
171. Trivedi DP, Doll R, Khaw KT 2003 Effect of four monthly oral vitamin D_3 (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: randomized double-blind controlled trial. BMJ **326** (7387):469.
172. Dawson-Hughes B, Harris SS, Krall EA, Dallal GE 1997 Effect of calcium and vitamin D supplementation on bone density in men and women 65 years of age or older. N Engl J Med **337**(10):670–676.
173. Lips P, van Ginkel FC, Jongen MJM, Rubertus F, van der Vijgh WJF, Netelenbos JC 1987 Determinants of vitamin D status in patients with hip fracture and in elderly control subjects. Am J Clin Nutr **46**:1005–1010.
174. Gallagher JC, Jerpbak CM, Jee WSS, Johnson KA, DeLuca HF, Riggs BL 1982 1,25-dihydroxyvitamin D_3: short- and long-term effects on bone and calcium metabolism in patients with postmenopausal osteoporosis. Proc Natl Acad Sci USA **79**:3325–3329.
175. Aloia JF, Vaswani A, Yeh JK, Ellis K, Yasumura A, Cohn SH 1988 Calcitriol in the treatment of postmenopausal osteoporosis. Am J Med **84**:401–408.
176. Gallagher JC, Riggs BL 1990 Action of 1,25-dihydroxyvitamin D_3 on calcium balance and bone turnover and its effect on vertebral fracture rate. Metabolism **39**(4 Suppl 1): 30–34.
177. Ott SM, Chesnut CH 1989 Calcitriol treatment is not effective in postmenopausal osteoporosis. Ann Int Med **110**:267–274.
178. Orimo H, Shiraki M, Hayashi Y, Hoshino T, Onaya T, Miyazaki S *et al.* 1994 Effects of 1-hydroxyvitamin D_3 on lumbar bone mineral density and vertebral fractures in patients with postmenopausal osteoporosis. Calcif Tissue Int **54**:370–376.
179. Christiansen C, Christiansen MS, Rodbro P, Hagen C, Transbol I 1981 Effect of 1,25-dihydroxyvitamin D_3 in itself or combined with hormone treatment in preventing postmenopausal osteoporosis. Eur J Clin Invest **11**:305–309.
180. Need AG, Nordin BEC, Horwitz M, Morris HA 1990 Calcium and calcitriol therapy in osteoporotic postmenopausal women with impaired calcium absorption. Metabolism **39**:53–54.
181. Gallagher JC, Riggs BL, Recker RR, Goldgar D 1989 The effect of calcitriol on patients with postmenopausal osteoporosis with special reference to fracture frequency. Proc Soc Exp Biol Med **191**:287–292.
182. Tilyard MW, Spears GF, Thomson J, Dovey S 1992 Treatment of postmenopausal osteoporosis with calcitriol or calcium [see comments]. N Engl J Med **326**(6):357–362.

183. Riggs BL, Nelson KI 1985 Effect of long term treatment with calcitriol on calcium absorption and mineral metabolism in postmenopausal osteoporosis. J Clin Endocrinol Metab **61**:457–461.
184. Shiraki M, Kushida K, Fukunaga M, Kishimoto H, Taga M, Nakamura T *et al.* 1999 A double-masked multicenter comparative study between alendronate and alfacalcidol in Japanese patients with osteoporosis. The Alendronate Phase III Osteoporosis Treatment Research Group. Osteoporos Int **10**(3):183–192.
185. Papadimitropoulos E, Wells G, Shea B, Gillespie W, Weaver B, Zytaruk N *et al.* 2002 Meta-analyses of therapies for postmenopausal osteoporosis. VIII: Meta-analysis of the efficacy of vitamin D treatment in preventing osteoporosis in postmenopausal women. Endocr Rev **23**(4):560–569.
186. Eastell R 1998 Treatment of postmenopausal osteoporosis. N Engl J Med **338**(11):736–746.
187. Heaney RP, Weaver CM 2003 Calcium and vitamin D. Endocrinol Metab Clin North Am **32**(1):181–viii.
188. Black DM, Cummings SR, Karpf DB, Cauley JA, Thompson DE, Nevitt MC *et al.* 1996 Randomized trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. Fracture Intervention Trial Research Group. Lancet **348**(9041):1535–1541.
189. Reginster J, Minne HW, Sorensen OH, Hooper M, Roux C, Brandi ML *et al.* 2000 Randomized trial of the effects of risedronate on vertebral fractures in women with established postmenopausal osteoporosis. Vertebral Efficacy with Risedronate Therapy (VERT) Study Group. Osteoporosis International **11**(1):83–91.
190. Liberman UA, Weiss SR, Broll J, Minne HW *et al.* 1995 Effect of oral alendronate on bone mineral density and the incidence of fractures in postmenopausal osteoporosis. New Eng J Med **333**(22):1437–1443.
191. Adami S, Frijlink WB, Bijvoet OL, O'Riordan JL, Clemens TL, Papapoulos SE 1982 Regulation of calcium absorption by 1,25,dihydroxy-vitamin D—studies of the effects of a bisphosphonate treatment. Calcif Tissue Int **34**(4):317–320.
192. Koster JC, Hackeng WH, Mulder H 1996 Diminished effect of etidronate in vitamin D deficient osteopenic postmenopausal women. European Journal of Clinical Pharmacology **51**(2):145–147.
193. Pfeifer M, Begerow B, Minne HW, Abrams C, Nachtigall D, Hansen C 2000 Effects of a short-term vitamin D and calcium supplementation on body sway and secondary hyperparathyroidism in elderly women. J Bone Miner Res **15**(6):1113–1118.
194. Bischoff HA, Stahelin HB, Dick W, Akos R, Knecht M, Salis C *et al.* 2003 Effects of vitamin D and calcium supplementation on falls: a randomized controlled trial. J Bone Miner Res **18**(2):343–351.
195. Hodgson SF, Watts NB, Bilezikian JP, Clarke BL, Gray TK, Harris DW *et al.* 2001 American Association of Clinical Endocrinologists 2001 Medical Guidelines for Clinical Practice for the Prevention and Management of Postmenopausal Osteoporosis. Endocr Pract **7**(4):293–312.
196. Shevde NK, Plum LA, Clagett-Dame M, Yamamoto H, Pike JW, DeLuca HF 2002 A potent analog of 1alpha,25-dihydroxyvitamin D_3 selectively induces bone formation. Proc Natl Acad Sci USA **99**(21):13487–13491.
197. Baeksgaard L, Andersen KP, Hyldstrup L 1998 Calcium and vitamin D supplementation increases spinal BMD in healthy, postmenopausal women. Osteoporos Int **8**(3):255–260.
198. Gallagher JC, Goldgar D 1990 Treatment of postmenopausal osteoporosis with high doses of synthetic calcitriol. A randomized controlled study. Ann Intern Med **113**(9): 649–655.
199. Guesens P, Dequecker J 1986 Long-term effect of nandrolone decanoate, 1-alpha hydroxyvitamin D_3, or intermittent calcium infusion therapy on bone mineral content, bone remodelling and fracture rate in symptomatic osteoporosis: a double blind controlled study. Bone and Mineral **1**:347–357.
200. Orimo H, Shiraki M, Hayashi T, Nakamura T 1987 Reduced occurrence of vertebral crush fractures in senile osteoporosis treated with 1-alpha(OH)-vitamin D_3. Bone and Mineral **47**:52.
201. Caniggia A, Delling G, Nuti R, Lore F, Vattimo A 1984 Clinical, biochemical and histological results of a double-blind trial with 1,25-dihydroxyvitamin D_3, estradiol, and placebo in post-menopausal osteoporosis. Acta Vitaminol Enzymol **6**:117–128.
202. Cummings SR, Black DM, Thompson DE, Applegate WB, Barrett-Connor E, Musliner TA *et al.* 1998 Effect of alendronate on risk of fracture in women with low bone density but without vertebral fractures: results from the Fracture Intervention Trial. JAMA **280**(24):2077–2082.
203. Harris ST, Watts NB, Genant HK, McKeever CD, Hangartner T, Keller M *et al.* 1999 Effects of risedronate treatment on vertebral and nonvertebral fractures in women with postmenopausal osteoporosis: a randomized controlled trial. Vertebral Efficacy with Risedronate Therapy (VERT) Study Group. JAMA **282**(14):1344–1352.
204. Chesnut CH, III, Silverman S, Andriano K, Genant H, Gimona A, Harris S *et al.* 2000 A randomized trial of nasal spray salmon calcitonin in postmenopausal women with established osteoporosis: the prevent recurrence of osteoporotic fractures study. PROOF Study Group. Am J Med **109**(4):267–276.
205. Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK *et al.* 1999 Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a three-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA **282**(7):637–645.

CHAPTER 68

Genetic Vitamin D Receptor Polymorphisms and Risk of Disease

ANDRÉ G. UITTERLINDEN, YUE FANG, JOYCE B.J. VAN MEURS, AND HUIBERT A.P. POLS

Genetic Laboratory, Department of Internal Medicine, Er asmus Medical Centre, NL-3015 GD Rotterdam, The Netherlands

I. Introduction

The secosteroid hormone vitamin D, its receptor (VDR), and the metabolizing enzymes involved in the formation of the biologically active form of the hormone, acting together, are major players in the vitamin D endocrine system. This system plays an important role in skeletal metabolism, including intestinal calcium absorption, but has also been shown to play an important role in other metabolic pathways, such as those involved in the immune response and cancer [1]. In the immune system, for example, vitamin D promotes monocyte differentiation and inhibits lymphocyte proliferation and secretion of cytokines, such as IL2, interferon-γ, and IL12. In several different types of cancer cells vitamin D has been shown to have antiproliferative effects. These aspects of the vitamin D endocrine system are extensively discussed elsewhere in this volume.

At the same time it is also widely known that large interindividual differences exist. One approach to understand interindividual differences in the vitamin D endocrine system is to study the influence of variations in the DNA sequence of important proteins of this system. For example, deleterious mutations in the VDR gene cause 1,25-dihydroxyvitamin D resistant rickets, a rare monogenetic disease (Chapter 72). More subtle sequence variations (polymorphisms) in the VDR gene occur much more frequently in the population, but they have not been systematically analyzed and their effects on VDR function are poorly understood. Their influence on the vitamin D endocrine system is currently under scrutiny in relation to a number of so-called complex diseases and traits, such as osteoporosis. This so-called candidate gene approach in the genetic dissection of complex traits is currently gaining increased importance over genome search approaches using linkage analysis [2,3].

The interpretation of polymorphic variations in the VDR gene is severely hindered by the fact that until now only a few polymorphisms in this large gene have been studied, and that most of these are anonymous restriction fragment length polymorphisms (RFLP). One expects them to be linked to truly functional polymorphisms elsewhere in the VDR gene (or in nearby gene(s)), which can then explain the associations observed. Thus, to understand the mechanisms underlying the associations one has to analyze the genomic organization of the VDR locus, to identify which genes are present in the chromosomal area, to categorize all relevant VDR polymorphisms, to determine the haplotypes across the gene, to determine their relationship with the RFLP markers used so far, and finally to perform association analyses with relevant phenotypic endpoints such as disease.

Below, we present a more detailed description of the genomic organization of the VDR gene, including discussion on polymorphisms, linkage disequilibrium, and haplotypes. We then describe association studies of VDR polymorphisms in relation to different diseases. Historically speaking, studies of VDR polymorphisms in relation to bone endpoints, including osteoporosis in particular, have received most attention while the analysis of VDR polymorphisms in relation to other diseases, including breast and prostate cancer and immune-related disorders, has reached the literature somewhat later on. This allows studies on associations with bone endpoints to be compared to a certain extent and to illustrate some of the difficulties in interpreting the results. This is much less possible for VDR polymorphism studies in relation to other disease endpoints, although similar interpretation problems exist. Essentially, these interpretation problems

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find their origin in the lack of knowledge of which polymorphisms are present in the VDR gene area and not knowing what the functional relevance is of these polymorphisms. Therefore, most attention in this review will be focused on these aspects rather than providing an exhaustive review of all the studies that have found their way into the literature on VDR polymorphisms and association with one or other disease endpoint.

II. STRUCTURE AND POLYMORPHISM OF THE VDR GENE

A. Genomic Structure of the 12q13 Locus

After the cloning of the human VDR cDNA in 1988 by Baker *et al*. [4], it took almost 10 years before major parts of the genomic structure of the human VDR gene became clear as described by Miyamoto *et al*. [5]. All of this happened, of course, before the Human Genome Project began to bear fruit in the form of easily accessible databases where genomic sequences could be found. Yet, these databases are still not complete and for particular genes efforts have to be made to determine their genomic structure. The location of the VDR gene on the physical map of chromosome 12 was elucidated first roughly by linkage mapping by Labuda *et al*. [6] and later on somewhat more refined by Fluorescent in Situ Hybridization (FISH) and radiation hybrid mapping by Taymans *et al*. [7]. However, these studies defined the position of the VDR gene in very general terms with a resolution of >100 kb and this is insufficient for understanding the role of VDR polymorphisms in disease. The major reason to know the exact gene content and distances of this gene area comes from genetic association studies. Since mostly anonymous polymorphisms have been studied so far, one of the explanations for the associations observed is that the effect is not due to the VDR gene itself, but rather the association is explained by another, nearby gene. It is important to know which genes are also present in the area, how close they are to the VDR gene, and how likely it is that these other genes play a role in explaining the associations.

Therefore, we have extended these physical mapping studies of the VDR gene to create a high-resolution physical map of the chromosomal 12q13 region in which the VDR gene is located. For this we applied thorough analysis of all available databases with genomic sequences and pulsed field gel electrophoresis to determine the exact location and order of the VDR gene and its neighboring genes (see Figs. 1–3). Our analysis of the genomic organization of the VDR locus at chromosome 12q13.1 has shown that the VDR gene itself is quite large (just over 100 kb; see Fig. 2; van Meurs *et al*., unpublished), has an extensive promoter region capable of generating multiple tissue-specific transcripts (8; see also Chapters 11–12 for more detailed information on this) and lies just downstream from the COL2A1 gene [9,10].

In Fig. 1 a summary is presented of the current mapping information of the VDR-COL2A1 region. On the genetic map [11] both genes are mapped around position 63 cM, between the markers D12S85 and D12S368. The radiation hybrid map [12] confirms the close conjunction of the two genes, since both genes are mapped close to the 210 centiRod position. The STS-based contig map [13] shows that VDR and COL2A1 are located on two separate PAC clones (P1057I20 and P228P16, respectively (see Fig. 1). Figure 2 shows a compilation of all the information from the databases and our own results with respect to the VDR-COL2A1 locus combined with information of the genomic organization of the VDR gene. Recently, most of this information can also be found in the draft of the human genome [14], but until now, a gap in the sequence was still present between COL2A1 and VDR. To elucidate the distance between the two PAC clones, P1057I20 and P228P16 were used as probes in a fiber-FISH experiment (see Fig. 3). The distance between the two PAC clones was estimated to be 50 kb. The distance between the two genes was subsequently estimated to be 30–60 kb, depending on the size of the first intron of VDR between exon 1f and 1e. Completion of the physical map was achieved by sequencing 2.5 kb of sequence upstream of exon 1f, which overlapped with the 3′-end of PAC clone P228P16. Based on this, we calculated the total sequence length between the end of exon 52 of COL2A1 and the beginning of VDR exon 1f to be 30.3 kb. Figure 2 shows the completed physical map of the region surrounding VDR. Besides already identified genes (phosphofructo kinase (PFKM), sentrin/SUMO specific protease (SENP1), and histone deacetylase (HDAC7), also some so far unknown genes were identified (information available at http://compbio.ornl.gov/channel/).

From these results, it appears that, in the absence of evidence for the role of the other genes in the area in, e.g., bone metabolism, the VDR gene is the obvious candidate gene to explain the associations found. The next step is to determine the polymorphisms in the relevant areas and determine how they interact with each other in genetic terms and in functional terms.

B. VDR Polymorphisms

Several genes in the human genome have been analyzed in detail for the occurrence of genetic variations which occur at a frequency of > 1 % in the population

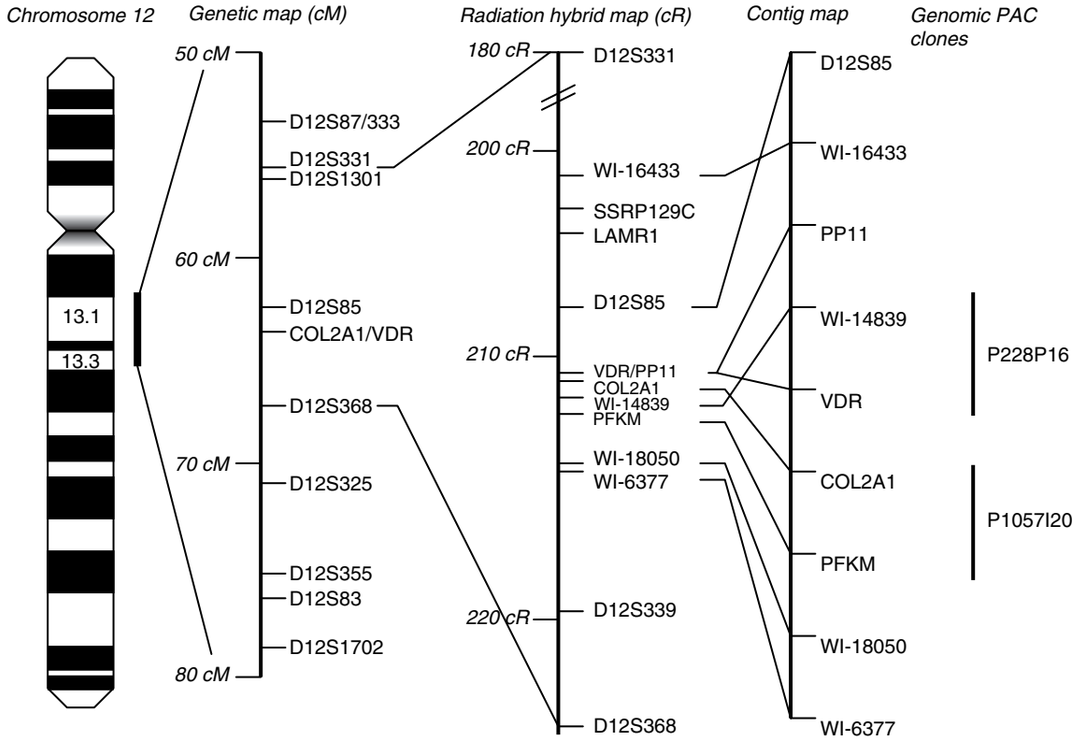

FIGURE 1 Genomic mapping of the VDR gene to chromosome 12q13.1. VDR and COL2A1 are located closely together and are shown amidst different types of genetic DNA markers on maps of increasing resolution going from left to right. From left to right is displayed: a karyotype with a high resolution banding pattern, the genetic map based on anonymous DNA markers, the more detailed radiation hybrid genetic map, the physical contig map and finally the rough map position of two PAC clones, used for the fiber-FISH experiment shown in Fig. 3.

(polymorphisms), such as the lipoprotein lipase gene [15] and the ACE gene [16]. From these and other analyses we now know that human polymorphisms occur on average at 1 out of every 300 bp. Some areas carry less polymorphism, such as the protein encoding regions (exons), where the occurrence can be as low as 1 in every 2000 bp of exonic sequence [17–19].

Information on the existence of VDR polymorphisms so far has come from analysis of only limited areas in the gene and by using rather insensitive techniques

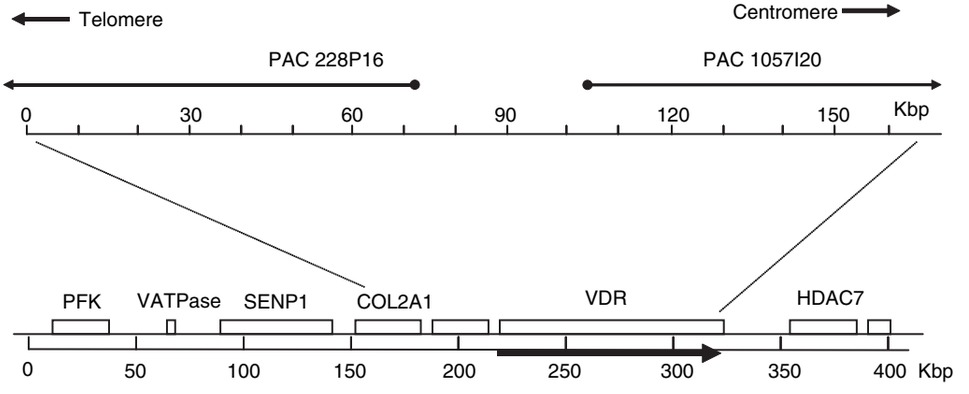

FIGURE 2 Genomic structure of the VDR-COL2A1 locus on chromosome 12q13.1 and detailed physical map position of the PAC clones used for the Fiber-FISH experiments shown in Figure 3. The arrow indicates direction of transcription of the VDR gene. PFK = Phosphofructokinase; VATPase = Vacuolar ATP-ase; SENP1 = sentrin/SUMO-specific protease; HDAC7 = Histone Deacetylase 7.

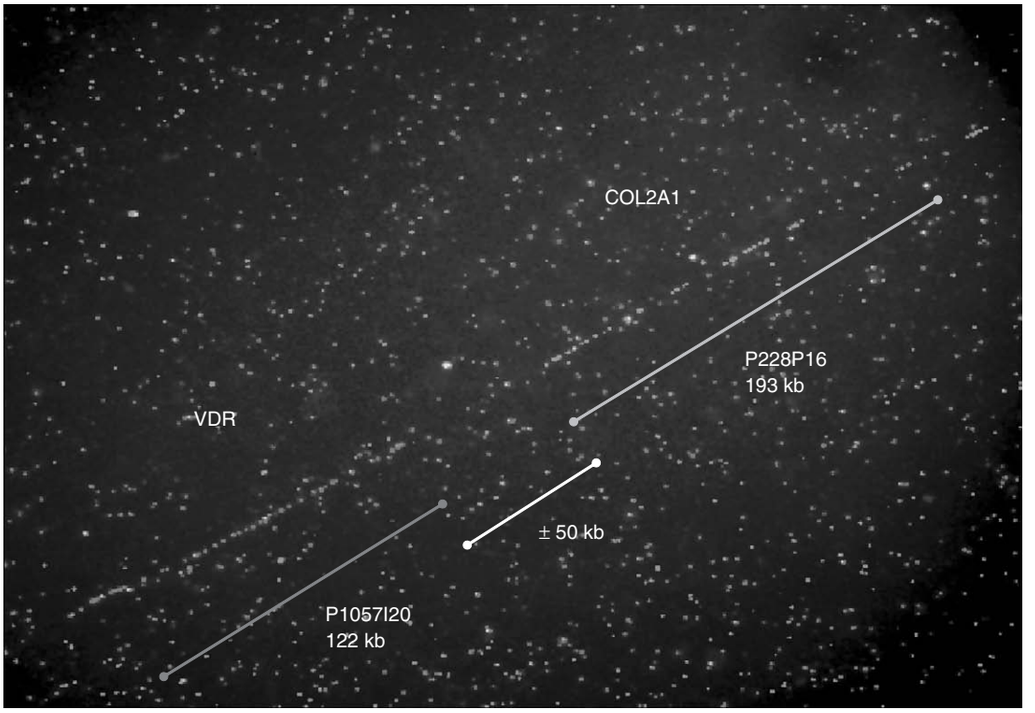

FIGURE 3 Fiber-FISH analysis of the VDR-COL2A1 locus. PAC-clone P1057120 was labeled with Texas red and P228P16 with FITC green and were used as probes in a fiber-FISH (fluorescent in situ hybridization) experiment. In this picture fluorescent signals are detected along a single DNA molecule. The known length of the PAC clone inserts (1057120 = 122 kb and P228P16 = 193 kb) allows to estimate the physical distance between them, i.e., in this case about 50 kb.

to find polymorphisms, such as screening with different restriction enzymes for polymorphic banding patterns in Southern blot hybridization experiments. Examples of this include the ApaI- [20], EcoRV- [21], BsmI- [21], TaqI- [22], and Tru9I- [23] restriction fragment length polymorphisms (RFLPs) discovered at the 3′ end of the VDR gene by this approach in the early 1990s. In Figs. 4 and 5, a number of the currently known VDR polymorphisms are depicted.

A special case in this respect is presented by the discovery of the so-called FokI RFLP. Upon comparison of the original Baker sequence of the VDR cDNA [4], two potential translation initiation start sites (ATG) were observed and subsequent sequence comparisons have shown that a T to C polymorphism exists (ATG to ACG) at the first potential start site [24–26]. This polymorphism, also referred to as the *Start Codon Polymorphism* or SCP, was later defined by using the FokI restriction enzyme in an RFLP test [27]. Thus, two protein variants can exist corresponding to the two available start sites: a long version of the VDR protein (the T-allele detected as the "f" allele; also referred to as the M1 form, i.e., the methionine at first position) and a protein shortened by three amino acids (the C-allele detected as the "F" allele; also referred to as the M4 form, i.e., the methionine at fourth position). Up until now, this is the only known polymorphism that alters the VDR protein.

A more informative approach to find polymorphisms is to simply determine the basepair sequence of the same part of the VDR sequence in a number of different individuals. This was applied by Morrison *et al.* for the 3.2 kb 3′ untranslated region (UTR) of the gene when searching for additional polymorphisms and possible functional variations to explain an association of BMD differences with the (supposedly nonfunctional) BsmI RFLP [22]. When they sequenced two subjects who were homozygous for the most frequent BsmI-ApaI-TaqI haplotypes (see below), i.e., BAt-BAt and baT-baT, they reported 13 polymorphisms in 3.2 kb, corresponding to the expected 1 in 300 bp to be variant. Among the sequence differences they reported

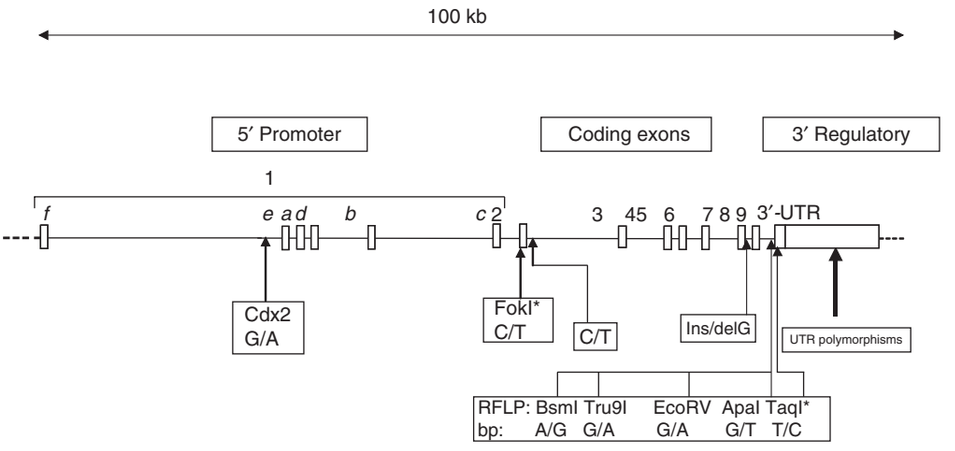

FIGURE 4 Exon-intron structure of the VDR gene and position of known polymorphisms. * indicates that these polymorphisms are in the coding sequence. See text for further details on the polymorphisms. The 3′UTR polymorphisms are shown in more detail in Figure 5.

was a polyA-tract with a varying number of A-residues with alleles that vary in length between 12–18 adenosines. Durrin *et al*. expanded this approach and sequenced the 3′UTR in five subjects homozygous for the baT-haplotype and three subjects homozygous for the BAt-haplotype and in total identified seven polymorphisms (see Fig. 5) of which 4 were common and 3 were rare in the eight subjects they analyzed [28]. Surprisingly, only two polymorphisms were found in both the sequence analyses by Durrin *et al*. [28] and Morrison *et al.* [22]. When considering the methods used and the number of subjects analyzed, it seems likely that the polymorphisms reported by Durrin *et al*. do not contain sequence errors and seem

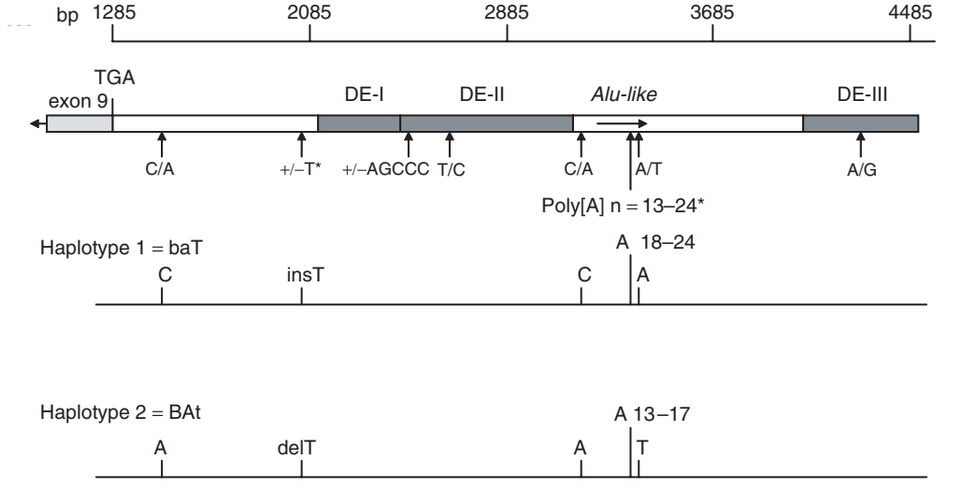

FIGURE 5 Structure and position of polymorphisms in the 3′UTR of the VDR gene based on Morrison *et al*. [22] and Durrin *et al*. [28]. The bp numbering is according to Baker *et al*. [4]. TGA indicates the stop codon in exon 9 where the 3′UTR starts. DE I–III refer to the so-called destabilizing elements as identified by Durrin *et al*. Below, the two most frequent BsmI-ApaI-TaqI haplotypes across this part of the gene are depicted, i.e., haplotype 1 (= baT) and haplotype 2 (= BAt), and the alleles of the 3′UTR polymorphisms to which they are linked based on eight subjects analyzed. The * indicates the polymorphisms that were identified in both studies.

to be more accurate. However, the number of subjects analyzed is still limited, and they were highly selected, so it is likely that more as yet uncovered polymorphisms exist in the 3.2 kb UTR (see below).

A similar sequence comparison approach was followed by Brown *et al*. when they analyzed the coding region of the VDR gene in 59 parathyroid tumors to find mutations [29]. Apart from the previously reported TaqI- and FokI-polymorphisms, they reported no polymorphisms in the coding region and found two intronic polymorphisms near exon 2 and 8 (shown in Fig. 4).

Another VDR polymorphism that was found through sequence analysis of a targeted area is the so-called Cdx2 polymorphism. Arai and colleagues reported a G to A sequence variation among Japanese women in (what they thought was) the VDR 1a promoter when characterizing this promoter area of the VDR gene [30]. The G to A polymorphism is in a binding site for an intestinal-specific transcription factor, called *Cdx2* [31]. Recently, our laboratory has shown the Cdx2 polymorphism to be in the VDR 1e promoter (see Fig. 4), to be present among Caucasians as well as other race groups, and we have developed an allele-specific genotyping assay for it [32].

Currently, it is possible to identify potential polymorphisms through bio-informatic approaches by mining of databases such as the NCBI SNP consortium (dbSNP; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp) or the Celera database (http://cds.celera.com). Indeed, many polymorphisms are usually reported in such databases for any gene of interest, but their validity has to be judged very carefully. For example, for the area which we analyzed in detail for the VDR gene, only 40% of the polymorphisms currently reported in these databases turn out to be real (Fang and van Meurs, unpublished). Although we can expect the databases to improve over time, for polymorphism finding in the near future it might still be necessary to generate reliable sequence information from multiple individuals.

We have recently conducted a more comprehensive study of the VDR gene and found 63 polymorphisms across 22 kb of sequenced parts the VDR gene (Fang *et al*., manuscript in preparation). Apart from the known polymorphisms shown in Figs. 4 and 5, also a number of novel polymorphisms were found in the promoter areas including in and around exons 1f-1c, in and around exons 2–9 and in the 3′UTR. The majority of polymorphisms we found to be in regulatory areas rather than in coding exons. In general, this does not seem to be unexpected since variation in the actual encoded protein itself, such as minor changes in the amino acid sequence, might result in drastic changes in function, such as affinity for the ligand and binding to DNA. More likely, one can assume that polymorphic variation and, thus, population variance, can exist in areas that regulate the level of expression of this gene, such as the 5′ promoter area and the 3′UTR region.

Once we know which polymorphisms are present in a certain candidate gene area, it is important to understand how they relate to each other, i.e., in a genetic sense as well as in a biologically functional sense. Genetically, we need to understand which alleles are linked to each other by studying the linkage disequilibrium between polymorphisms. Functionally, we have to determine how certain combinations of alleles across such a candidate gene might augment or diminish certain effects on gene function.

C. Linkage Disequilibrium and Haplotypes

Linkage Disequilibrium (LD) measures describe the association (or co-occurrence) of alleles of adjacent polymorphisms with each other [33]. This means in practice that one polymorphism can predict the other adjacent "linked" one because very little recombination has occurred between them over the time of evolution and population history. High levels of LD in a certain area will coincide with a limited number of "haplotypes" in that area. Haplotypes are blocks of linked alleles of adjacent polymorphisms, whereby the length of such a block coincides with the strength of LD across the area. By analysis of polymorphisms across 51 autosomal areas in the human genome, it has become evident that indeed such haplotype blocks exist [34]. The haplotype block size can vary between 5 kb to >50 kb with an average around 10–20 kb. That means that frequent haplotype alleles can be found that encompass the polymorphic variation in such areas. In practice, this also means that relatively few polymorphisms have to be genotyped to "cover" the variance in a certain area. Therefore, a massive effort is currently under way to determine a haplotype map of the human genome [33,34].

It follows that the LD and haplotype structure of a certain candidate gene, such as the VDR, is important for association analyses to understand how the polymorphic variation in such a gene can contribute to risk of disease and population variance of certain phenotypes of interest. When a certain allele of a polymorphism has been found to be associated with—say—risk of fracture, it follows that this association might be explained by the effect of this particular allele, but also—because of LD and the haplotype structure—by one or more other alleles that happen to be linked to this allele

within the haplotype. Once we know which haplotype carries this risk allele, we can determine by cell biological and molecular biological functional analyses which of the variants on that haplotype allele truly cause this effect.

Based on some of the known polymorphisms shown in Fig. 4, several studies have analyzed the extent of Linkage Disequilibrium (LD) across the VDR gene. Since these analyses have used only a small number of polymorphisms, accurate information on LD and haplotypes so far has been very limited. Nevertheless, strong LD at the 3′ end of the gene has been observed for the BsmI-, ApaI-, EcoRV-, and TaqI-RFLPs [21,22]. This information was taken a step further by our laboratory to describe molecular haplotypes for these RFLPs [35]. We identified five haplotype alleles in a large Caucasian population of which haplotype 1 (baT; 48%) and 2 (BAt; 40%) were the most frequent and corresponded to the ones identified by Morrison *et al*. [22]. In line with this, strong LD was also observed between the BsmI RFLP and the polyA variable number of tandem repeats (VNTR) in the 3′UTR [36]. This latter polymorphism has at least 12 different alleles (in 627 subjects analyzed in [36] across five ethnic groups) but the allele size distribution of the poly-A VNTR essentially follows a bimodal distribution. This pattern is such that this marker can be characterized as bi-allelic and that subjects can be classified as having alleles with short or long polyA stretches. Ingles *et al*. reported strong linkage between the "b" allele and a long poly-A stretch and the "B" allele and a short poly-A stretch. Combined with the results of Morrison *et al*. [22], Durrin *et al*. [28], and our results [35], it follows that the Bsm-Apa-Taq haplotype 1 (baT) is linked to a large number of As in the poly-A VNTR (n = 18–24, Long or L alleles), while haplotype 2 (BAt) is linked to a smaller number of As (n = 13–17, short or S alleles). See Fig. 5. Interestingly, several studies have shown that the FokI polymorphism showed no linkage to any of the other VDR polymorphisms.

We have recently determined the LD pattern across the VDR gene using the polymorphisms we have recently discovered (see above; Fang *et al*., manuscript in preparation). For this we determined the genotype for 41 polymorphisms across the VDR gene in a group of 235 middle-aged Caucasians and then analyzed their association or co-occurrence by calculating D′ values using the PHASE program [37]. By plotting these pair wise measures of LD (so-called D′ values) between each of the polymorphisms using the GOLD program [38], a graphical display of LD across a certain area is obtained (shown in Fig. 6). This shows that at least 4–5 areas of high LD with an average size of 10–20 kb can be recognized in the 100 kb of genomic sequence that encompasses the VDR gene, using these 41 polymorphisms. This is very much in line with what has been found in relation to LD blocks elsewhere in the human genome [33,34]. Importantly, this also predicts that in these regions of high LD, a limited number of frequent haplotypes will encapsulate all the polymorphic variation in that area. For the 3′ region we already know that this is the case because the haplotypes we previously identified in this region using the BsmI-ApaI-TaqI RFLPs [35] correspond closely to the haplotypes predicted when using all available polymorphisms in this area (see Figs. 5 and 6; Fang *et al*., manuscript in preparation). For the other areas of high LD, we are currently determining the haplotype structure and composition.

D. Ethnic Variation in Polymorphisms

VDR polymorphisms have been identified and analyzed so far mostly in Caucasians and to a lesser extent in other ethnic groups. For example, the Cdx2 polymorphism was discovered in Japanese [30] and has only recently been analyzed in Caucasians [32]. For the most widely studied VDR polymorphisms, sometimes substantial differences have been noted between races and/or ethnic groups (see Table I and ref. 39). For example, the f allele of the FokI RFLP, corresponding with the 427 *aa* long VDR protein variant, occurs with lower frequency in Africans when compared to Caucasians and Asians, while the B allele of the BsmI RFLP has a lower frequency in Asians compared to Caucasians and Africans. Similarly, Ingles *et al*. showed differences between ethnic groups for the polyA VNTR in the 3′UTR [36]. We have found the frequency of the Cdx2 A allele to vary widely across different ethnic groups with the A allele being lowest in Caucasians, at about 19% population frequency, and highest (74%) in Africans [32].

Why do we see such differences? In general, all polymorphisms start as mutations which occur perhaps due to a DNA damage event, and then can grow in frequency in the population and become true polymorphisms. Thus, allele frequency differences between ethnic groups most likely result from evolutionary processes and population genetic behavior. The same holds true for the LD between the polymorphisms and the haplotype structure. In Table I the frequencies are presented of the Bsm-Apa-Taq haplotypes, which we have recently determined in different ethnic groups (Fang *et al*., manuscript in preparation). They show complex patterns across ethnic groups, which cannot

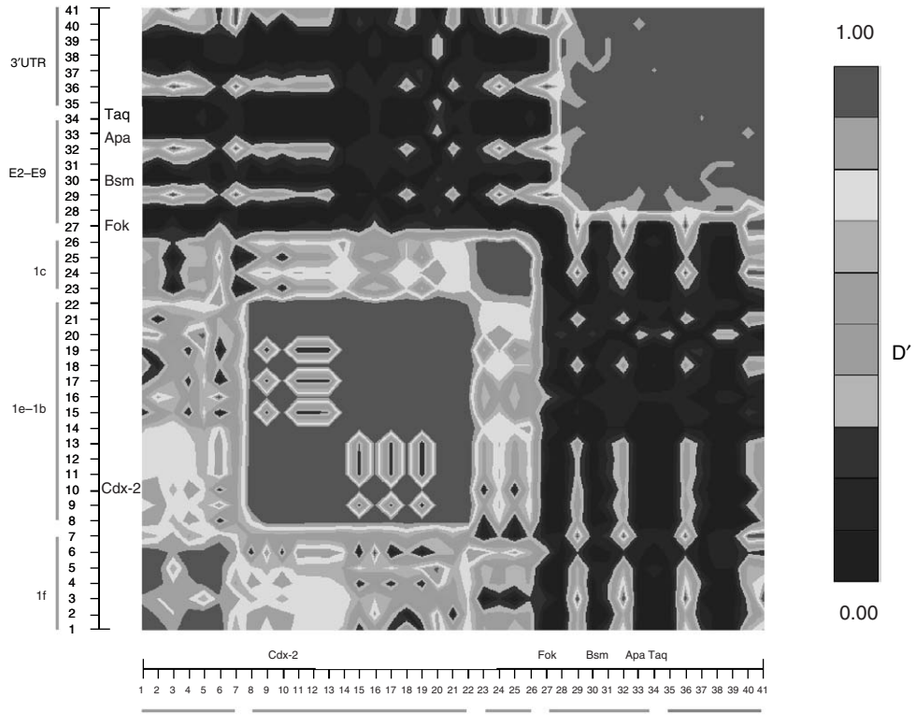

FIGURE 6 Graphic display of a measure of strength of Linkage Disequilibrium (LD), the D′ value, across the VDR gene in Caucasians (Fang *et al*., unpublished). Shown are pairwise D′ values between 41 SNPs across the VDR gene, plotted at equal distance from each other. On the left and at the bottom, the position of some known VDR-SNPs is presented. The color bar indicates the strength of D′, with red representing very strong LD and blue representing very weak LD. Analysis is based on genotypes generated for 41 SNPs in 235 Caucasian subjects using the PHASE and GOLD programs [37,38]. (See color plate.)

TABLE I Comparison of VDR Allele Frequencies Across the Three Major Ethnic Groups for the Most Widely Studied Polymorphisms*

		Ethnic group		
VDR polymorphism	Minor allele**	Caucasian (%)	Asian (%)	African (%)
Individual polymorphisms				
Cdx2	A	19	43	74
FokI	f	34	51	24
BsmI	B	42	7	36
ApaI	A	44	74	31
TaqI	T	43	8	31
polyA	Short n = 13–17	41	12	29
Bsm-Apa-Taq haplotypes				
	baT: haplotype 1	48	75	26
	BAt: haplotype 2	39	7	16
	bAT: haplotype 3	11	17	59

The data are allele frequencies based on the total number of chromosomes. Haplotypes of very low frequency are omitted.
*Data are from various sources [20–28,32,35,36] and from Fang *et al*. (manuscript in preparation).
**Minor allele in Caucasians refers to the less common allele; see text for details on definition of alleles.

be derived from the frequency differences of the individual composite polymorphisms. This is probably due to the existence of different sets of alleles linked together, to form haplotypes across the 3′ end of the VDR gene. In other words, the haplotype "1" (or baT) in Caucasians is not defining the same set of linked alleles as the haplotype 1 in Asians or Africans. The set of haplotypes will reflect the out-of-Africa theory describing the origin of human populations across the globe, and perhaps also be due to gene-environment interactions in which certain variants might have had survival and/or reproductive advantages. One can assume that relatively "old" polymorphisms show little variation between different ethnic groups, whereas relatively new polymorphisms might display large differences. In this respect, for example the Cdx2 polymorphism appears relatively new and the FokI RFLP rather old.

For the interpretation of association studies, we can assume that individual functional polymorphisms might have the same functional effect in different ethnic groups, because the physiological role of the vitamin D endocrine system will not be vastly different between ethnic groups. However, the individual polymorphisms will explain more or less of the population variance given their difference in frequency between these ethnic groups. This is also the basis for interpretation of ecological studies where allele frequencies across (ethnic) groups are correlated with the different incidence of disease/phenotype between such groups. For nonfunctional or anonymous polymorphisms, the situation is different because here we rely on the LD that is detected by the polymorphism to explain its association with a disease. And as shown for the Bsm-Apa-Taq haplotypes in Table I, the frequencies of these "marker haplotypes" (consisting of nonfunctional polymorphisms which are markers for truly functional alleles elsewhere) are very different between ethnic groups. More importantly, the particular alleles that will be linked in the individual haplotypes is likely to be very different. This means that haplotype 1 (baT) in Caucasians is not describing the same set of linked alleles as in Asians or Africans. Thus, if haplotype 1 is found associated in Caucasians and also in Africans, this can be due to linkage with a totally different functional allele. Alternatively, if an association of such a marker-haplotype is seen in Caucasians but, for example, not in Asians, this can be explained because the LD between the marker and the functional allele is different between these groups. It is therefore essential to elucidate the genetic structure of the linked alleles in such haplotype groups across the complete VDR gene in the different ethnic groups, if we want to compare genetic association results between these groups. As indicated before, we are currently performing such a study (Fang *et al*., manuscript in preparation).

At this time it is difficult to fully understand the consequences of ethnic allele variation, because of the vastly different environmental factors between such ethnic groups, such as diet and exercise. In addition, there are very different "genetic backgrounds" (i.e., the remainder of the genome and genetic variation therein) between ethnic groups in which these VDR polymorphisms interact with each other and with other genetic variants of other genes. In understanding this it is therefore important to start by defining the haplotype structure of VDR polymorphisms in different ethnic groups. This will reveal which VDR alleles occur together on certain haplotypes and what their frequency is in different ethnic groups. Subsequently, together with the information on differences in environmental factors between ethnic groups, we can start to understand the relevant gene-environment interactions.

E. Functionality of Polymorphisms

The interpretation of the association studies using VDR polymorphisms is severely hindered by the fact that most of the polymorphisms used are anonymous, i.e., have an unknown functional effect. The likely explanation for any observed association is then to assume the presence of a truly functional sequence variation elsewhere in the gene which is—to a certain extent—in linkage with an allele of the anonymous polymorphism used. As can be understood from the complex organization of the VDR gene (see Fig. 4), the identification of these functional polymorphisms in the VDR gene is a challenging enterprise. While these results are still eagerly awaited, several investigators have—nevertheless—over the past years analyzed multiple bio-response parameters using the anonymous polymorphisms, including the BsmI RFLP, and Bsm-Apa-Taq haplotypes, and the polyA VNTR in the 3′UTR. These studies include *in vitro* cell biological and molecular biological studies, but also *in vivo* measurements of biochemical markers and response to treatments with vitamin D, calcium, and even HRT or bisphosphonates. In Fig. 7 a schematic representation is shown of how functionality of polymorphisms can be tested at different levels, i.e., at the mRNA level, the protein level, cell level, etc. In the optimal situation, the polymorphism is expected to show similar directions of the allelic effects at all these levels and, allow a connection of mechanisms at the molecular level with epidemiological findings at the population level. For the Cdx2 and the FokI polymorphism, this has now been established to a large extent, while for the

BsmI-ApaI-TaqI RFLPs and the polyA VNTR polymorphism, this is less certain and controversy still exists. Although one might argue that it is useless to perform any functional study with an anonymous polymorphism, results from such studies might still be informative because of the (strong) LD that is present between the marker polymorphisms and the truly functional polymorphism. An overview of the functional studies for each of these VDR polymorphisms is presented in Table II and these are discussed below.

1. CDX2 POLYMORPHISM

The Cdx2 polymorphism has been well-characterized by the studies of Arai *et al*. and Yamamoto *et al*. [30,31]. The G to A polymorphism is located in the Cdx2 binding site in the 1e promoter region, and this site is suggested to play an important role in intestinal-specific transcription of the VDR gene. As the intestine is the site where the calcium absorption predominantly takes place, the Cdx2 site is thought to influence the vitamin D regulation of calcium absorption. The A-allele has been demonstrated to be more "active" than the G-allele by binding the Cdx2 transcription factor more strongly, and by having more transcriptional activity [30]. Thus, the A-allele is thought to cause increased VDR expression in the intestine and, thereby, can increase the transcription of calcium transport proteins such as calbindin-9K and -28K, TRPV5, TRPV6 (Chapters 24 and 25). This could enhance the intestinal absorption of calcium and result in increased BMD. Although this increased BMD has indeed been demonstrated for Japanese women who carry the A-allele [30], this was not found in Caucasian women [32]. Yet, the A-allele of this polymorphism was indeed found to be associated with decreased fracture risk (as would be expected from having an increased BMD) in a large study of Caucasian women, but independently of BMD [32]. Therefore, although the functionality of this polymorphism has indeed been convincingly demonstrated, the exact mechanism whereby the A-allele would confer lower risk for fracture has not been elucidated yet and requires further study.

2. FOKI POLYMORPHISM

From the genetic perspective, it is important to note that the FokI RFLP can be considered an independent marker in the VDR gene since there is no LD with any of the other VDR polymorphisms and the LD area surrounding this polymorphism seems to be very small (<2 kb; see Fig. 6). Therefore, LD with another polymorphism is not a likely explanation for the associations observed with this polymorphism and so functional studies should be focused on the polymorphism itself. In a study by Arai *et al*. [27], evidence for the functionality of the FokI polymorphism was obtained. Results from transcriptional activation studies in transfected HeLa cells using a reporter construct under the control of a short portion of the rat 24-hydroxylase gene promoter region (−291–+9) containing a VDRE, suggested the short 424 amino acid VDR protein variant (corresponding with the C-allele or "big F" allele) to be more active than the long 427 aa variant, with a 1.7-fold difference between the two variants. Gross *et al*. [40] were not able to confirm these results. They also analyzed FokI allelic differences in the transcription activation characteristics of the VDR protein, but now in COS7 cells and using the rat 24 hydroxylase promoter although containing a slightly larger region of the promoter (−1399–+76). In addition, they analyzed VDREs from the human osteocalcin gene and the rat osteopontin gene, but could not see FokI allelic differences in these systems. Yet, the authors noted that it might be difficult to observe the relatively small effects of this polymorphism in these test systems. Moreover, small differences in cell type, promoter area, and gene-specificity of the VDRE might be crucial to see an allelic difference or not. Jurutka *et al*. [42] demonstrated the 424 aa VDR variant to interact more efficiently with the transcription factor TFIIB, using reporter constructs containing 1100 bp of a rat osteocalcin promoter in COS7-, HeLa, and ROS2/3 cells. The authors concluded the 424 aa short VDR variant to represent a more transcriptionally potent VDR protein. This notion was corroborated by the same authors in an analysis of 20 fibroblast cell lines of different endogenous FokI VDR genotype using a reporter construct containing four copies of the rat osteocalcin gene [43]. Results from our own laboratory [44] seem to confirm the higher activity of the 424 aa short VDR variant while using a different readout of functionality. We tested capacity for growth inhibition by vitamin D in PBMCs of different genotype for the FokI RFLP in cells derived from 72 postmenopausal women. We observed that the PBMCs carrying the "F"-allele (corresponding to the 424 aa short variant) had a lower ED50 and, thus, had a more active VDR variant in inhibiting the (PHA induced) cell growth [44].

In conclusion, we can state that the FokI polymorphism seems to be functional and that the 424 aa VDR variant (F-allele) is somewhat more active than the 427 aa variant (f-allele) in terms of its transactivation capacity as a transcription factor. There might be a gene-specific effect in that some promoter areas of vitamin D target genes might be more sensitive to this VDR genotype-dependent difference in activity, while others may not. Together with cell type specific interaction

TABLE IIA Functional Effects of VDR Polymorphisms: *In vitro* Studies

Polymorphism	Study [Reference]	Cell type	Allelic differences				
			VDR expression	VDR mRNA stability	Transactivation by VDR	Cell growth inhibition	Mechanism
Cdx2; A to G	Arai *et al*., 2001 [30,31]	Caco-2 intestinal cell line	A > G	–	A > G	–	The G allele has diminished binding of the Cdx2 transcription factor
FokI; C (F; 424 aa; M4) to T (f; 427 aa; M1)	Arai *et al*., 1997 [27]	Hela cell line	–	–	424 aa > 427 aa	–	
	Gross *et al*., 1998 [40]	GMK-Cos7	No Difference	–	No Difference	–	
		Human fibroblasts	No Difference	–	No Difference		
	Correa *et al*., 1999 [41]	Parathyroid adenoma's	No Difference	–	–	–	
	Jurutka *et al*., 2000 [42]	COS7, Hela, ROS 2/3	No Difference	–	424 aa > 427 aa	–	The 424 aa VDR variant (M4) interacts more efficiently with transcription factor TFIIB
	Whitfield *et al*., 2001 [43]	Human fibroblasts (n = 20)	–	–	424 aa > 427 aa	–	
	Collin *et al*., 2000 [44]	Human PBMC (n = 72)	–	–	–	424 aa > 427 aa	
	Ogunkolade *et al*. [211a]	Human PBMC (n = 41)	424 aa > 427 aa	–	–	–	
Bsm, Apa, Taq, UTR (polyA VNTR)	Morrison *et al*., 1994 [22]	GMK Cos-7	BAt> baT	–	–	–	mRNA stability of BAt is larger than of baT
		Rat ROS 17/2.8	BAt > baT	–	–	–	
	Beaumont *et al*., 1998 [45]	GMK Cos-7	No Difference	No Difference	–	–	
		Human osteoblast	baT > BAt	baT > BAt	–	–	
	Durrin *et al*., 1999 [28]	Mouse NIH3T3 cells	No Difference	No Difference	–	–	
	Crofts *et al*., 1996 [46]	Human fibroblasts (n = 9), PBL (n = 13)	(ba)T > (BA)t	–	–	–	
	Gross *et al*., 1998 [47]	Human fibroblasts (n = 9)	No Difference	–	No Difference	–	

Continued

TABLE IIA Functional Effects of VDR Polymorphisms: *In vitro* Studies—Cont'd

Polymorphism	Study [Reference]	Cell type	Allelic differences				
			VDR expression	VDR mRNA stability	Transactivation by VDR	Cell growth inhibition	Mechanism
Bsm, Apa, Taq, UTR (Cont'd)	Whitfield *et al*., 2001 [43]	Human fibroblasts (n = 11)	–	–	L > S (baT > BAt)	–	
	Yamagata *et al*., 1999 [48]	Human PBMC (n = 24; Japanese)	(BA)t > (ba)T	–	–	–	
	Mocharla *et al*., 1997 [49]	Human PBMC (n = 38)	No Difference	–	–	–	
	Collin *et al*., 2000 [44]	Human PBMC (n = 72)	–	–	–	No Difference	
	Verbeek *et al*., 1997 [50]	Human PBL, leukemia cell line, prostate cell line	(ba)T > (BA)t	No Difference	–	–	
	Carling *et al*., 1997, 1998 [51,52]	Human parathyroid adenoma's (n = 42)	BAt > baT	–	–	BAt > baT	
	Ohtera *et al*., 2001 [53]	Human osteoblasts (n = 18; Japanese)	–	–	baT > BAt	–	
	Ogunkolade *et al*. [211a]	Human PBMC (n = 41)	baT > BAt				

GMK, Green Monkey Kidney.
PBMC, Peripheral blood mononuclear cells.
PBL, Peripheral blood lymphocytes.
–, Not analyzed.

TABLE IIB Functional Effects of VDR Polymorphisms: *In vivo* Studies*

Polymorphism	Study [Reference]	Marker	Allelic effect: serum concentration
FokI	Ogunkolade *et al*. [211a]	Insulin secretion after OGTT** (n = 143 Bangladeshi)	F > f for insulin secretion index (in 25(OH)D deficient subjects)
Bsm, Apa, EcoRV	Morrison *et al*., 1992 [21]	Osteocalcin (n = 91)	BA(t) > ba(T)
Bsm	Morrison *et al*., 1994 [22]	Calcitriol ((n = 117)	B(At) > b(aT)
Bsm	Kroger *et al*., 1995 [56]	Osteocalcin, ICTP (n = 23)	b(aT) > B(At)
Bsm	Fleet *et al*., 1995 [57]	Osteocalcin, calcitriol (n = 154)	No Difference
Bsm Apa Taq	Garnero *et al*., 1995 [58]	Osteocalcin, 25(OH), etc. (n = 189)	B(At) > b(aT): NS trend***
Bsm	Krall *et al*., 1995 [59]	$1,25(OH)_2D$ (n = 229)	No Difference
Bsm	Howard *et al*., 1995 [60]	Osteocalcin, calcitriol, ICTP (n = 21)	B(At) > b(aT)
Bsm Apa Taq	Tokita *et al*., 1996 [61]	Osteocalcin, BAP, $1,25(OH)_2D$ (n = 159; Japanese)	BAt > baT
Bsm Apa Taq	Tsai *et al*., 1996 [62]	Osteocalcin, BAP, PICP, NTX (n = 268; Chinese)	No Difference
Bsm	Mocharla *et al*., 1997 [49]	Osteocalcin, calcitriol (n = 38)	No Difference
Bsm	Rauch *et al*., 1997 [63]	Osteocalcin, AlkPhosp, PICP (n = 50)	B(At) > b(aT): NS trend
Bsm Apa Taq	McClure *et al*., 1997 [64]	Osteocalcin, calcitriol, PTH (n = 103)	BAt > baT: NS trend for PTH , calcitriol baT > BAt: NS trend for osteocalcin
Bsm	Graafmans *et al*., 1997 [65]	Osteocalcin, calcitriol	No Difference
Bsm	Hansen *et al*., 1998 [66]	Osteocalcin, 25(OH), BAP (n = 200)	No Difference
Bsm, FokI	Willing *et al*., 1998, 1999 [67,68]	Osteocalcin, PTH, 25(OH) (n = 372; n = 261)	No Difference
Bsm	Ferrari *et al*. 1999 [69]	Osteocalcin, PTH (n = 72 young men)	B(At) > b(aT)
Bsm Apa Taq	Bell *et al*., 2001 [70]	Osteocalcin, PTH, $1,25(OH)_2D$ (n = 39 African men, n = 44 Caucasian men)	(b)a(T) > (B)A(t) for PICP
Bsm Apa Taq	Hitman *et al*., 1998 [211,250]	Insulin secretion after OGTT** (n = 143 Bangladeshi)	BAt > baT for insulin secretion index (in 25(OH)D deficient subjects)

*Studies used either one or all of the 3′ polymorphisms, i.e., BsmI, ApaI, EcoRV, and TaqI. To relate these results to what is described in this chapter for sequence variation at the 3′end of the VDR gene, the genotypes and allelic effects are presented as haplotypes 1 (baT) or 2 (BAt). Letters in brackets refer to polymorphisms not actuallly tested in the study but inferred by this author based on VDR haplotype structure in the 3′ area. All studies are in Caucasians unless stated otherwise.

** OGTT = Oral glucose tolerance test.

*** NS trend: non–significant trend.

TABLE IIC Functional Effects of VDR Polymorphisms: *In vivo* Studies of Response to Treatment*

Polymorphism	Study [Reference]	Treatment	Response	
			Serum markers	BMD
FokI	Kurabayashi *et al*., 1999 [73]	1 year HRT (n = 82)	–	No Difference
Bsm	Howard *et al*., 1995 [60]	7 days calcitriol (n = 21)	b(aT) > B(At)	–
Bsm Apa Taq	Matsuyama *et al*., 1995 [71]	12 month 1αOHD$_3$ (n = 115)	–	baT > BAt
Bsm	Graafmans *et al*., 1997 [65]	2 year calcitriol (n = 81)	No Difference	B(At) > b(aT)
Bsm	Krall *et al*., 1995 [59]	2 year calcium (n = 229)	–	B(At) > b(aT)
Bsm	Deng *et al*., 1998 [72]	3.5 year HRT (n = 108)	–	B(aT) > B(At)
Apa Taq	Kurabayashi *et al*., 1999 [73]	1 year HRT (n = 82)	–	(ba)T > (BA)t
Bsm Taq	Ho *et al*., 1999 [74]	>1 year corticosteroids (n = 263)	–	No Difference
Bsm	Marc *et al*., 1999 [75]	1 year etidronate (n = 24)	–	B(At) > b(aT)

*Studies used either one or all of the 3′ polymorphisms, i.e., BsmI, ApaI, EcoRV, and TaqI. To relate these results to what is described in this chapter for sequence variation at the 3′end of the VDR gene, the genotypes and allelic effects are presented as haplotypes 1 (baT) or 2 (BAt). Letters in brackets refer to polymorphisms not actuallly tested in the study but inferred by this author based on VDR haplotype structure in the 3′ area. All studies are in Caucasians unless stated otherwise.

** OGTT = Oral glucose tolerance test.

*** NS trend: non–significant trend.

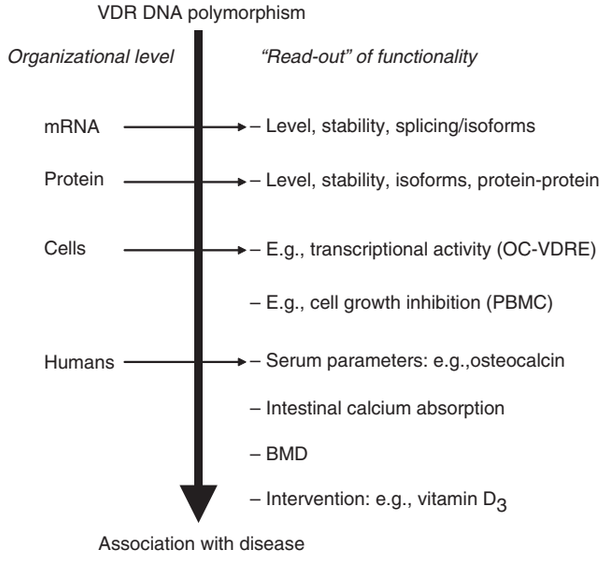

FIGURE 7 Schematic depiction of the different organizational levels in physiology which determines the relation between a DNA polymorphism and the association with an endpoint in an epidemiological study, which are at the same time the levels at which functionality of VDR polymorphisms can be determined. Such functionality can be established using different types of read-outs of test systems indicated on the right of the arrow. OC VDRE = osteocalcin vitamin D responsive element. PBMC = peripheral blood mononuclear cells.

with co-transcription factors, this might result in a cell type-specific and organ-specific expression of the genotype-dependent differences.

3. BSM-APA-TAQ AND 3′UTR POLYMORPHISMS

Most efforts to identify functional sequence variations in the VDR gene have focused on the 3′ regulatory region because this is close to the anonymous markers used mostly up until now in association studies (see Figs. 4 and 5). While the BsmI, ApaI, and TaqI RFLPs are located near the 3′ end of the gene, the LD extends into the 3′ regulatory region containing the UTR. We have already discussed the fact that the 3′UTR of the VDR gene contains many polymorphisms and, thus, through strong LD, these other polymorphisms might explain associations observed with Bsm-Apa and/or Taq RFLPs. The 3′UTR of genes is known to be involved in regulation of gene expression, especially through regulation of mRNA stability. This finding includes the steroid receptor genes which contain extensive 3′UTRs, such as the glucocorticoid receptor α [54]. For the latter receptor, polymorphisms in the 3′UTR have been described in the so-called AUUUA-motifs which influence the mRNA stability [55].

Morrison and colleagues provided evidence of differential luciferase activity for the two 3′UTR-variants that are linked to the two most frequent haplotypes, i.e., "baT" (haplotype 1 according to ref. 35) and "BAt" (haplotype 2). Durrin and colleagues have shown that certain parts of the UTR, so-called destabilizing elements, are involved in determining stability of the VDR-mRNA [28]. However, the UTRs linked to "baT" and "BAt" haplotype were not found by them to differ with respect to mRNA stability [28]. Furthermore, heterologous constructs (human VDR-UTR sequences coupled to a rabbit β-globin gene) and cell types (mouse NIH3T3 cells) were used to test for functionality. Especially, since it is known that 3′-UTRs display cell-type specific effects on mRNA stability, this could be important in demonstrating functionality of sequence variations in the UTR. Although it is assumed that mRNA stability differences might underly the allelic differences, alternative explanations should still be considered. Other studies have analyzed differences in expression levels according to the 3′ polymorphisms, and although there is a tendency for the BAt haplotype to display overall somewhat higher levels of mRNA expression than baT, the results have not been consistent (see Table IIA).

Recently, Whitfield and colleagues demonstrated functional significance of the translation initiation codon polymorphism (detected as FokI RFLP) and the poly(A) stretch in the 3′UTR [43]. In a series of 20 fibroblast cell lines of different VDR genotype, the relative transcription efficiency was measured of the endogenous VDR-protein. The VDRs differed by genotype at both the FokI RFLP (F and f alleles) and the poly(A) stretch with Long (L) and Short (S) alleles. The endogenous VDR protein is then acting as a transcription factor for a 1,25-dihydroxyvitamin D_3-responsive reporter gene (containing the rat osteocalcin gene VDRE), which is transfected in the cell. This study provided evidence for so-called high VDR activity (the "FL" genotype) and low VDR activity (the "fS" genotype). One of the possible explanations mentioned included differences in translational activity (rather than mRNA stability) of the different mRNA-3′UTR variants. However, further research is necessary to prove that assumption. In any case, this study also illustrated the importance of analyzing multiple polymorphisms in the VDR gene in relation to each other (as is illustrated in Fig. 8).

At another level, the responses by VDR genotype have been analyzed as differences in serum markers (Table IIB). VDR is then thought to act on vitamin D responsive genes, e.g., through gene-specific VDREs, which results in certain protein/protein fragments being secreted into the circulation. These 17 studies

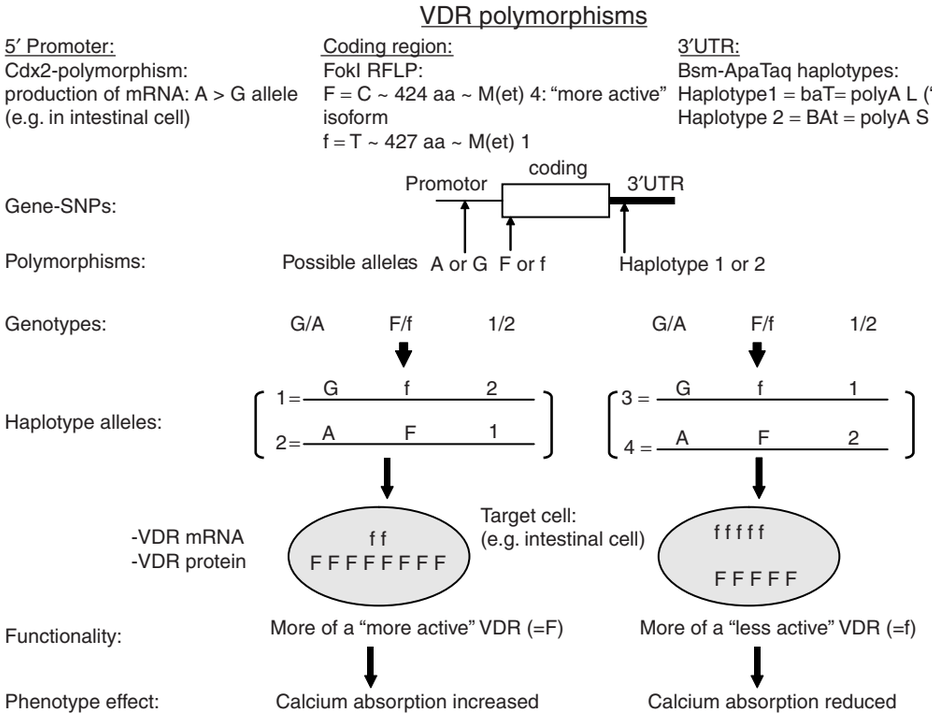

FIGURE 8 The importance of gene-wide haplotypes in the VDR gene. Three adjacent SNPs in different parts of the VDR gene are shown for two individuals (A and B indicated at the bottom). The subjects A and B have identical genotypes, i.e., they are both heterozygous for all three SNPs. However, they have different haplotype-combinations: 1+2 for subjects A and 3+4 for subjects B. The promoter area regulates production of mRNA while the 3′UTR is involved in stability/degradation of mRNA and their interaction/combined effects regulates the net availability of the mRNA for translation into the VDR protein. In this case, the example is shown for the Cdx-2 promoter polymorphism which has two alleles A and G, of which the A allele is the more active variant in intestinal cells. For the 3′UTR the two different variants, haplotype 1 and haplotype 2, are presented consisting of haplotypes of the Bsm-Apa-Taq RFLPs. The haplotype 1 is supposedly the more active/less unstable 3′UTR resulting in more VDR protein being produced. The VDR protein can occur in two variants: "little f " (less active, M1, 427 aa) and "big F" (more active, M4, 423 aa) and both individual A and B are heterozygous for this polymorphism. The result of the particular haplotype combinations is that individual A has less of the "risk" VDR protein, i.e., the little f variant (M1, 427 aa long), than individual B in the target cell. This could not have been predicted by analyzing single SNPs and/or only looking at genotypes of individual SNPs, but is only evident upon analysis of the gene-wide haplotypes.

include several different serum markers thought to be vitamin D specific, such as osteocalcin, PTH, and bone-formation—and resorption markers (see Table IIB for references). In particular osteocalcin has been analyzed because this is a highly vitamin D responsive gene, and it is frequently measured in clinical practice to monitor bone metabolism (for bone formation activity). Although 7 (out of 17) studies were not able to detect genotype-dependent differences in serum levels of osteocalcin (or other bone markers), 8 studies reported individuals with the BAt haplotype to have higher osteocalcin levels than those with the baT haplotype, while 3 (out of 17) studies observed an opposite trend.

A similar pattern can be distinguished when we analyze the studies which determined the *in vivo* response to treatment by VDR genotype (Table IIC). Studies included the analysis of response to treatment with calcitriol, calcium, corticosteroids, HRT, and etidronate and responses mostly involved measuring changes in BMD. Four out of 9 studies reported the response for BAt to be better than for baT. However, 3 out of 9 showed the opposite effect, while 3 showed no effect. Complicating factors to interpret these studies are, of course, the different endpoints being measured and the different polymorphisms in usually (and understandably) small studies most likely lacking power to demonstrate subtle effects.

Thus, when we examine Tables IIA–C, the picture is still complicated but there seems to be a trend for the BAt-haplotype (linked to Short polyA VNTR alleles in the 3′UTR) to display somewhat better responses than the baT-haplotype (linked to Long polyA VNTR alleles). Together with the *in vitro* studies there is therefore some argument to state that it might indeed be haplotype 2 (BAt) which—in general—confers a better response than haplotype 1 (baT). It is tempting to speculate that perhaps this is due to a slightly better mRNA stability and half life. This would theoretically result in higher numbers of VDR being present in the target cell giving this target cell a better response to vitamin D.

In view of what has been discussed previously, it is not very surprising that these "functional" studies have shown alleles of only few polymorphisms being consistently associated with all of the different parameters of functionality such as displayed in Fig. 7. This holds true for the FokI polymorphism and the Cdx2 polymorphism. Yet, the studies of the 3′ polymorphisms have been hampered. Major caveats applied to these studies are: (a) the use of the anonymous rather than functional polymorphisms to group subjects and cells by genotype, and (b) the use of different types of bioresponses and different cell types and cell culture conditions in which the vitamin D response might not be evident under the conditions of the experiment. Therefore, the identification of truly functional polymorphisms in the 3′UTR and the use of different well-defined cell types will help in clarifying the molecular mechanisms underlying the associations observed especially for the 3′ polymorphisms such as BsmI, ApaI, and TaqI.

In addition, it is very important to analyze all known VDR polymorphisms and their inter-relationships in such experiments since they will interact with each other to determine VDR expression and activity (see Fig. 8). This was indicated by the work of Whitfield *et al*. [43], and this is further illustrated in Fig. 8 where the interaction of promoter-, coding-, and 3′UTR-polymorphisms is highlighted. In a normally active cell, certain promoter polymorphisms will "join forces" with certain 3′UTR polymorphisms in regulating the amount of VDR mRNA being available in a certain target cell. Together, they determine the expression of the known Fokl variants, F and f, which are the (functionally different) VDR proteins that act, e.g., on VDREs to activate certain vitamin D responsive genes to be expressed. In the example, subjects A and B have identical genotypes at three polymorphisms (the Cdx2, the FokI, and the Bsm-Apa-Taq 3′UTR polymorphisms), but only differ in their particular combinations of alleles on chromosomes (i.e., their haplotypes). This ultimately results in less "high activity" VDR proteins (i.e., having the "F"-allele) being expressed in cells of subject B, which is then expected to display lower responses to vitamin D. In this case, the Cdx2 promoter variant is involved and differences between subjects are then expected at the level of calcium absorption in intestinal cells. However, when only one of the polymorphisms would have been tested in cell biological experiments, this would not have been detected (unless very large genotype groups would have been analyzed to detect the individual subtle differences). Moreover, if only the three individual polymorphisms would have been analyzed and the haplotypes would not have been taken into account, these effects would not have been noticed. Thus, not controlling for the underlying complexities in VDR polymorphisms, i.e., by not analyzing multiple polymorphisms and analyzing their haplotypes, can help to explain contradictory results from *in vitro* and *in vivo* functional experiments.

III. ASSOCIATION ANALYSIS IN DISEASE STATES

As many chapters in this book illustrate, the vitamin D endocrine system has been shown to be involved in a number of endocrine pathways related to calcium metabolism, immune-modulation, regulation of cell growth and differentiation (of keratinocytes, osteoblasts, cancer cells, T-cells), etc. [1]. Thus, for a pleiotropic "master" gene such as the VDR, one can expect to find associations of this gene with multiple traits and disease phenotypes. Indeed, VDR polymorphisms have been found associated with a number of different phenotypes of which several are supported by multiple independent and large studies reporting similar associations. However, inconsistencies have also been noted between these studies as well. This can be due to a number of factors and they are briefly summarized below.

1. Studies are mostly small and therefore of limited statistical power in order to detect differences of small effect size. This leads to spurious results occurring by chance rather than due to biological effects. It is important to realize that the effects of the VDR gene are expected to be small and, thus, will require large studies to demonstrate them. This holds true for clinical studies designed to find association with disease, as well as for molecular biological studies aiming to demonstrate molecular and cellular effects. In addition, investigators sometimes are too strict in claiming there is NO effect simply because the magic $p < 0.05$ is not reached and instead, for example, the p value = 0.08. This could mean that in that particular study there is a real

effect to be seen, but it simply needs more power to be detected. Thus, ultimately, meta-analyses (combining the results of a large number of individual association studies) will be crucial to estimate the effect size of a particular (set of) polymorphism(s). We will here refer to a number of excellent reviews on the topic of meta-analyses and their application in the field of complex genetics [76–78] for further information.

2. The polymorphisms that have been examined in the association studies are **anonymous**, i.e., they have no known function. This means that the polymorphism itself cannot explain the association due to some differential functional effect of the alleles but rather, that it merely serves as a marker for a truly functional polymorphism located elsewhere, but relatively close by, in the VDR gene. As a result different alleles can be reported to be the risk allele. A good example of this are the association studies (still) using the individual BsmI-, ApaI-, or TaqI-RFLP. The phenomenon underlying this is called *Linkage Disequilibrium* (LD), and it is described under Section II.C. Analysis of the LD over this 5.5 kb region at the 3′UTR of the VDR gene in different ethnic population groups indicated that the LD differed among populations [28]. A single RFLP, such as the BsmI RFLP which is the most frequently used in association studies of the VDR gene, is therefore not a good marker for the LD with other sequence variations and, thus, the use of the BsmI RFLP might contribute to heterogeneity among association studies. We have now established the LD and haplotype structure of the VDR gene and defined the borders of the genomic area to look for functional polymorphisms once an association has been observed. LD is different between populations and between ethnic groups and, thus, the linkage between the marker allele and the truly functional allele can be different, leading to allelic heterogeneity in the associations.
3. Polymorphisms within a gene are interacting with each other (**intragenic interaction**) to determine how that gene functions in a given cell and/or subject. This is illustrated for the VDR gene in Fig. 8. The 5′ promoter and 3′UTR polymorphisms determine how much of a given VDR mRNA will be expressed in which cell, while the coding variations determine how well that VDR protein does its job. When only one polymorphism is selected for study, this intragenic interaction is ignored. This can result in unrecognized heterogeneity between experiments/studies when different 5′ promoter variants and 3′UTR variants are interacting (see Fig. 8). Yet, if studies are of sufficient size, this type of intragenic interaction heterogeneity might be averaged out across the genotype groups being compared and the effect of an individual polymorphism can still be distinguished.
4. The misconception is that different endpoints (phenotypes) in a complex disease are expected to show similar, if not identical associations, for a given polymorphism. If, for example, an association with fracture risk has been observed, this does not necessarily imply that associations with BMD will be seen as well. Low BMD is only one of several risk factors for fracture. Also, the effect size of a polymorphism on BMD can be so minimal that it will be very difficult to demonstrate an effect on fracture risk: a decrease of 1 standard deviation (SD) in BMD is necessary to see a 2.5-fold increase in fracture risk, while the likely biological effects of individual polymorphisms will be about 0.1–0.3 SD. The associations of VDR polymorphisms with osteoporosis could be explained by a number of processes in which vitamin D plays a role (through the VDR) and which are all important for osteoporosis and fracture risk, including calcium absorption, osteoblast regulation, BMD, and muscle control. Similar arguments can be made for risk factors for other diseases (Kellgren score and joint replacement in osteoarthritis; hypertension and myocardial infarction in cardiovascular disease; insulin secretion and glucose level in diabetes; etc.).
5. The potential confounding effects, which arise from the pleiotropic effects of the vitamin D endocrine system and the VDR as a central player in this, can influence the associations observed. For example, VDR gene variants can influence calcium metabolism through differential absorption in the intestine and, at the same time, influence bone turnover. However, in the same individuals, the occurrence of osteophytosis (as a characteristic of osteoarthritis) can be influenced by the VDR gene variants, together with multiple other factors resulting in a net effect on BMD measured at a certain site, at a certain age, and in a subject with a certain dietary intake of calcium and with a certain disease history. Only with carefully designed studies that control for such factors and are of sufficient power, can such intricate interacting effects be disentangled.

Below, a brief review of association studies of VDR polymorphisms in relation to several disease states is presented. The discussion does not seek to be complete, but rather to illustrate the pleiotropic character of the vitamin D endocrine system, and in particular the effect of VDR gene polymorphisms. Several excellent reviews have appeared elsewhere which can be used as further reference on this topic [e.g., 39]. In Table III

TABLE III Pleiotropic Effects of VDR Alleles*

	Cdx2				FokI				BsmI-ApaI-TaqI-polyA			
	Number of studies	Number of subjects in association study		Overall allele effect***	Number of studies	Number of subjects in association study		Overall allele effect***	Number of studies	Number of subjects in association study		Overall allele effect***
		Positive	Negative			Positive	Negative			Positive	Negative	
PHENOTYPE/DISEASE												
Calcium handling												
Intestinal calcium absorption	–	–	–	–	1	72	–	F > f	9	435	375	baT > BAt
Calcium stone formation	–	–	–	–	–	–	–	–	5	721	283	baT > BAt
Bone												
Osteocalcin	–	–	–	–	7	–	1103	–	15	708	1136	BAt > baT
BMD	2	56	2848	A>G	14	1581	1151	F > f		Meta**		BAt > baT
Anthropometry	–	–	–	–	1	159	–	F > f	9	1463	209	baT >BAt
Muscle strength	–	–	–	–	–	–	–	–	1	264	–	baT > BAt
Fracture protection	1	2848	–	A>G	2	564	263	F > f	10	2073	1664	baT > BAt
Osteoarthritis												
Protection for knee OA/disc degeneration	–	–	–		1	160	–	F > f	9	1832	1634	BAt > baT
Cancer/proliferative disease												
Prostate cancer protection	–	–	–	–	4	895	237	f > F	19	3691	2537	BAt > baT
Prostate cancer protection**						Meta**		No effect		Meta**		No effect
Breast cancer protection	–	–	–	–	4	–	2013	F=f	10	2594	1759	BAt > baT
Colon cancer protection	–	–	–	–	3	1847	467	F > f	2	799	59	BAt > baT
Melanoma protection	–	–	–	–	1	424	–	F > f	3	?	1495	BAt > baT
Renal cell carcinoma protection	–	–	–	–	–	–	–	–	1	306	–	BAt > baT
Hyperparathyroidism protection	–	–	–	–	1	120	–	F > f	7	1443	370	BAt > baT
Psoriasis protection	–	–	–	–	–	–	–	–	4	528	175	baT > BAt

? = Inconclusive.

Continued

Table III Pleiotropic Effects of VDR Alleles*—Cont'd

	Cdx2				FokI				BsmI-ApaI-TaqI-polyA			
	Number of studies	Number of subjects in association study		Overall allele effect***	Number of studies	Number of subjects in association study		Overall allele effect***	Number of studies	Number of subjects in association study		Overall allele effect***
		Positive	Negative			Positive	Negative			Positive	Negative	
Immuno-modulation												
Diabetes mellitus type1 protection	–	–	–	–	6	1344	842	F > f	11	3138	314	BAt > baT
Diabetes mellitus type 2 protection	–	–	–	–	–	–	–	–	7	4171	189	BAt > baT
Sarcoidosis protection	–	–	–	–	–	–	–	–	3	206	265	?
Multiple sclerosis protection	–	–	–	–					1	172	–	BAt > baT
Rheumatoid arthritis	–	–	–	–	1	–	102	–	3	120	470	?
Crohn's disease protection	–	–	–	–	1	–	567	–	2	874	–	baT > BAt
Grave's disease protection	–	–	–	–	1	281	–	F > F	1	375	–	baT > BAt
Addison's disease protection	–	–	–	–	1	315	–	F > f	1	315	–	baT > BAt
Infection protection (TBC, leprosy mycobacterium, hepatitis B, HIV)	–	–	–	–	4	611	464	F > f	6	2961	464	BAt > baT
Periodontal disease protection	–	–	–	–	–	–	–	–	2	309	–	?
Cardiovascular disease												
Hypertension protection	–	–	–	–	–	–	–	–	3	1523	247	?
Atherosclerosis protection	–	–	–	–	–	–	–	–	3	241	3441	?
Congestive heart failure protection	–	–	–	–	–	–	–	–	1	–	88	–
Myocardial infarction protection	–	–	–	–	–	–	–	–	1	1978	–	BAt > baT
Total numbers	3	2904	2848		453	8373	7209		149	32,532	16,074	
Overall effect				**A > G**				**F > f**				**?**
		Risk allele:		**G**		**Risk allele:**		**f**		**Risk allele: ?**		**9 × baT** **14 × BAt**

*See text for further details on the associations.

** Meta-analysis has been performed; see text for further details.

***For measurements of quantitative traits (such as osteocalcin level or BMD), e.g., F > f means that the mean value for the measurement was higher in the F-group than in the f-group. For dichotomous traits such as disease we present the extent of protection against the disease (i.e., the inverse of the risk for disease). So, in that case F > f means that the risk for disease was higher in the f group.

? = Inconclusive.

the results of the association studies are summarized for the three most widely studied polymorphisms.

So far, the vast majority of association studies have been performed in Caucasians (including European and American Whites, Hispanics, and Arabics), while occasional reports have also appeared on studies in Asian populations (including Japanese, Chinese, and Korean subjects) and in African populations (i.e., African-Americans). Although, as discussed above (3 and 4), ethnic variation is an important source of heterogeneity between association studies, and we have not presented them separately here, because statistical power is usually far too low to draw definite conclusions on ethnic-specific effects of polymorphisms. However, once sufficient power is obtained in this respect, it will be necessary to interpret association studies separately for each ethnic group (see Section 3 below).

A. Osteoporosis

A plausible line of reasoning to implicate the vitamin D endocrine system in osteoporosis and fracture suggests that vitamin D regulates absorption of calcium from the intestine, thereby influencing bone mineral density (BMD), which in turn is an important determinant of the risk of osteoporotic fracture. However, apart from BMD, other risk factors for osteoporosis, such as anthropometry (bone size) and muscle control, are also under the influence of the vitamin D endocrine system (see Chapter 102). After the initial observation by Morrison and colleagues on the association between the VDR BsmI RFLP and BMD [22], some of these other risk factors for osteoporosis have been analyzed in association studies, and they are discussed below. However, only a few association analyses have focused on the clinically most relevant endpoint of osteoporosis: fractures. Most attention has gone to BMD while the number of studies on calcium absorption, anthropometry, and muscle strength have been rather limited. This reflects the widespread availability of BMD data, the most widely used diagnostic criterion for osteoporosis.

1. Intestinal Calcium Absorption

Several studies indicated that the relationship between VDR polymorphisms and BMD is modified by dietary calcium intake. In particular, Krall and colleagues [59] demonstrated that the association between the VDR 3′ polymorphisms and rates of bone loss became more evident at low calcium intake (i.e., mean < 400 mg/day). In addition, Kiel and colleagues [79] demonstrated that the relation between calcium intake and BMD was dependent on VDR genotype (for the 3′ BsmI RFLP). Taken together these studies suggest a gene-environment interaction in the relation between dietary calcium intake and BMD mediated by VDR genotype. Furthermore, these data suggest that the intestinal absorption of calcium is dependent on VDR genotype. Such a relationship was indeed demonstrated in five studies (with a total of 435 subjects) using the laborious isotope tests of intestinal absorption [80–84]. Four of these showed the B(At) haplotype allele to be associated with decreased calcium absorption compared to the b(aT) haplotype allele [80–82]. In line with this, in a study of 84 Thai women (in whom the frequency of the BsmI RFLP B-allele is lower (B < 10%) than in Caucasians; B = 42%), the b-allele was found to have higher urinary calcium excretion, which the authors believed to be due to higher intestinal calcium absorption [83]. One study of 72 children (7–12 yrs) showed the F-allele of the FokI RFLP (demonstrated to be the "more active" VDR; see above) to be associated with higher calcium absorption than the f-allele [84]. Nevertheless, these findings are not universal since four studies (with a total of 375 subjects) could not find any significant association between VDR genotype and calcium absorption [85–88].

Another VDR polymorphism of great interest in this respect is the Cdx2-polymorphism in the 1e/1a promoter area of the VDR gene (see also Section II,E,1 and references 30–32). This polymorphism is a G to A substitution in a binding site for the intestinal-specific transcription factor Cdx2 and has been shown to have differential binding affinity for Cdx2 (A > G; [31]). It is now thought that this polymorphism leads to different levels of expression of the VDR in the intestine, whereby the A-allele has higher expression levels than the G-allele. VDR regulates the expression of several calcium transport-related genes (e.g., calbindin and calcium transporters ECAC1 and ECAC2), which thereby would lead to higher calcium-absorption for the A-allele compared to the G-allele. This sequence of VDR mediated actions in the intestine is thought to ultimately result in increased BMD [31] and lower risk for fracture [32]. While several aspects of this hypothesis have indeed been shown to be the case, the direct influence of this polymorphism on the process of vitamin D–regulated calcium absorption in intestinal cells, has not been demonstrated yet.

The above discussion indicates that probably there is a modest contribution of the 3′ polymorphisms (defined as the BsmI-ApaI-TaqI haplotypes) and the FokI polymorphism to explain some of the interindividual variability in calcium absorption. These effects may be more pronounced in subjects with low dietary calcium intake (< 500 mg/day). Adding the likely (but so far undisclosed) effect of the Cdx2 polymorphism on

calcium absorption, the mechanisms shown in Fig. 8 act together to determine the contribution of genetic variations in the complete VDR gene to intestinal calcium absorption.

2. Calcium Stone Formation

Idiopathic calcium nephrolithiasis, or calcium stone formation, is related to the excretion of excessive amounts of calcium in the urine, or idiopathic hypercalciuria. Hypercalciuria is frequently associated with increased intestinal absorption of calcium, which in turn is regulated by the vitamin D endocrine system (Chapter 77). This rationale explains why several studies have analyzed the relation between the VDR gene and calcium stone formation.

A study to implicate the VDR gene in calcium stone formation was based on linkage analysis in French-Canadian sib-pairs, collected from renal stone clinics on the basis of the presence of at least one calcium stone episode [192]. This study demonstrated suggestive evidence for linkage of this trait to markers in and near the VDR gene, but VDR polymorphisms were not assessed in this study. This linkage analysis approach can implicate a gene in a certain phenotype. Following this study, six association studies have now appeared [193–198]. Four studies with a total of 721 Caucasian subjects showed the baT haplotype allele to be associated with either: (a) increased urinary calcium excretion [193], or (b) more aggressive stone disease [194,196], or (c) more hypocitruria, a risk factor for calcium nephrolithiasis [195]. One study with 214 Chinese subjects could not find evidence for a different distribution of BsmI RFLP alleles between stone formers and controls [197]. The initial study to assess the role of VDR polymorphisms in hypercalciuria did not observe a different distribution of BsmI alleles between 33 hypercalciuric patients and 36 race/age-matched controls [198].

Together with the previous studies on calcium absorption (see Section III,A,1 above) and urinary calcium excretion [83], these data suggest that the VDR baT haplotype allele is associated with increased calcium absorption and higher urinary calcium excretion, resulting in a somewhat increased risk for calcium stone formation. In view of the frequently observed decreased BMD in hypercalciuric patients, this also suggests a possible mechanism explaining the associations of VDR genotype with BMD.

3. Bone Mineral Density

In the initial studies on VDR polymorphisms, Morrison *et al*. reported that the BsmI RFLP in the last intron of the VDR gene was related to serum osteocalcin concentration [21]. These authors subsequently found the BsmI RFLP to be associated with differences in BMD in a twin study and in postmenopausal women [22]. Although the initial observations on the twin study have been withdrawn [89], in the following years dozens of papers were published analyzing the same RFLP in relation to BMD. Some of these confirmed the initial observation, while others could not find an association or found another allele associated. In the largest single cohort study published so far and which analyzed 1782 Dutch elderly men and women, no effect of single RFLPs on BMD was observed, but a small effect was detected employing haplotypes constructed of the three adjacent 3′ RFLPs [35]. A meta-analysis of 29 studies (excluding the Dutch cohort) on the relationship of VDR genotype with BMD [90] concluded that VDR BsmI RFLP genotype is associated with BMD in elderly subjects but with only 1–2% difference between extreme genotypes with the B allele being associated with 1–2% lower BMD. In addition, Gong and colleagues analyzed 75 articles and abstracts on the relation between VDR genotype and BMD [91], and in particular tested whether either of the b-, a-, T-, or F-alleles were associated with increased BMD, BMC, cortical area, thickness and/or diameter, higher response to vitamin D treatment, increased calcium absorption, and/or low bone turnover rate, bone loss, and frequency of fractures. They concluded that increased BMD is indeed associated with the b/a/T/F-allele, especially in females before the menopause thus contributing to peak bone mass. However, the effect on BMD of the 3′VDR polymorphisms by themselves is small and, thus, difficult to consistently detect.

When analyzing individual effects of the FokI RFLP on BMD, the "f" allele (corresponding to the 427 aa longer VDR protein with somewhat lower activity) has been found associated with a lower BMD in nine study populations [26,27,69,84,92–96] with a total of 1581 subjects, mostly women. Five other studies, however, with a total of 1151 subjects, have not detected a significant effect on BMD [68,97–100]. Taken together, the results indicate that there probably is a real effect of the FokI RFLP on BMD and that the effect size of this polymorphism is probably similar to what is seen for the 3′ polymorphisms (1–3%).

The FokI RFLP is not in linkage disequilibrium with the 3′ polymorphisms (see above) and can therefore not "explain" the association results of the BsmI-, ApaI-, and TaqI polymorphisms. Rather, it should be treated as a separate marker with individual effects. In line with what is shown in Fig. 8, these polymorphisms are likely to have intragenic interaction resulting in combined effects, but this has not been extensively analyzed so far. However, suggestive evidence for such intragenic interaction between the FokI RFLP and the 3′BsmI, ApaI, and TaqI RFLPs has been obtained by Ferrari

and colleagues with respect to effects on BMD [93]. Also see the work of Haussler and colleagues with respect to functional molecular and cellular effects of combined VDR polymorphisms discussed in Chapter 13 and Section II,E,2.

The recently described G to A sequence variation in the Cdx-2 binding element just upstream of exon 1e/1a has also been found to be associated with BMD (see Section II,E,1). Arai and colleagues reported that the G-allele had a decreased transactivation capacity and was associated with a 10% decrease in lumbar spine BMD in 58 postmenopausal Japanese women, but not in premenopausal women. More recently, we have extended the association analysis of this polymorphism with osteoporosis to a large group of >2200 postmenopausal Caucasian women [32], but we could not observe Cdx2 genotype-related differences in BMD. Perhaps this is due to the relatively high dietary calcium intake in these subjects and/or differences in vitamin D serum levels between (ethnic) populations. Further research is necessary to understand these differences between studies.

4. ANTHROPOMETRY

Bone size is an important determinant of bone strength, and thus, a risk factor for osteoporosis. Vitamin D is known to regulate the proliferation, differentiation, and maturation of cells responsible for skeletal growth, i.e., the chondrocytes of the epiphysial growth plate, and osteoblasts (see Chapter 33). Hence, genetic variations in the VDR gene might contribute to interindividual differences in bone dimensions, growth and skeletal size characteristics, expressed as difference in height/stature, vertebral area, or femur shaft diameter. Indeed, six studies (with a total of 1463 subjects) have shown associations between the 3′ end polymorphisms and anthropometric measurements [101–106]. While most studies found the b-allele (in the baT haplotype) to be associated with increased height, two studies found the opposite allele associated [107,108], while one study could not find a significant association [109]. So far, only one study has analyzed the FokI RFLP in relation to height in 159 Japanese young adults, with the f-allele being associated with a lower height [110]. Altogether, the data indicate several polymorphisms across the VDR gene to be associated with anthropometric differences.

5. MUSCLE STRENGTH

VDRs are expressed in striated muscles, and decreased serum 25(OH)D3 has been found to correlate with decreased muscle strength (see Chapters 55 and 102). However, only one study has analyzed the association between VDR polymorphisms and muscle strength [111]. In 264 nonobese (BMI < 30) Caucasian women (>70 yrs), the b(aT) allele was found associated with increased muscle strength measured as quadriceps and grip strength. The association of the VDR Bsm RFLP with muscle strength explained the association with femoral neck BMD also observed in these (nonobese) women. BMD is known to be correlated with muscle strength. Together, these associations suggest that VDR polymorphisms might play a role in determining muscle strength, and, through variation in fall risk, might influence the risk of osteoporotic fracture rather than by influencing BMD. However, studies that include assessments of muscle strength together with incidence of fractures and measurements of BMD are necessary to prove this assumption. This hypothesis is further discussed in Chapter 102.

6. FRACTURES

After the initial findings of Morrison *et al*. [22,89], it was thought that the VDR genotype-dependent differences in BMD would translate into VDR genotype-dependent differences in fracture risk. This has initiated both ecological as well as epidemiological studies on the relation between VDR genotype and fracture risk. An ecological analysis of 14 studies suggested that higher population frequencies of the (BA)t allele were correlated with higher age-adjusted hip fracture rates [112,113]. Eleven epidemiological studies on the relation of VDR polymorphisms and fracture risk have appeared. Six studies of a total of 2073 Caucasian men and women [114–119] suggested that the VDR genotype was associated with increased fracture risk, including both hip and vertebral fractures. This effect was mostly independent of VDR genotype-related differences in BMD [118], reflecting the relatively small effect of VDR genotype on BMD (see also the results of the meta-analysis on this relationship). Although five of the studies indicated the B-allele (as present in the BAt haplotype allele) was associated with increased fracture risk, one large study of 1004 women [118] showed the baT haplotype to be the risk allele. Four other studies with a total of 1664 Caucasian women did not find a relationship between fracture risk and VDR genotype as defined by the Bsm, Apa, and/or Taq polymorphism [120–123].

The FokI RFLP has been analyzed in two studies of which one reported an increased fracture risk for the f-allele seen in 564 postmenopausal women [124] while the other study did not see an effect in 263 elderly men and women [117]. These results might indicate a small risk associated with the f-allele (corresponding to the 427 aa longer VDR protein with a somewhat lower transactivation activity (see Section II,E,2).

The Cdx2-polymorphism has been analyzed in only one study cohort, a large group of 2848 Caucasian women from the Rotterdam Study [32]. In this cohort, we found the A-allele (18% frequency in Caucasians) to be associated with a protective effect on fracture risk, and so the G-variant (82% population frequency in Caucasians) was associated with increased fracture risk in Caucasians. Although this polymorphism was reported to be associated with BMD differences in a small cohort of Japanese postmenopausal women (see above), we have not seen BMD differences related to this polymorphism in our cohort of Caucasian women. The lack of an effect could be due to interaction with the relatively high calcium intake in this group of Caucasian women, and/or differences in circulating vitamin D levels between Caucasian and Asian subjects, but this requires further study.

When we view the data on Cdx2, FokI, and the Bsm-Apa-Taq polymorphisms together, these data suggest that there probably is a VDR genotype-dependent increased fracture risk. Again, intragenic interaction mechanisms such as depicted in Fig. 8 might underly this effect. It is also clear that the effect on fracture risk is caused by other factors in addition to the small effects on BMD. In view of what has been discussed above, the genotype-dependent differences in fracture risk could be explained, in part by VDR genotype-dependent differences in bone geometry, or muscle strength, or combinations of these. Studies controlling for all of these factors will be necessary to disentangle these effects and determine the combined effects of the VDR polymorphisms. Only one meta-analysis has been done [90] but others, using more subjects and focusing on endpoints other than just BMD, are eagerly awaited.

B. Osteoarthritis

The radiographic "Kellgren score" used to diagnose osteoarthritis (OA) is composed of asessment of osteophytosis (bony outgrowths) as well as of cartilage damage by measuring joint space narrowing (JSN). The vitamin D endocrine system has been implicated in the etiology of osteoarthritis/osteoarthrosis by demonstration of expression of the VDR in chondrocytes, which produce the cartilage and are also present in osteophytes [125,126]. In addition, observations of low vitamin D levels in subjects with radiographic osteoarthritis of the knee lended further support to the notion that vitamin D can play a role in the etiology of OA [127] and that perhaps polymorphisms in the VDR play a role in explaining some of the interindividual variability of risk for OA. Five studies with a total of 1832 men and women found indeed an association between the VDR Bsm-, Apa-, or Taq-polymorphism with aspects of osteoarthritis, assessed either as radiographically determined OA (ROA) of the knee, hip dysplasia, or spinal/intervertebral disc degeneration [128–132]. However, four other studies involving 1634 subjects did not find significant evidence for such an association [133–136]. One study that also analyzed the FokI polymorphism in 160 twin males, found evidence to suggest that the f allele (corresponding to the 427 aa longer VDR protein with a somewhat lower activity) was associated with increased risk of intervertebral disc degeneration [132]. Interestingly, also here allelic heterogeneity and controversy exists for the Bsm-Apa-Taq polymorphisms in that two studies found the "baT" haplotype to be associated with increased risk for OA, while three other studies found the "BAt" haplotype to be the risk allele.

A problem with interpreting the studies of VDR polymorphisms and OA is the diversity in OA phenotypes. This ranges from subphenotypes in the Kellgren score (osteophytosis and JSN) to the clinically relevant phenotype requiring joint replacement. Similar to the situation for osteoporosis, we have to realize that finding an association with the Kellgren score does not necessarily mean that the association will translate to risk for joint replacement. Joint replacement is dependent on many other risk factors as well, such as joint pain, and it is well-known that the Kellgren score does not correlate well with joint pain.

An interesting observation in this line of research is that the VDR gene happens to be located very near the COL2A1 gene (see Fig. 2), which encodes the major constituent of cartilage, i.e., the collagen type 2 protein. Thus, one possible explanation for the associations found for the VDR gene with OA is that these were explained by LD of the VDR (marker) polymorphisms with (functional) polymorphisms in the COL2A1 gene. By analyzing polymorphisms in both genes in relation to OA in the same population as was used to find the VDR associations, we were able to exclude this explanation [137]. Rather, it seems that both genes are associated with OA, but with different aspects of the disease. The association with VDR polymorphisms seems to be driven by osteophytosis, the bony characteristic of OA, while the association with COL2A1 seems to be driven by JSN, which reflects cartilage loss. Also in view of the function of these gene products, such associations would make sense.

It is interesting to note that osteophytosis is also regarded as a loss of growth inhibition for cells present in the subchondral bone [126]. In this respect, the involvement of the VDR in osteophytosis could be very similar to the involvement of the VDR in cancer (see below) and in fact might reflect a similar mechanism

(variation in the control of cell growth by VDR) underlying the association.

Taken together, both the 3′ polymorphisms, as well as the FokI polymorphism, seem to be associated with OA. However, similar to what we discussed for the other disease phenotypes, meta-analyses will be necessary to determine the true effect size of the individual polymorphisms, while intragenic interaction of the different polymorphisms will have to be taken into account.

C. Cancer and Hyperproliferative Disease

The role of vitamin D in cancer etiology has been widely studied and is the subject of separate chapters in this book (see all chapters in Section IX). It is therefore not surprising that the role of VDR polymorphisms in relation to interindividual variability of risk for different types of cancers has been studied, including prostate cancer, breast cancer, melanoma, colon cancer, lung cancer, renal cell carcinoma, and some hyperplastic syndromes including hyperparathyroidism.

1. Prostate Cancer

Because of striking relationships between the incidence of prostate cancer and sunlight/UV exposure (expressed as latitude on earth), suggesting a relationship with vitamin D production, the study of VDR polymorphisms in prostate cancers has received the most attention with now 21 studies having analyzed 7360 subjects (see Table III), including mostly Caucasian men but also African (n = 325) and Japanese (n = 1902) subjects.

Nine studies reported an association between the BsmApaTaq polymorphisms and prostate cancer risk, seven of which identified the baT haplotype as the risk allele [138–144] and two studies the BAt haplotype allele [145,146]. Ten studies did not find a significant association between these polymorphisms and prostate cancer [147–156]. Four studies including 1132 subjects analyzed the FokI polymorphisms, two of which found the f-allele (corresponding to the 427 aa longer VDR protein with a somewhat lower activity) to be associated with decreased risk of prostate cancer or decreased aggressiveness [156,157], while one identified the f-allele as a risk allele [158]. One study did not find a significant association with FokI [146]. Several indications for gene-environment interactions have been observed, including augmentation of the association in subjects with low vitamin D levels [140]. A formal meta-analysis of 17 studies, including Caucasian, Asian, and African study populations, could not find significant evidence for any of the four VDR polymorphisms analyzed, i.e., FokI, BsmI, TaqI, and polyA, to be a major determinant of susceptibility to prostate cancer [158a]. This meta-analysis did not address haplotypes of the 3′ polymorphisms, while some weak and nonsignificant trends corresponding to a slight protective effect of the B(A)t-haplotype allele could be observed. Although such an effect would correspond to what is noted in Table III for the effect of the 3′ VDR polymorphisms on prostate cancer, the robust design of this meta-analysis excludes the polymorphisms analyzed to have large effects, if any, on prostate cancer.

2. Breast Cancer

Numerous studies have appeared showing similar patterns for breast cancer as for prostate cancer. Eight studies including 2594 subjects, mostly Caucasian women, observed an association of the Bsm-, Apa-, or Taq-polymorphisms with increased risk for breast cancer, defined as either sporadic breast cancers, breast cancer progression, or risk for (bone) metastasis. Again, there is some evidence for allelic heterogeneity with five studies with 1719 women, identifying the baT haplotype allele as the risk allele [159–163], and three studies, with 875 women, identifying the BAt haplotype as the risk allele [164–166]. Two large studies of a total of 1759 Caucasian women [167,168] did not find evidence for significant association, although one study suggested interaction with hormone replacement therapy (HRT), with the baT haplotype increasing breast cancer risk only in HRT users [168].

Somewhat surprisingly, four large studies with a total of 2013 women and derived from the same study populations in which the 3′VDR polymorphisms were analyzed have not found any significant evidence to suggest the FokI polymorphism modifies the risk for breast cancer [160,162–164].

3. Colon Cancer

Two studies on the relation between Bsm-Apa-Taq polymorphisms and colorectal carcinoma have appeared. One large study involving 799 subjects showed the baT haplotype allele to be associated with increased risk for colon cancer [169], while a smaller study of 59 subjects could not find a significant association [170]. Of particular interest was the observation that the risk was increased in subjects with lower dietary vitamin D intake, suggesting a gene-environment interaction [169].

Four studies on the FokI polymorphism and colorectal cancer have appeared with a total of 2341 subjects [171–174]. Two studies (with a total of 1847 subjects) found an association albeit that allelic heterogeneity was evident: one study including 767 Caucasian subjects found the F-allele to be the risk allele [171], while a study with 1107 Chinese subjects found the f-allele to be the risk allele [172]. A study with 467 Caucasian

subjects found no significant association between the FokI polymorphisms and risk for colorectal cancer [173].

4. Melanoma and Basal Cell Carcinoma

Three studies have analyzed VDR polymorphisms in relation to malignant melanoma and basal cell carcinoma (BCC). One study observed an increased risk for BCC [175] while two others, involving 1495 subjects, could not find significant evidence to support this [176,177]. Perhaps this is due to the choice of a particular subphenotype, the multiple presentation phenotype, in the latter association study [177]. One study analyzing 424 subjects [176] observed the f-allele of the FokI polymorphism to be associated with increased risk of malignant melanoma in a hospital-based case-control study. In addition, they found evidence for intragenic interaction between the FokI and Bsm-Apa-Taq polymorphisms in that subjects homozygous for the f-t combination of alleles had the highest risk for increased Breslow thickness, a measure to express thickness of the tumors [176].

5. Renal Cell Carcinoma

A single study reported an association of the baT haplotype allele with increased risk for the aggressive renal cell carcinoma, observed in 306 Japanese subjects [178].

6. Hyperparathyroidism

Vitamin D has a strong influence on parathyroid hormone (PTH) secretion and proliferation of parathyroid cells (see Chapters 30, 76, 78, and 103). Several studies have suggested that VDR polymorphisms influence the risk of primary and secondary hyperparathyroidism. Both diseases are characterized by benign parathyroid adenomas or parathyroid hyperplasia, and are accompanied by substantial elevations in the secretion of PTH. While four studies, with a total of 1443 subjects, have observed the baT haplotype allele to be associated with increased risk for primary [179–181], as well as secondary hyperparathyroidism [182], three other studies, with a total of 370 subjects, could not find significant evidence to support this [183–185]. Only one study analyzed the FokI polymorphism [184] and found in 120 women that the f-allele was associated with increased risk for primary hyperparathyroidism.

7. Psoriasis

Psoriasis is a common skin disease characterized by hyperproliferation of keratinocytes and inflammation. It has also immunological elements and could be discussed under Immune Related Diseases. VDRs have been demonstrated in keratinocytes and vitamin D is known to inhibit proliferation and to induce terminal differentiation of cultured keratinocytes (see Chapters 35 and 101). Four studies have analyzed the association of VDR polymorphisms with the risk of psoriasis [186–188]. Three studies (of Korean and Japanese subjects) observed an association of the Bsm-Apa-Taq polymorphisms, but with evidence for allelic heterogeneity. Two studies, with a total of 344 subjects, found the BAt allele associated with increased risk [186,187], while one study with 184 subjects found the baT allele to be the risk allele [188]. One study of 175 Caucasians could not find evidence for association with the BsmI polymorphism [189].

Four studies have addressed the VDR genotype-dependent differences in response to treatment of psoriasis with calcipotriol cream, a potent vitamin D analog used for topical treatment of the disease. Three studies of 166 patients could not find significant evidence for a VDR genotype-dependent difference in response to calcipotriol [189–191], while a recent study analyzing the FokI polymorphism found the f-allele to be increased in frequency among nonresponders [188].

D. Immune-related Diseases

1. Diabetes

Vitamin D has important immunomodulatory properties (see Chapters 36, 98, and 99). $1,25(OH)_2D_3$ inhibits T-cell proliferation and can suppress both TNFα as well as IL-1 production. The vitamin D endocrine system can inhibit pancreatic insulin secretion while in the NOD mouse administration of $1,25(OH)_2D_3$ can prevent the development of insulin dependent diabetes mellitus (IDDM), as well as the autoimmune insulinitis. Furthermore, vitamin D levels were found to be reduced in subjects at risk for development of noninsulin dependent diabetes mellitus (NIDDM). Thus, the vitamin D endocrine system can play a role in the etiology of diabetes type 1 (IDDM) as well as type 2 (NIDDM). This explains why the role of VDR polymorphisms was analyzed in both type 1 (T1DM) and type 2 diabetes mellitus (T2DM). Studies involving mostly Caucasians, but also Indian and Japanese subjects have been performed.

Both linkage analysis of families and population-based association studies with VDR polymorphisms have been done. Three Transmission Disequilbrium Testing (TDT) studies with a total of 449 families (corresponding to roughly 1500 subjects) have found initial evidence to suggest that VDR polymorphisms influence risk for T1DM [199–201]. Two studies found the baT haplotype allele associated with increased risk for T1DM [199,200], while one study found the BAt haplotype allele as the risk allele [201]. The involvement

of VDR polymorphisms in T1DM etiology was soon confirmed by association studies [202–207]. Similarly, three studies with a total of 747 subjects found the baT allele as the risk allele for T1DM [202–204], while three other studies encompassing 891 subjects, identified the BAt as the risk allele [205–207]. Two studies could not find evidence for association of the BsmI, ApaI, or TaqI polymorphisms with T1DM [208,209].

In partly the same study populations, the contribution of the FokI polymorphism to the risk for T1DM has been analyzed. Three studies, including one TDT analysis, with a total of 1344 subjects demonstrated significant association between the F-allele and increased risk for T1DM [200,209,210], while three other studies, also including one TDT analysis, found no significant evidence for a contribution of the FokI polymorphism to a risk of T1DM [201,203,204]. The mechanism whereby the association of VDR polymorphisms with T1DM is explained remains unclear. The selective beta-cell destruction seen in T1D is caused by a T-cell mediated autoimmune process. Vitamin D has been shown to inhibit T-cell activation both *in vitro* and *in vivo* and inhibits the secretion of IL-1, IL-2, IL-6, IL-12, TNFα, and IFNγ. These cytokines play an important role in the development of T-cells, which are thought to be involved in the pathogenesis of several chronic inflammatory autoimmune diseases. VDR genotype-dependent differences in regulation of cytokine production and/or cell growth inhibition could therefore be the basis for the observed association of VDR polymorphisms with T1DM. However, more molecular and cell biological experiments will be neccesary to prove this.

To assess the involvement of VDR polymorphisms in type 2 diabetes mellitus (T2DM) seven studies have been performed on a total of 4360 subjects, mostly in Caucasians although the initial study was done in Bangladeshi Asians. Four studies with a total of 2339 subjects reported the baT allele to be associated with increased risk for T2DM [211–214], sometimes expressed as decreased insulin secretion [211,211a] or an increased risk for obesity in T2DM subjects [213], while two other studies involving 1832 subjects reported the BAt allele to be the risk allele [215,216], sometimes expressed as higher fasting glucose levels [216]. One study (of 189 subjects) could not find a significant association [217]. The mechanism whereby VDR polymorphism associations with T2DM were explained remains unclear. It could involve direct regulation of insulin secretion [211,211a] or perhaps inhibition of growth of pancreatic beta-cells. As explained earlier in this chapter, LD with another gene is a very unlikely explanation.

2. OTHER AUTOIMMUNE DISORDERS

In line with what has been observed for diabetes, other autoimmune disorders also have been reported to be associated with altered vitamin D levels and VDR polymorphisms, including sarcoidosis, multiple sclerosis, Crohn's disease, Graves disease, and Addison's disease. (See also Chapter 98.)

For sarcoidosis, a systematic granulomatous disorder of unknown etiology, three studies were reported of which one found the BAt haplotype to be associated with increased risk [218], while two other studies could not find an association [219,220].

Multiple sclerosis (MS) is an autoimmune disorder directed against the myelin sheath around axons in the central nervous system. For multiple sclerosis, two studies on the relation with VDR polymorphisms have appeared that describe the same association in the same population, i.e., the baT haplotype was associated with increased risk for multiple sclerosis in 172 Japanese subjects [221,222]. However, a linkage study of 187 Canadian families encompassing 236 sibling pairs analyzed the ApaI and TaqI polymorphisms in the VDR gene and a microsatellite (D12S85) near the VDR gene, by Transmission Disequilibrium Testing (TDT), and could not find evidence for linkage or association of the VDR gene with MS [222a]. In addition, this later study was unable to demonstrate linkage or association with MS for polymorphisms in other genes of the vitamin D endocrine pathway, i.e., the gene encoding the vitamin D–binding protein (4q12) and the gene encoding the 1α-hydroxylase (12q13). While TDT is rather insensitive to detect the more subtle effects of polymorphisms, this study excludes major effects of variations in these vitamin D–related genes to play a role in the etiology of MS.

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease of unknown etiology manifested by inflammation of joints and resulting in progressive joint destruction. Three studies analyzed the distribution of VDR polymorphisms between RA cases and controls with a total of 590 subjects, of which 368 were Korean [222 b,c,d]. The study of a Spanish population observed a slightly earlier onset of RA in the B(A)t-haplotype carriers [222b], the two other studies could not find association for the 3′ polymorphisms with RA. Only the German study analyzed the contribution of the FokI polymorphism but could not find no evidence for an association with RA [222d].

For Crohn's disease [Inflammatory Bowel Disease or IBD], which is characterized by aberrant regulation of mucosal immune response, two studies appeared with a total of 874 subjects which both found the BAt haplotype allele to be associated with increased risk for Crohn's disease [223,224]. For the FokI polymorphism no association was observed [223].

Graves' disease (GD) is an autoimmune thyroid disease in which TSH receptor autoantibodies cause hyperthyroidism. Two studies of the relation between VDR polymorphisms and GD of a total of 656 Japanese subjects found an association, one with the BAt haplotype allele [225] and one with the F allele of the FokI polymorphism [226].

Finally, Addison's disease (AD) is an uncommon disorder that results from the T-cell mediated destruction of adrenocortical cells and shares a number of genetic susceptibility markers with T1DM, GD, and Hashimoto's thyroiditis, including the 12q12 region where the VDR is located. Only one study on the relation between VDR polymorphisms and AD has been conducted and found the fallele of the FokI polymorphism and the BAt-haplotype allele of the Bsm-Apa-Taq RFLPs to be associated with increased risk for AD [227].

3. SUSCEPTIBILITY TO INFECTION

Susceptibility to infectious disease caused by bacteria and/or virus is influenced by environmental factors and by genetic factors in the bacterial/viral agents (determining its ability to infect). Also important are host factors that determine the susceptibility of that host for infection by the infectious agents. In view of what is known about the role of vitamin D in the immune response (see above and Chapters 36 and 98) and what has been described above on the VDR role in autoimmune disorders, it is not very surprising that the role of VDR polymorphisms has also been examined in relation to susceptibility to a number of different infectious diseases. These include tuberculosis [228,229,232,233], leprosy [230], *Mycobacterium malmoense* [231], as well as the hepatitis B and HIV virus [232,234]. The results are summarized in Table III and similar to what has been seen before, the results vary in the particular allele being associated (baT or BAt and F or f as the risk allele) and some studies not showing an association. In line with this, associations also have been reported between VDR Bsm-Apa-Taq alleles and periodontal disease as well as the intra-oral inflammation seen after bacterial infection [235,236].

E. Cardiovascular Disease

A number of studies have analyzed serum vitamin D levels and VDR genotypes in relation to heart disease. The observed relationships suggest an involvement of the vitamin D endocrine system in the etiology of several aspects of heart disease (see also Chapters 54 and 56). Two studies of a total of 1523 subjects observed Bsm-Apa-Taq haplotypes to be associated with increased risk of high blood pressure or hypertension, albeit with evidence for allelic heterogeneity with one study finding the baT as the risk allele [237], while another study found the BAt allele as the risk allele [238]. One study (in 247 Japanese) could not find evidence for an association of BsmI polymorphism with hypertension, although the B-allele/BAt haplotype was associated with decreased serum calcium levels [239].

In view of the involvement of vitamin D in calcium handling and the occurrence of calcification processes (including expression of many bone genes such as osteocalcin) at atherosclerotic plaques, the involvement of VDR genotype in atherosclerosis has been investigated. Two studies with a total of 241 subjects found association, again with evidence for allelic heterogeneity. One study observed the baT as the risk allele [240], while another study of 200 subjects found the BAt haplotype as the risk allele [241]. However, one large study of 3441 subjects could not find an association [242].

Congestive heart failure (CHF) is a cardiac dysfunction syndrome characterized by a reduced left ventricular ejection fraction, with muscle weakness and fatigue as major symptoms. An alteration in intracellular calcium handling seems to play a role in the impaired contractility of the myocardium. Several studies have found vitamin D deficiency in CHF patients. VDR is expressed in cardiac muscle and is known to activate calcium channels in cardiac muscle cells. However, one study of 88 CHF patients and controls could not find evidence for an association of VDR genotype with CHF [243].

Finally, we have observed in a large sample of 1978 men and women from the Rotterdam Study increased risk for myocardial infarction (MI) for carriers of the baT haplotype. Although the overall effect was modest (only 20% more risk), the relationship was modified by dietary calcium intake with those with high dietary calcium intake (>1200 mg/day) having three- to fourfold increased risk for MI [244]. The relationship seemed to be independent of known risk factors such as atherosclerosis and hypertension.

In view of the above associations, and the clear involvement of the vitamin D endocrine system in cardiac function, it seems probable that VDR genotype influences risk of heart disease, but the mechanism whereby this occurs remains unclear and, thus, more studies are necessary.

IV. CONCLUSIONS

When we survey the data in Tables II and III, it seems that the different VDR polymorphisms studied until now (Cdx2, FokI, and the 3′ Bsm-Apa-Taq RFLPs) are associated with differences in biological responses and risk of disease. The findings are, however, far from

universal as is clear from the fact that the studies finding positive associations are mostly balanced by those that are not. More formal meta-analyses will be necessary to deal with this and to assess the magnitude of effects, but they are likely to be small. The first meta-analyses, on BMD, indeed demonstrated this to be the case. This is not surprising because most of the time we are looking at complex traits and diseases in which multiple genes play a role, of which the VDR gene is likely to be just one of many.

A major limitation of the association studies using VDR polymorphisms in relation to disease endpoints has been the limited number of polymorphisms that have been analyzed and, thus, the lack of control of intragenic interactions between polymorphisms. In addition, the lack of statistical power of most studies to detect the expected subtle effects and misconceptions about how such small biological effects could be translated to the risk of disease has led to a number of controversies in the field. Interactions among genes and interactions with environmental factors also play a role in the action of this pleiotropic steroid hormone receptor transcription factor. For example, dietary Ca-intake is known to differ substantially between countries and populations, while circulating serum vitamin D levels, which are determined by several metabolizing enzymes, also differ between populations. Consequently, gene-gene and gene-environment interactions can then differ between different populations and will seriously affect interpretation of association results. Only very large studies that can control for these factors are able to overcome this difficulty.

It is likely that still more polymorphisms, including functional ones, will be discovered in the complex promoter region of the VDR gene, and large population studies will be necessary to document the LD over the region and to evaluate the associations with relevant disease endpoints. In particular, studies should be undertaken in which the VDR gene is systematically scanned for sequence variations, such as has been done for other candidate genes. Haplotype analyses should be used to identify groups of SNPs linked together and, thus, simplify the association analyses and increase understanding of the associations observed. Until clearly functional polymorphisms are identified in the VDR gene, interpretation of meta-analyses to evaluate consistency of associations and estimate effective size of a polymorphism will be cumbersome. This is especially true for the 3′ polymorphisms where we still do not know which polymorphism(s) in linkage with these variations are driving certain associations. For the Cdx2 promoter polymorphism evidence seems convincing to conclude this to be a functional polymorphism while more association studies are required to demonstrate its effects, particularly in intestinal calcium metabolism. Also for the FokI polymorphism, evidence seems convincing to conclude this is a functional polymorphism.

Taken together, it is clear that multiple polymorphic variations exist in the VDR gene that could each have different types of consequences (as is illustrated in Fig. 8). Thus, 5′ promoter variations can affect mRNA expression patterns and VDR levels while 3′UTR sequence variations can affect the mRNA stability and/or protein translation efficiency. In combination, these genotypic differences are likely to affect the VDR protein concentration and/or function, depending on the cell type, developmental stage, and activation status.

In summary, one can conclude that VDR gene variants seem to influence a number of biological endpoints. Yet, the associations have different magnitudes with BMD probably being one of the weaker effects. In different study populations, different alleles of the anonymous RFLPs can be found associated with the same endpoint. This probably reflects that linkage disequilibrium, between the anonymous marker alleles and the causative alleles in the VDR gene, is likely to be different between populations. Finding functional sequence variants that matter, establishing the phase of alleles across the entire VDR gene, and defining haplotype patterns is therefore required to put the associations observed with VDR gene polymorphisms in biological perspective. Meta-analyses of association studies are the way forward to determine the effect size of the small but true effects on disease risk.

References

1. Haussler MR, Whitfield GK, Haussler CA, Hsieh J-C, Thompson PD, Selznick SH, Encinas Dominguez C, Jurutka PW 1998 The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res **13**:325–349.
2. Lander ES, Schork NJ 1994 Genetic dissection of complex traits. Science **265**:2037–2048.
3. Risch N, Merikangas K 1996 The future of genetic studies of complex human diseases. Science **273**:1516–1517.
4. Baker AR, McDonell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW 1988 Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci USA **85**: 3294–3298.
5. Miyamoto K-I, Kesterson RA, Yamamoto H, Taketani Y, Nishiwaki E, Tatsumi S, Inoue Y, Morita K, Takeda E, Pike JW 1997 Structural organization of the human vitamin D receptor chromosomal gene and its promotor. Mol Endocrinol **11**:1165–1179.
6. Labuda M, Fujiwara TM, Ross MV, Morgan K, Garcia-Heras J, Ledbetter D, Hughes MR, Glorieux FH 1992 Two hereditary

defects related to vitamin D metabolism map to the same region of human chromsome 12q13-14. J Bone Miner Res **7**:1447–1453.
7. Taymans SE, Pack S, Pak E, Orban Z, Barsony J, Zhuang Z, Stratakis CA The human vitamin D receptor gene (VDR) is localized to region 12cen-q12 by Fluorescent In Situ Hybridization and Radiation Hybrid Mapping: Genetic and Physical VDR map. J Bone Miner Res **14**:1163–1166.
8. Crofts LA, Hancock MS, Morrison NA, Eisman JA 1998 Multiple promotors direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. Proc Natl Acad Sci USA **95**:10529–10534.
9. Takahashi E, Hori T, Sutherland GR 1990 Mapping of the human type II collagen gene (COL2A1) proximal to fra(12)(q13.1) by nonisotopic in situ hybridization. Cytogenet Cell Genet **54**:84–85.
10. Huang MC, Seyer JM, Thompson JP, Spinella DG, Cheah KS, Kang AH 1991 Genomic organization of the human procollagen alpha 1(II) collagen gene. Eur J Biochem **195**:593–600.
11. http://www.marshfieldclinic.org/research/genetics/default
12. http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/maps.cgi?org=hum&chr=12
13. http://sequence.aecom.yu.edu/chr12
14. http://genome.ucsc.edu
15. Nickerson DA, Taylor SL, Weiss KM, Clark AG, Hutchinson RG, Stengard J, Salomaa V, Vartiainen E, Boerwinkle E, Sing CF 1998 DNA sequence diversity in a 9.7-kb region of the human lipoprotein lipase gene. Nature Genet **19**:233–240.
16. Rieder MJ, Taylor SL, Clark AG, Nickerson DA 1999 Sequence variation in the human angiotensin converting enzyme. Nature Genet **22**:59–62.
17. Cambien F, Poirier O, Nicaud V, Herrman S, Mallet C, Ricard S, Behague I, Hallet V, Blanc H, Loukaci V, Thillet J, Evans A, Ruidavets J-B, Arveiler D, Luc G, Tiret L 1999 Sequence diversity in 36 candidate genes for cardiovascular disorders. Am J Hum Genet **65**:183–191.
18. Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N, Lane CR, Lim EP, Kalyanaraman N, Nemesh J, Ziaugra L, Friedland L, Rolfe A, Warrington J, Lipshutz R, Daley GQ, Lander ES 1999 Characterization of single-nucleotide polymorphisms in coding regions of human genes. Nature Genet **22**:231–238.
19. Wang DG , Fan J-B, Siao C-J, Berno A, Young P, Sapolsky R, Ghandour G, Perkins N, Winchester E, Spencer J, Kruglyak L, Stein L, Hsie L, Topaloglou T, Hubbell E, Robinson E, Mittmann M, Morris MS, Shen N, Kilburn D, Rioux J, Nusbaum C, Rozen S, Hudson TJ, Lipshutz R, Chee M, Lander ES 1998 Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. Science **280**:1077–1082.
20. Faraco JH, Morrison NA, Baker A, Shine J, Frossard PM 1989 ApaI dimorphism at the human vitamin D receptor gene locus. Nucl Acids Res **17**:2450.
21. Morrison NA, Yeoman R, Kelly PJ, Eisman JA 1992 Contribution of trans-acting factor alleles to normal physiological variability: vitamin D receptor gene polymorphisms and circulating osteocalcin. Proc Natl Acad Sci USA **89**:6665–6669.
22. Morrison NA, Qi JJC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA 1994 Prediction of bone density from vitamin D receptor alleles. Nature **367**:284–287.
23. Ye W-Z, Reis AF, Velho G 2000 Identification of a novel Tru9I polymorphism in the human vitamin D receptor gene. J Hum Genet **45**:56–57.
24. Saijo T, Ito M, Takeda E, Huq AH, Naito E, Yokota I, Sone T, Pike JW, Kuroda Y 1991 A unique mutation in the vitamin D receptor gene in three Japanese patients with vitamin D–dependent rickets type II: Utility of single strand conformation polymorphism analysis for heterozygous carrier detection. Am J Hum Genet **49**:668–673.
25. Sturzenbecker L, Scardaville B, Kratzeisen C, Katz M, Abarzua P, McLane J 1997 Isolation and analysis of cDNA encoding a naturally-occurring truncated form of the human vitamin D receptor. In: Bouillon R, Norman AW, Thomasset M (eds) Vitamin D: a pluripotent steroid hormone: structural studies, molecular endocrinology, and clinical applications. W De Gruyter, New York, NY, USA, pp. 253–257.
26. Gross C, Eccleshall TR, Malloy PJ, Villa ML, Marcus R, Feldman D 1996 The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. J Bone Miner Res **11**:1850–1855.
27. Arai H, Miyamoto K-I, Taketani Y, Yamamoto H, Iemori Y, Morita K, Tonai T, Nishisho T, Mori S, Takeda E 1997 A vitamin D receptor polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. J Bone Miner Res **12**:915–921.
28. Durrin LK, Haile RW, Ingles SA, Coetzee GA 1999 Vitamin D receptor 3′-untranslated region polymorphisms: lack of effect on mRNA stability. Biochim Biophys Acta **1453**:311–320.
29. Brown SB, Brierley TT, Palanisamy N, Salusky IB, Goodman W, Brandi ML, Drueke TB, Sarfati E, Urena P, Chaganti RSK, Pike JW, Arnold A 2000 Vitamin D receptor as a candidate tumor-suppressor gene in severe hyperparathyroidism of uremia. J Clin Endocrinol Metab **85**:868–872.
30. Arai H, Miyamoto KI, Yoshida M, Yamamoto H, Taketani Y, Morita K, Kubota M, Yoshida S, Ikeda M, Watabe F, Kanemasa Y, Takeda E 2001 The polymorphism in the caudal-related homeodomain protein Cdx-2 binding element in the human vitamin D receptor gene. J Bone Miner Res **16**:1256–1264.
31. Yamamoto H, Miyamoto K-I, Bailing L, Taketani Y, Kitano M, Inoue Y, Morita K, Pike JW, Takeda E 1999 The caudal-related homeodomain protein Cdx-2 regulates vitamin D receptor gene expression in the small intestine. J Bone Miner Res **14**:240–247.
32. Fang Y, van Meurs JB, Bergink AP, Hofman A, van Duijn CM, van Leeuwen JP, Pols HA, Uitterlinden AG 2003 Cdx-2 polymorphism in the promoter region of the human vitamin D receptor gene determines susceptibility to fracture in the elderly. J Bone Miner Res **18**:1632–1641.
33. Wall JD, Pritchard JK 2003 Haplotype blocks and linkage disequilibrium in the human genome. Nature Reviews Genetics **4**:587–597.
34. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Codero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D 2002 The structure of haplotype blocks in the human genome. Science **296**:2225–2229.
35. Uitterlinden AG, Pols HAP, Burger H, Huang Q, van Daele PLA, van Duijn CM, Hofman A, Birkenhäger JC, van Leeuwen JPTM 1996 A large-scale population-based study of the association of vitamin D receptor gene polymorphisms with bone mineral density. J Bone Miner Res **11**:1242–1248.
36. Ingles SA, Haile RW, Henderson BE, Kolonel LN, Nakaichi G, Shi C-Y, Yu MC, Ross RK, Coetzee GA 1997 Strength of linkage disequilibrium between two vitamin D receptor markers in five ethnic groups: implications for association studies. Cancer Epidemiol Biomark Prevent **6**:93–98.

37. http://archimedes.well.ox.ac.uk/pise/PHASE
38. http://www.sph.umich.edu/csg/abecasis/GOLD
39. Zmuda JM, Cauley JA, Ferrell RE 2000 Molecular epidemiology of vitamin D receptor gene variants. Epidemiol Rev **22**:203–217.
40. Gross C, Krishnan AV, Malloy PJ, Ross Eccleshall T, Zhao X-Y, Feldman D 1998 The vitamin D receptor gene start codon polymorphism: a functional analysis of FokI variants. J Bone Miner Res **13**:1691–1699.
41. Correa P, Rastad J, Schwarz P, Westin G, Kindmark A, Lundgren E, Akerstrom G, Carling T 1999 The vitamin D receptor (VDR) start codon polymorphism in primary hyperparathyroidism and parathyroid VDR messenger ribonucleic acid levels. J Clin Endocrinol Metab **84**:1690–1694.
42. Jurutka PW, Remus LS, Whitfield GK, Thompson PD, Hsieh JC, Zitzer H, Tavakkoli P, Galligan MA, Dang HTL, Haussler CA, Haussler MR 2000 The polymorphic N terminus in human vitamin D receptor isoforms influences transcriptional activity by modulating interaction with transcription factor IIB. Mol Endocrinol **14**:401–420.
43. Whitfield GK, Remus LS, Jurutka PW, Zitzer H, Oza AK, Dang HTL, Haussler CA, Galligan MA, Thatcher ML, Dominguez CE, Haussler MR 2001 Functionally relevant polymorphisms in the human nucelar vitamin D receptor gene. Mol Cell Endocrinol **177**:145–159.
44. Collin EM, Weel AEAM, Uitterlinden AG, Buurman CJ, Birkenhager JC, Pols HAP, van Leeuwen JPTM 2000 Consequences of vitamin D receptor gene polymorphisms for growth inhibition of cultured human peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D_3. Clin Endocrinol **52**:211–216.
45. Beaumont M, Bennett AJ, White DA, Hosking DJ 1998 Allelic differences in the 3′ untranslated region of the vitamin D receptor gene affect mRNA levels in bone cells. Osteop Int **8**:37 (abstract P081).
46. Crofts LA, Morrison NA, Dudman N, Eisman JA 1996 Differential expression of VDR gene alleles. J Bone Miner Res **11**:S208 (abstract S473).
47. Gross C, Musiol IM, Eccleshall TR, Malloy PJ, Feldman D 1998 Vitamin D receptor gene polymorphisms: analysis of ligand binding and hormone responsiveness in cultured skin fibroblasts. Biochem Biophys Res Comm **242**:467–473.
48. Yamagata M, Nakajima S, Tokita A, Sakai N, Yanagihara I, Yabuta K, Ozono K 1999 Analysis of stable levels of messenger RNA derived from different polymorphic alleles in the vitamin D receptor gene. J Bone Miner Metab **17**: 164–170.
49. Mocharla H, Butch AW, Pappas AA, Flick JT, Weinstein RS, de Togni P, Jilka RL, Roberson PK, Parfitt AM, Manolagas SC 1997 Quantification of vitamin D receptor mRNA by competitive polymerase chain reaction in PBMC: lack of correspondence with common allelic variants. J Bone Miner Res **12**:726–733.
50. Verbeek W, Gombart AF, Shiohara M, Campbell M, Koeffler HP 1997 Vitamin D receptor: no evidence for allele-specific mRNA stability in cells which are heterozygous for the TaqI restriction enzyme polymorphism. Biochem Biophys Res Comm **238**:77–80.
51. Carling T, Ridefelt P, Hellman P, Rastad J, Akerstrom G 1997 Vitamin D receptor polymorphisms correlate to parathyroid cell function in primary hyperparathyroidism. J Clin Endocrinol Metab **82**:1772–1775.
52. Carling T, Rastad J, Akerstrom G, Westin G 1998 Vitamin D receptor (VDR) and parathyroid hormone messenger ribonucleic acid levels correspond to polymorphic VDR allelels in human parathyroid tumors. J Clin Endocrinol Metab **83**: 2255–2259.
53. Ohtera K, Ishii S, Matsuyama T 2001 Influence of the vitamin D receptor alleles on human osteoblast-like cells. J Bone Joint Surg **83-B**:134–138.
54. Decker CJ, Parker R 1995 Diversity of cytoplasmic functions for the 3′ untranslated region of eukaryotic transcripts. Curr Opin Cell Biol **7**:386–392.
55. Schaaf MJM, Cidlowski JA 2002 AUUUA motifs in the 3′UTR of human glucocorticoid receptor alpha and beta mRNA destabilize mRNA and decrease receptor protein expression. Steroids **67**:627–636.
56. Kroger H, Mahonen A, Ryhanen S, Turunen A-M, Alhava E, Maenpaa P 1995 Vitamin D receptor genotypes and bone mineral density. Lancet **345**:1238.
57. Fleet JC, Harris SS, Wood RJ, Dawson-Hughes B 1995 The BsmI vitamin D receptor restriction fragment length polymorphism (BB) predicts low bone density in premenopausal black and white women. J Bone Miner Res **10**: 985–990.
58. Garnero P, Borel O, Sornay-Rendu E, Delmas PD 1995 Vitamin D receptor gene polymorphisms do not predict bone turnover and bone mass in healthy premenopausal women. J Bone Miner Res **10**:1283–1288.
59. Krall EA, Parry P, Lichter JB, Dawson-Hughes B 1995 Vitamin D receptor alleles and rates of bone loss: influence of years since menopause and calcium intake. J Bone Miner Res **10**:978–984.
60. Howard G, Nguyen T, Morrison N, Watanabe T, Sambrook P, Eisman J, Kelly PJ 1995 Genetic influences on bone density: physiological correlates of vitamin D receptor gene alleles in premenopausal women. J Clin Endocrinol Metab **80**: 2800–2805.
61. Tokita A, Matsumoto H, Morrison NA, Tawa T, Miura Y, Fukamauchi K, Mitsuhashi N, Irimoto M, Yamamori S, Miura M, Watanabe T, Kuwabara Y, Yabuta K, Eisman JA 1996 Vitamin D receptor allelels, bone mineral density, and turnover in premenopausal Japanese women. J Bone Miner Res **11**:1003–1009.
62. Tsai KS, Hsu SHJ, Cheng WC, Chen CK, Chieng PU, Pan WH 1996 Bone mineral density and bone markers in relation to vitamin D receptor gene polymorphisms in Chinese men and women. Bone **19**:513–518.
63. Rauch F, Radermacher A, Danz A, Schiedermaier U, Golucke A, Michalk D, Schonau E 1997 Vitamin D receptor genotypes and changes of bone density in physically active German women with high calcium intake. Exp Clin Endocrinol Diabetes **105**:103–108.
64. McClure L, Eccleshall TR, Gross C, Villa ML, Lin N, Ramaswamy V, Kohlmeier L, Kelsey JL, Marcus R, Feldman D 1997 Vitamin D receptor polymorphisms, bone mineral density, and bone metabolism in postmenopausal Mexican-American women. J Bone Miner Res **12**:234–240.
65. Graafmans WC, Lips P, Ooms ME, van Leeuwen JPTM, Pols HAP, Uitterlinden AG 1997 The effect of vitamin D supplementation on the bone mineral density of the femoral neck is associated with vitamin D receptor genotype. J Bone Miner Res **12**:1241–1245.
66. Hansen TS, Abrahamsen B, Henriksen FL, Hermann AP, Jensen LB, Horder M, Gram J 1998 Vitamin D receptor alleles do not predict bone mineral density or bone loss in Danish perimenopausal women. Bone **22**:571–575.
67. Willing M, Sowers M, Aron D, Clark MK, Burns T, Bunten C, Crutchfield M, D'Ágostino D, Jannausch M 1998 Bone mineral density and its change in white women: estrogen and

vitamin D receptor genotypes and their interaction. J Bone Miner Res **13**:695–705.
68. Sowers M, Willing M, Burns T, Deschenes S, Hollis B, Crutchfield M, Jannausch M 1999 Genetic markers, bone mineral density, and serum osteocalcin levels. J Bone Miner Res **14**:1411–1419.
69. Ferrari S, Manen D, Bonjour J-P, Slosman D, Rizzoli R 1999 Bone mineral mass and calcium and phosphate metabolism in young men: relationships with vitamin D receptor alleleic polymorphisms. J Clin Endocrinol Metab **84**:2043–2048.
70. Bell NH, Morrison NA, Nguyen TV, Eisman JA, Hollis BW 2001 ApaI polymorphisms of the vitamin D receptor predict bone density of the lumbar spine and not racial difference in bone density in young men. J Lab Clin Med **137**:133–140.
71. Matsuyama T, Ishii S, Tokita A, Yabuta K, Yamamori S, Morrison NA, Eisman JA 1995 Vitamin D receptor genotypes and bone mineral density. Lancet **345**:1238–1239.
72. Deng HW, Li J, Li J-L, Johnson M, Gong G, Davis KM, Recker RR 1998 Change of bone mass in postmenopausal Caucasian women with and without hormone replacement therapy is associated with vitamin D receptor and estrogen receptor genotypes. Hum Genet **103**:576–585.
73. Kurabayashi T, Tomita M, Matsushita H, Yahata T, Honda A, Takakuwa K, Tanaka K 1999 Association of vitamin D and estrogen receptor gene polymorphism with the effect of hormone replacement therapy on bone mineral density in Japanese women. Am J Obstet Gynecol **180**:1115–1120.
74. Ho YV, Briganti EM, Duan Y, Buchanan R, Hall S, Seeman E 1999 Polymorphisms of the vitamin D receptor gene and corticoid-related osteoporosis. Osteop Int **9**:134–138.
75. Marc J, Prezelj J, Komel R, Kocijancic A 1999 VDR genotype and response to etidronate therapy in late postmenopausal women. Osteop Int **10**:303–306.
76. Ioannidis JP, Lau J 1999 Pooling research results: benefits and limitations of meta-analysis. Jt Comm J Qual Improv **25**:462–469.
77. Ioannidis JP, Trikalinos TA, Ntzani EE, Contopoulos-Ioannidis DG 2001 Replication validity of genetic association studies. Nat Genet **29**:306–309.
78. Ioannidis JP 2003 Genetic associations: false or true? Trends Mol Med **9**:135–138.
79. Kiel DP, Myers RH, Cupples LA, Kong XF, Zhu XH, Ordovas J, Schaeffer EJ, Felson DT, Rush D, Wilson PW, Eisman JA, Holick MF 1997 The BsmI vitamin D receptor restriction fragment length polymorphism (bb) influences the effect of calcium intake on bone mineral density. J Bone Miner Res **12**:1049–1057.
80. Dawson-Hughes B, Harris SS, Finneran S 1995 Calcium absorption on high and low calcium intakes in relation to vitamin D receptor genotype. J Clin Endocrinol Metab **80**:3657–3661.
81. Gennari L, Becherini L, Masi L, Gonelli S, Cepollaro C, Martini S, Mansani R, Brandi ML 1997 Vitamin D receptor genotypes and intestinal calcium absorption in postmenopausal women. Calc Tissue Int **61**:460–463.
82. Wishart JM, Horowitz M, Need AG, Scopacasa F, Morris HA, Clifton PM, Nordin BEC 1997 Relations between calcium intake, calcitriol, polymorphisms of the vitamin D receptor gene, and calcium absorption in premenopausal women. Am J Clin Nutr **65**:798–802.
83. Ongphiphadhanakul B, Rajatanavin R, Chanprasertyothin S, Chailurkit L, Piaseu N, Teerarungsikul K, Sirisriro R, Komindr S, Puavilai G 1997 Vitamin D receptor gene polymorphism is associated with urinary calcium excretion but not with bone mineral density in postmenopausal women. J Endocrinol Invest **20**:592–596.
84. Ames SK, Ellis KJ, Gunn SK, Copeland KC, Abrams SA 1999 Vitamin D receptor gene FokI polymorphism predicts calcium absorption and bone mineral density in children. J Bone Miner Res **14**:740–746.
85. Francis RM, Harrington F, Turner E, Papiha SS, Datta HK 1997 Vitamin D receptor gene polymorphism in men and its effect on bone density and calcium absorption. Clin Endocrinol **46**:83–86.
86. Kinyamu HK, Gallagher JC, Knezetic JA, DeLuca HF, Prahl JM, Lanspra SJ 1997 Effect of vitamin D receptor genotypes on calcium absorption, duodenal vitamin D receptor concentration, and serum 1,25 dihydroxyvitamin D levels in normal women. Calc Tissue Int **60**:491–495.
87. Wolf RL, Cauley JA, Baker CE, Ferrell RE, Charron M, Cagiulla AW, Salamone LM, Heany RP, Kuller LH 2000 Factors associated with calcium absorption efficiency in pre- and perimenopausal women. Am J Nutr **72**:466–471.
88. Laaksonen M, Karkkainen M, Outila T, Vanninen T, Ray C, Lamberg-Allardt C 2002 Vitamin D receptor gene BsmI-polymorphism in Finnish premenopausal and postmenopausal women: its association with bone mineral density, markers of bone turnover, and intestinal calcium absorption, with adjustment for lifestyle factors. J Bone Miner Metab **20**:383–390.
89. Morrison NA, Qi JJC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA 1997 Prediction of bone density from vitamin D receptor alleles (correction). Nature **387**:106.
90. Cooper GS, Umbach DM 1996 Are vitamin D receptor polymorphisms associated with bone mineral density? A meta-analysis. J Bone Miner Res **11**:1841–1849.
91. Gong G, Stern HS, Cheng S-C, Fong N, Mordeson J, Deng H-W, Recker RR 1999 The association of bone mineral density with vitamin D receptor gene polymorphisms. Osteop Int **9**:55–64.
92. Harris SS, Eccleshall TR, Gross C, Dawson-Hughes B, Feldman D 1997 The vitamin D receptor start codon polymorphism (FokI) and bone mineral density in premenopausal American black and white women. J Bone Miner Res **12**:1043–1048.
93. Ferrari S, Rizzoli R, Manen D, Slosman D, Bonjour JP 1998 Vitamin D receptor gene start codon polymorphism (FokI) and bone mineral density: interaction with age, dietary calcium, and 3′ end region polymorphisms. J Bone Miner Res **13**:925–930.
94. Gennari L, Becherini L, Mansani R, Masi L, Falchetti A, Morelli A, Colli E, Gonelli S, Cepollaro C, Brandi ML 1999 FokI polymorphism at translation initiation site of the vitamin D receptor gene predicts bone mineral density and vertebral fractures in postmenopausal Italian women. J Bone Miner Res **14**:1379–1386.
95. Lucotte G, Mercier G, Burckel A 1999 The vitamin D receptor FokI start codon polymorphism and bone mineral density in osteoporotic postmenopausal French women. Clin Genet **56**:221–224.
96. Kanan RM, Varanasi SS, Francis RM, Parker L, Datta HK 2000 Vitamin D receptor gene start codon polymorphism (FokI) and bone mineral density in healthy male subjects. Clin Endocrinol **53**:93–98.
97. Eccleshall TR, Garnero P, Gross C, Delmas PD, Feldman D 1998 Lack of correlation between start codon polymorphism of the vitamin D receptor gene and bone mineral density in premenopausal French women: the OFELY study. J Bone Miner Res **13**:31–35.

98. Zmuda JM, Cauley JA, Danielson ME, Theobald TM, Ferell RE 1999 Vitamin D receptor translation initiation codon polymorphism and markers of osteoporotic risk in older African-American women. Osteop Int **9**:214–219.
99. Cheng WC, Tsai KS 1999 The vitamin D receptor start codon polymorphism (FokI) and bone mineral density in premenopausal women in Taiwan. Osteop Int **9**:545–549.
100. Wynne F, Drummond F, O'Sullivan K, Daly M, Shanahan F, Molloy MG, Quane KA 2002 Investigation of the genetic influence of the OPG, VDR (FokI), and COLIA1 Sp1 polymorphisms on BMD in the Irish population. Calc Tissue Int **71**:26–35.
101. Heany RP, Barger-Lux MJ, Davies KM, Ryan RA, Johnson ML, Gong G 1997 Bone dimensional change with age: interactions of genetic, hormonal, and body size variables. Osteop Int **7**:426–431.
102. Tao C, Yu T, Garnett S, Briody J, Knight J, Woodhead H, Cowell CT 1998 Vitamin D receptor alleles predict growth and bone density in girls. Arch Dis Child **79**:488–494.
103. Suarez F, Rossignol C, Garabedian M 1998 Interactive effects of estradiol and vitamin D receptor gene polymorphisms as a possible determinant of growth in male and female infants. J Clin Endocrinol Metab **83**:3563–3568.
104. Lorentzon M, Lorentzon R, Nordstrom P 2000 Vitamin D receptor gene polymorphism is associated with birth height, growth to adolescence, and adult stature in healthy Caucasian men: a cross-sectional and longitudinal study. J Clin Endocrinol Metab **85**:1666–1670.
105. Dennison EM, Arden NK, Keen RW, Syddall H, Day INM, Spector TD, Cooper C 2001 Birthweight, vitamin D receptor genotype, and the programming of osteoporosis. Pediatr Perinat Epidemiol **15**:211–219.
106. van der Sluis IM, de Muinck Keizer-Schrama SMPF, Krenning EP, Pols HAP, Uitterlinden AG 2003 Vitamin D receptor gene polymorphisms predict height and bone size, rather than bone density in children and young adults. Calc Tissue Int. **73**:332–338.
107. Keen RW, Egger P, Fall C, Major PJ, Lanchbury JS, Spector TD, Cooper C 1997 Polymorphisms of the vitamin D receptor, infant growth, and adult bone mass. Calc Tissue Int **60**:233–235.
108. Suarez F, Zegoud F, Rossignol C, Walrant O, Garabedian M 1997 Association between vitamin D receptor gene polymorphisms and sex-dependent growth during the first two years of life. J Clin Endocrinol Metab **82**:2966–2970.
109. Baroncelli GI, Federico G, Bertelloni S, Ceccarelli C, Cupelli D, Saggese G 1999 Vitamin D receptor genotype does not predict bone mineral density, bone turnover, and growth in prepubertal children. Horm Res **51**:150–156.
110. Minamitani K, Takahashi Y, Minagawa M, Yasuda T, Niimi H 1998 Difference in height associated with a translation start site polymorphism in the vitamin D receptor gene. Ped Res **44**:628–632.
111. Geussens P, VandeVyver C, VanHoof J, Cassiman J-J, Boonen S, Raus J 1997 Quadriceps and grips strength are related to vitamin D receptor genotype in elderly nonobese women. J Bone Miner Res **12**:2082–2088.
112. Beaven S, Prentice A, Yan L, Dibba B, Ralston S 1996 Differences in vitamin D receptor genotype and geographical variation in osteoporosis. Lancet **348**:136–137.
113. Young RP, Lau EM, Birjandi Z, Critchley JA, Woo J 1996 Interethnic differences in hip fracture rate and the vitamin D receptor polymorphism. Lancet **348**:688–689.
114. Berg JP, Falch JA, Haug E 1996 Fracture rate, pre- and postmenopausal bone loss are not associated with vitamin D receptor genotype in a high-endemic area of osteoporosis. Eur J Endocrinol **135**:96–100.
115. Feskanich D, Hunter DJ, Willett WC, Hankinson SE, Hollis BW, Hough HL, Kelsey KT, Colditz GA 1998 Vitamin D receptor genotype and the risk of bone fractures in women. Epidemiol **9**:535–539.
116. Gomez C, Naves ML, Barrios Y, Diaz JB, Fernandez JL, Salido E, Torres A, Cannata JB 1999 Vitamin D receptor gene polymorphisms, bone mass, bone loss, and prevalence of vertebral fracture: differences in postmenopausal women and men. Osteop Int **10**:175–182.
117. Langdahl BL, Gravholt CH, Brixen K, Eriksen EF 2000 Polymorphisms in the vitamin D receptor gene and bone mass, bone turnover, and osteoporotic fractures. Eur J Clin Invest **30**:608–617.
118. Uitterlinden AG, Weel AEAM, Burger H, Fang Y, van Duijn CM, Hofman A, van Leeuwen JPTM, Pols HAP 2001 Interaction between the vitamin D receptor gene and collagen type Iα1 gene in susceptibility for fracture. J Bone Miner Res **16**:379–385.
119. White CP, Nguyen TV, Jones G, Morrison NA, Gardiner EM, Kelly PJ, Sambrook PN 1994 Vitamin D receptor alleles predict osteoporotic fracture risk. J Bone Miner Res S263 (abstract).
120. Houston LA, Grant SFA, Reid DM, Ralston SR 1996 Vitamin D receptor polymorphism, bone mineral density, and osteoporotic vertebral fracture: studies in a UK population. Bone **18**:249–252.
121. Ramalho AC, Lazaretti-Castro M, Hauache O, Kasamatsu T, Brandao C, Reis AF, Takata E, Cafalli F, Tavares F, Gimeno SG, Vieira JG 1998 Fractures of the proximal femur: correlation with vitamin D receptor gene polymorphism. Braz J Med Biol Res **31**:921–927.
122. Ensrud KE, Stone K, Cauley JA, White C, Zmuda JM, Nguyen TV, Eisman JA, Cummings SR 1999 Vitamin D receptor gene polymorphisms and the risk of fractures in older women. For the Study of Osteoporotic Fractures Research Group. J Bone Miner Res **14**:1637–1645.
123. Valimaki S, Tahtela R, Kainulainen K, Laitinen K, Loyttyniemi E, Sulkava R, Valimaki M, Kontula K 2001 Relation of collagen type I alpha 1 (COLIA1) and vitamin D receptor genotypes to bone mass, turnover, and fractures in early postmenopausal women and to hip fractures in elderly people. Eur J Intern Med **12**:48–56.
124. Gennari L, Becherinni L, Mansani R, Masi L, Falchetti A, Morelli A, Colli E, Gonelli S, Cepollaro C, Brandi ML 1999 FokI polymorphism at translation initiation site of the vitamin D receptor gene predicts bone mineral density and vertebral fractures in postmenopausal Italian women. J Bone Miner Res **14**:1379–1386.
125. Balmain N, Hauche corne M, Pike JW, Cuisinier-Gleizes P, Mathieu P 1993 Distribution and subcellular immunolocalization of the 1,25 dihydroxy vitamin D3 receptors in rat epiphysial cartilage. Cell Mol Biol **39**:339–350.
126. Dodds RA, Gowen M 1994 The growing osteophyte: a model system for the study of human bone development and remodelling in situ. J Histotechnol **17**:37–45.
127. McAlindon TE, Felson DT, Zhang Y, Hannan MT, Aliabadi P, Weissman B, Rush D, Wilson PWF, Jacques P 1996 Relation of dietary intake and serum levels of vitamin D to progression of osteoarthritis of the knee among participants in the Framingham Study. Ann Intern Med **125**:353–359.
128. Uitterlinden AG, Burger H, Huang Q, Odding E, van Duijn CM, Hofman A, Birkenhager JC, van Leeuwen JPTM, Pols HAP

1997 Vitamin D receptor genotype is associated with radiographic osteoarthritis at the knee. J Clin Invest **100**:259–263.
129. Keen RW, Hart DJ, Lanchbury JS, Spector TD 1997 Association of early osteoarthritis of the knee with a Taq I polymorphism of the vitamin D receptor gene. Arthr Rheum **40**:1444–1449.
130. Granchi D, Stea S, Sudanese A, Toni A, Baldini N, Giunti A 2002 Association of two gene polymorphisms with osteoarthritis secondary to hip dysplasia. Clin Orthop **403**:108–117.
131. Jones G, White C, Sambrook P, Eisman J 1998 Allelic variation in the vitamin D receptor, lifestyle factors and lumbar spinal degenerative disease. Ann Rheum Dis **57**:94–99.
132. Videman T, Leppavuori J, Kaprio J, Battie M, Gibbons LE, Peltonen L, Koskenvuo M 1998 Intragenic polymorphisms of the vitamin D receptor gene associated with intervertebral disc degeneration. Spine **23**:2477–2485.
133. Aerssens J, Dequeker J, Peeters J, Breemans S, Boonen S 1998 Lack of association between osteoarthritis of the hip and gene polymorphisms of VDR, COLIA1, and COL2A1 in postmenopausal women. Arthr Rheum **41**:1946–1950.
134. Huang J, Ushiyama T, Inoue K, Kawasaki T, Hukuda S 2000 Vitamin D receptor gene polymorphisms and osteoarthritis of the hand, hip, knee: a case-control study in Japan. Rheumatology **39**:79–84.
135. Loughlin J, Sinsheimer JS, Mustafa Z, Carr AJ, Clipsham K, Bloomfield VA, Chitnavis J, Bailey A, Sykes B, Chapman K 2000 Association of the vitamin D receptor gene, the type I collagen gene COLIA1, and the estrogen receptor gene in idiopathic osteoarthritis. J Rheum **27**:779–784.
136. Baldwin CT, Cupples LA, Joost O, Demissie S, Chaisson C, McAlindon T, Myers RH, Felson D 2002 Absence of linkage or association for osteoarthritis with the vitamin D receptor/type II collagen locus: the Framingham Osteoarthritis Study. J Rheum **29**:161–165.
137. Uitterlinden AG, Burger H, van Duijn CM, Huang Q, Hofman A, Birkenhager JC, van Leeuwen JPTM, Pols HAP 2000 Adjacent genes for COL2A1 and the vitamin D receptor are associated with separate features of radiographic osteoarthritis of the knee. Arthr Rheum **43**:1456–1464.
138. Taylor JA, Hirvonen A, Watson M, Pittman G, Mohler JL, Bell DA 1996 Association of prostate cancer with vitamin D receptor gene polymorphism. Cancer Res **56**:4108–4110.
139. Ingles SA, Ross RK, Yu MC, Irvine RA, La Pera G, Haile RW, Coetzee GA 1997 Association of prostate cancer risk with genetic polymorphisms in vitamin D receptor and androgen receptor. J Natl Cancer Inst **89**:166–170.
140. Ma J, Stampfer MJ, Gann PH, Hough HL, Giovannucci E, Kelsey KT, Hennekens CH, Hunter DJ 1998 Vitamin D receptor polymorphisms, circulating vitamin D metabolites, and risk of prostate cancer in United States physicians. Cancer Epidemiol Biomarkers Prev **7**:385–390.
141. Habuchi T, Suzuki T, Sasaki R, Wang L, Sato K, Satoh S, Akao T, Tsuchiya N, Shimoda N, Wada Y, Koizumi A, Chihara J, Ogawa O, Kato T 2000 Association of vitamin D receptor gene polymorphism with prostate cancer and benign prostatic hyperplasia in a Japanese population. Cancer Res **60**:305–308.
142. Medeiros R, Morais A, Vasconcelos A, Costa S, Pinto D, Oliveira J, Lopes C 2002 The role of vitamin D receptor gene polymorphisms in the susceptibility to prostate cancer of a southern European population. J Hum Genet **47**: 413–418.
143. Hamasaki T, Inatomi H, Katoh T, Ikuyama T, Matsumoto T 2002 Significance of vitamin D receptor gene polymorphism for risk and disease severity of prostate cancer and benign prostatic hyperplasia in Japanese. Urol Int **68**:226–231.
144. Hamasaki T, Inatomi H, Katoh T, Ikuyama T, Matsumoto T 2001 Clinical and pathological significance of vitamin D receptor gene polymorphism for prostate cancer, which is associated with a higher mortality in Japanese. Endocr J **48**:543–549.
145. Ingles SA, Coetzee GA, Ross RK, Henderson BE, Kolonel LN, Crocitto L, Wang W, Haile RW 1998 Association of prostate cancer with vitamin D receptor haplotypes in African-Americans. Cancer Res **58**:1620–1623.
146. Correa-Cerro L, Berthon P, Haussler J, Bochum S, Drelon E, Mangin P, Fournier G, Paiss T, Cussenot O, Vogel W 1999 Vitamin D receptor polymorphisms as markers in prostate cancer. Hum Genet **105**:281–287.
147. Kibel AS, Isaacs SD, Isaacs WB, Bova GS 1998 Vitamin D receptor polymorphisms and lethal prostate cancer. J Urol **160**:1405–1409.
148. Furuya Y, Akakura K, Masai M, Ito H 1999 Vitamin D receptor gene polymorphism in Japanese patients with prostate cancer. Endocr J **46**:467–470.
149. Watanabe M, Fukutome K, Murata M, Uemura H, Kubota Y, Kawamura J, Yatani R 1999 Significance of vitamin D receptor gene polymorphism for prostate cancer risk in Japanese. Anticancer Res **19**:4511–4514.
150. Blazer DG, III, Umbach DM, Bostick RM, Taylor JA 2000 Vitamin D receptor polymorphisms and prostate cancer. Mol Carcinog **27**:18–23.
151. Bousema JT, Bussemakers MJ, van Houwelingen KP, Debruyne FM, Verbeek AL, de la Rosette JJ, Kiemeney LA 2000 Polymorphisms in the vitamin D receptor gene and the androgen receptor gene and the risk of benign prostatic hyperplasia. Eur Urol **37**:234–238.
152. Schatzl G, Gsur A, Bernhofer G, Haidinger G, Hinteregger S, Vutuc C, Haitel A, Micksche M, Marberger M, Madersbacher S 2001 Association of vitamin D receptor and 17 hydroxylase gene polymorphisms with benign prostatic hyperplasia and benign enlargement. Urology **57**:567–572.
153. Suzuki K, Matsui H, Ohtake N, Nakata S, Takei T, Koike H, Nakazato H, Okugi H, Hasumi M, Fukabori Y, Kurokawa K, Yamanaka H 2003 Vitamin D receptor gene polymorphism in familial prostate cancer in a Japanese population. Int J Urol **10**:261–266.
154. Tayeb MT, Clark C, Haites NE, Sharp L, Murray GI, McLeod HL 2003 CYP3A4 and VDR gene polymorphisms and the risk of prostate cancer in men with benign prostate hyperplasia. Br J Cancer **88**:928–932.
155. Gsur A, Madersbacher S, Haidinger G, Schatzl G, Marberger M, Vutuc C, Micksche M 2002 Vitamin D receptor gene polymorphism and prostate cancer risk. Prostate **51**:30–34.
156. Chokkalingam AP, McGlynn KA, Gao YT, Pollak M, Deng J, Sesterhenn IA, Mostofi FK, Fraumeni JF, Hsing AW 2001 Vitamin D receptor gene polymorphisms, insulin-like growth factors, and prostate cancer risk: a population-based, case-control study in China. Cancer Res **61**:4333–4336.
157. Xu Y, Shibata A, McNeal JE, Stamey TA, Feldman D, Peehl DM 2003 Vitamin D receptor start codon polymorphism (FokI) and prostate cancer progression. Cancer Epidemiol Biomarkers Prev **12**:23–27.
158. Luscombe CJ, French ME, Liu S, Saxby MF, Jones PW, Fryer AA, Strange RC 2001 Outcome in prostate cancer associations with skin type and polymorphism in pigmentation-related genes. Carcinogenesis **22**:1343–1347.

158a. Ntais C, Polycarpou A, Ioannidis JPA 2003 Vitamin D receptor polymorphisms and risk of prostate cancer: A meta-analysis. Cancer Epidemiol Biomark Prevent **12**: 1395–1402.
159. Ruggiero M, Pacini S, Aterini S, Fallai C, Ruggiero C, Pacini P 1998 Vitamin D receptor gene polymorphism is associated with metastatic breast cancer. Oncol Res **10**:43–46.
160. Curran JE, Vaughan T, Lea RA, Weinstein SR, Morrison NA, Griffiths LR 1999 Association of a vitamin D receptor polymorphism with sporadic breast cancer development. Int J Cancer **83**:723–726.
161. Lundin AC, Soderkvist P, Eriksson B, Bergman-Jungestrom M, Wingren S 1999 Association of breast cancer progression with a vitamin D receptor gene polymorphism. Southeast Sweden Breast Cancer Group. Cancer Res **59**:2332–2334.
162. Bretherton-Watt D, Given-Wilson R, Mansi JL, Thomas V, Carter N, Colston KW 2001 Vitamin D receptor gene polymorphisms are associated with breast cancer risk in a UK Caucasian population. Br J Cancer **85**:171–175.
163. Guy M, Lowe LC, Bretherton-Watt D, Mansi JL, Colston KW 2003 Approaches to evaluating the association of vitamin D receptor polymorphisms with breast cancer risk. Recent Results Cancer Res **164**:43–54.
164. Ingles SA, Garcia DG, Wang W, Nieters A, Henderson BE, Kolonel LN, Haile RW, Coetzee GA 2000 Vitamin D receptor genotype and breast cancer in Latinas (United States). Cancer Causes Control **11**:25–30.
165. Hou MF, Tien YC, Lin GT, Chen CJ, Liu CS, Lin SY, Huang TJ 2002 Association of vitamin D receptor gene polymorphism with sporadic breast cancer in Taiwanese patients. Breast Cancer Res Treat **74**:1–7.
166. Schondorf T, Eisberg C, Wassmer G, Warm M, Becker M, Rein DT, Gohring UJ 2003 Association of the vitamin D receptor genotype with bone metastases in breast cancer patients. Oncology **64**:154–159.
167. Dunning AM, McBride S, Gregory J, Durocher F, Foster NA, Healey CS, Smith N, Pharoah PD, Luben RN, Easton DF, Ponder BA 1999 No association between androgen or vitamin D receptor gene polymorphisms and risk of breast cancer. Carcinogenesis **20**:2131–2135.
168. Newcomb PA, Kim H, Trentham-Dietz, Farin F, Hunter D, Egan KM 2002 Vitamin D receptor polymorphism and breast cancer risk. Cancer Epidemiol Biomark Prev **11**:1503–1504.
169. Kim HS, Newcomb PA, Ulrich CM, Keener CL, Bigler J, Farin FM, Bostick RM, Potter JD 2001 Vitamin D receptor polymorphism and the risk of colorectal adenomas: evidence of interaction with dietary vitamin D and calcium. Cancer Epidemiol Biomarkers Prev **10**:869–874.
170. Speer G, Dworak O, Cseh K, Bori Z, Salamon D, Torok I, Winkler G, Vargha P, Nagy Z, Takacs I, Kucsera M, Lakatos P 2000 Vitamin D receptor gene BsmI polymorphism correlates with erbB-2/HER-2 expression in human rectal cancer. Oncology **58**:242–247.
171. Ingles SA, Wang J, Coetzee GA, Lee ER, Frankl HD, Haile RW 2001 Vitamin D receptor polymorphisms and risk of colorectal adenomas (United States). Cancer Causes Control **12**:607–614.
172. Wong HL, Seow A, Arakawa K, Lee HP, Yu MC, Ingles SA 2003 Vitamin D receptor start codon polymorphism and colorectal cancer risk: effect modification by dietary calcium and fat in Singapore Chinese. Carcinogenesis **24**:1091–1095.
173. Peters U, McGlynn KA, Chatterjee N, Gunter E, Garcia-Closas M, Rothman N, Sinha R 2001 Vitamin D, calcium, and vitamin D receptor polymorphism in colorectal adenomas. Cancer Epidemiol Biomarkers Prev **10**:1267–1274.
174. Slatter ML, Yakumo K, Hoffman M, Neuhausen S 2001 Variants of the VDR gene and risk of colon cancer (United States). Cancer Causes Control **12**:359–364.
175. Ramachandran S, Fryer AA, Lovatt TJ, Smith AG, Lear JT, Jones PW, Stange RC 2003 Combined effects of gender, skin type, and polymorphic genes on clinical phenotype: use of rate of increase in numbers of basal cell carcinoma as a model system. Cancer Lett **189**:175–181.
176. Hutchinson PE, Osborne JE, Lear JT, Smith AG, Bowers PW, Morris PN, Jones PW, York C, Strange RC, Fryer AA 2000 Vitamin D receptor polymorphisms are associated with altered prognosis in patients with malignant melanoma. Clin Cancer Res **6**:498–504.
177. Ramachandran S, Fryer AA, Smith AG, Lear JT, Bowers B, Hartland AJ, Whiteside JR, Jones PW, Strange RC 2001 Basal cell carcinomas: association of allelic variants with a high-risk subgroup of patients with the multiple presentation phenotype. Pharmacogenetics **11**:247–254.
178. Ikuyama T, Hamasaki T, Inatomi H, Katoh T, Muratani T, Matsumoto T 2002 Association of vitamin D receptor gene polymorphism with renal cell carcinoma in Japanese. Endocr J **49**:433–438.
179. Carling T, Kindmark A, Hellman P, Lundgren E, Ljunghall S, Rastad J, Akerstrom G, Melhus H 1995 Vitamin D receptor genotypes in primary hyperparathyroidism. Nat Med **1**: 1309–1311.
180. Carling T, Kindmark A, Hellman P, Holmberg L, Akerstrom G, Rastad J 1997 Vitamin D receptor alleles b, a, and T: risk factors for sporadic primary hyperparathyroidism (HPT), but not HPT of uremia or MEN1. Biochem Biophys Res Commun **231**:329–332.
181. Carling T, Ridefelt P, Hellman P, Juhlin C, Lundgren E, Akerstrom G, Rastad J 1998 Vitamin D receptor gene polymorphism and parathyroid calcium sensor protein (CAS/gp330) expression in primary hyperparathyroidism. World J Surg **22**:700–706.
182. Nagaba Y, Heishi M, Tazawa H, Tsukamoto Y, Kobayashi Y 1998 Vitamin D receptor gene polymorphisms affect secondary hyperparathyroidism in hemodialyzed patients. Am J Kidney Dis **32**:464–469.
183. Menarguez J, Goicoechea M, Crist bal E, Arribas B, Martinez ME, Alcazar JA, Carri n R, Polo JR 1999 Lack of relationship betwen BsmI vitamin D receptor polymorphism and primary hyperparathyroidism in a Spanish female population. Calc Tissue Int **65**:214–216.
184. Sosa M, Torres A, Martin N, Salido E, Liminana JM, Barrios Y, De Miguel E, Betancor P 2000 The distribution of two different vitamin D receptor polymorphisms (BsmI and start codon) in primary hyperparathyroidism. J Intern Med **247**:124–130.
185. Pacheo D, Menarguez J, Cristobal E, Arribas B, Alcazar JA, Carrion R, Polo JR 2000 BsmI vitamin D receptor polymorphism and pathogenesis of parathyroid adenoma. Med Sci Monit **6**:658–660.
186. Park B-S, Park J-S, Lee D-Y, Youn J-I, Kim I-G 1999 Vitamin D receptor polymorphism is associated with psoriasis. J Invest Derm **112**:113–116.
187. Okita H, Ohtsuka T, Yamakage A, Yamazaki S 2002 Polymorphism of the vitamin D(3) receptor in patients with psoriasis. Arch Dermatol Res **294**:159–162.

188. Saeki H, Asano N, Tsunemi Y, Takekoshi T, Kishimoto M, Mitsui H, Tada Y, Torii H, Komine M, Asahina A, Tamaki K 2002 Polymorphisms of the vitamin D receptor gene in Japanese patients with psoriasis vulgaris. J Derm Sci **30**:167–171.
189. Mee JB, Cork MJ 1998 Vitamin D receptor polymorphism and calcipotriol response in patients with psoriasis. J Invest Derm **110**:301–302.
190. Kontula K, Valimaki S, Kainulainen K, Viitanen A-M, Keski-Oja J 1997 Vitamin D receptor polymorphism and treatment of psoriasis with calcipotriol. Br J Derm **136**:977–978.
191. Lee DY, Park BS, Choi KH, Jeon JH, Cho KH, Song KY, Kim IG, Youn JI 2002 Vitamin D receptor genotypes are not associated with clinical response to calcipotriol in Korean psoriasis patients. Arch Dermatol Res **294**:1–5.
192. Scott P, Ouimet D, Valiquette L, Guay G, Proulx Y, Trouvé M-L, Gagnon B, Bonnardeaux A 1999 Suggestive evidence for a susceptibility gene near the vitamin D receptor locus in idiopathic calcium stone formation. J Am Soc Nephrol **10**:1007–1013.
193. Ruggiero M, Pacini S, Amato M, Aterini S, Chiarugi V 1999 Association between vitamin D receptor gene polymorphism and nephrolithiasis. Miner Electrolyte Metab **25**:185–190.
194. Jackman SV, Kibel AS, Ovuworie CA, Moore RG, Kavoussi LR, Jarrett TW 1999 Familial calcium stone disease: TaqI polymorphism and the vitamin D receptor. J Endourol **13**:313–316.
195. Mosetti G, Vuotto P, Rendina D, Numis FG, Viceconti R, Girodano F, Cioffi M, Scopacasa F, Nunziata V 2003 Association between vitamin D receptor gene polymorphisms and tubular citrate handling in calcium nephrolithiasis. J Intern Med **253**:194–200.
196. Ozkaya O, Soylemezoglu M, Gonen S, Buyan N, Hasanoglu E 2003 Polymorphisms in the vitamin D receptor gene and the risk of calcium nephrolithiasis in children. Eur Urol **44**:150–154.
197. Chen WC, Chen HY, Hsu CD, Wu JY, Tsai FJ 2001 No association of vitamin D receptor gene BsmI polymorphisms with calcium oxalate stone formation. Mol Urol **5**:7–10.
198. Zerwekh JE, Hughes MR, Reed BY, Breslau NA, Heller HJ, Lemke M, Nasonkin I, Pak CY 1995 Evidence for normal vitamin D receptor messenger ribonucleic acid and genotype in absorptive hypercalciuria. J Clin Endocrinol Metab **80**:2960–2965.
199. McDermott MF, Ramachandran A, Ogunkolade BW, Aganna E, Curtis D, Boucher BJ, Snehalatha C, Hitman GA 1997 Allelic variation in the vitamin D receptor influences susceptibility to IDDM in Indian Asians. Diabetologica **40**:971–975.
200. Guja C, Marshall S, Welsh K, Merriman M, Smith A, Todd JA, Ionescu-Tirgoviste C 2002 The study of CTLA-4 and vitamin D receptor polymorphisms in the Romanian type 1 diabetes population. J Cell Mol Med **6**:75–81.
201. Pani MA, Knapp M, Donner H, Braun J, Baur J, Baur MP, Usadel KH, Badenhoop K 2000 Vitamin D receptor allele combinations influence genetic susceptibility to type 1 diabetes in Germans. Diabetes **49**:504–507.
202. Chang TJ, Lei HH, Yeh JI, Chiu KC, Lee KC, Chen MC, Tai TY, Chuang LM 2000 Vitamin D receptor gene polymorphisms influence susceptibility to type 1 diabetes mellitus in the Taiwanese population. Clin Endocrinol **52**:575–580.
203. Fassbender WJ, Goertz B, Weismuller K, Steinhauer B, Stracke H, Auch D, Linn T, Bretzel RG 2002 VDR gene polymorphisms are overrepresented in German patients with type 1 diabetes compared to healthy controls without effect on biochemical parameters of bone metabolism. Horm Metab Res **34**:330–337.
204. Gyorffy B, Vasarhelyi B, Krikovszky D, Madacsy L, Tordai A, Tulassay T, Szabo A 2002 Gender-specific association of vitamin D receptor polymorphism combinations with type 1 diabetes mellitus. Eur J Endocrinol **147**:803–808.
205. Taverna MJ, Sola A, Guyot-Argenton C, Pacher N, Bruzzo F, Slama G, Reach G, Selam JL 2002 Taq I polymorphism of the vitamin D receptor and risk of severe retinopathy. Diabetologica **45**:436–442.
206. Skrabic V, Zemunik T, Situm M, Terzic J 2003 Viatmin D receptor polymorphism and susceptibility to type 1 diabetes in the Dalmatian population. Diabetes Res Clin Prac **59**: 31–35.
207. Motohashi Y, Yamada S, Yanagawa T, Maruyama T, Suzuki R, Niino M, Fukazawa T, Kasuga A, Hirose H, Matsubara K, Shimada A, Saruta T 2003 Vitamin D receptor gene polymorphism affects onset pattern of type 1 diabetes. J Clin Endocrinol Metab **88**:3137–3140.
208. Aterini S, Pacini S, Amato M, Ruggiero M 2000 Vitamin D receptor gene polymorphism and diabetes mellitus prevalence in hemodialysis patients. Nephron **84**:186.
209. Yokota I, Satomura S, Kitamura S, Taki Y, Naito E, Ito M, Nishisho K, Kuroda Y 2002 Association between vitamin D receptor genotype and age of onset in juvenile Japanese patients with type 1 diabetes. Diabetes Care **25**:1244.
210. Ban Y, Taniyama M, Yanagawa T, Yamada S, Maruyama T, Kasuga A, Ban Y 2001 Vitamin D receptor initiation codon polymorphism influences genetic susceptibility to type 1 diabetes mellitus in the Japanese population. BMC Medical Genetics 2:7 (http://www.biomedcentral.com/1471-2350/2/7).
211. Hitman GA, Mannan N, McDermott MF, Aganna E, Ogunkolade BW, Hales CN, Boucher BJ 1998 Vitamin D receptor gene polymorphisms influence insulin secretion in Bangladeshi Asians. Diabetes **47**:688–690.
211a. Ogunkolade BW, Boucher BJ, Prahl JM, Bustin SA, Burrin JM, Noonan K, North BV, Mannan N, McDermott MF, DeLuca HF, Hitman GA 2002 Vitamin D receptor (VDR) mRNA and VDR protein levels in relation to vitamin D status, insulin secretory capacity, and VDR genotype in Bangladeshi Asians. Diabetes **51**:2294–2300.
212. Speer G, Cseh K, Winkler G, Vargha P, Braun E, Takacs I, Lakatos P 2001 Vitamin D and estrogen receptor gene polymorphisms in type 2 diabetes mellitus and in android type obesity. Eur J Endocrinol **144**:385–389.
213. Ye WZ, Reis AF, Dubois-Laforgue D, Bellane-Chantelot C, Timsit J, Velho G 2001 Vitamin D receptor gene polymorphisms are associated with obesity in type 2 diabetic subjects with early age of onset. Eur J Endocrinol **145**: 181–186.
214. Oh JY, Barett-Connor E 2002 Association between vitamin D receptor polymorphism and type 2 diabetes or metabolic syndrome in community dwelling older adults: the Ranch Bernardo Study. Metabolism **51**:356–359.
215. Ortlepp JR, Lauscher J, Hoffman R, Hanrath P, Joost HG 2001 The vitamin D receptor gene variant is associated with the prevalence of type 2 diabetes mellitus and coronary artery disease. Diabet Med **18**:842–845.
216. Ortlepp JR, Metrikat J, Albrecht M, von Korff A, Hanrath P, Hoffman R 2003 The vitamin D receptor gene variant and physical activity predicts fasting glucose levels in healthy young men. Diabet Med **20**:451–454.
217. Boullu-Sanchis S, Lepretre F, Hedelin G, Donnet JP, Schaffer P, Froguel P, Pinget M 1999 Type 2 diabetes mellitus: association study of five candidate genes in an Indian population of Guadeloupe, genetic contribution of FAB2 polymorphism. Diabetes Metab **25**:150–156.

218. Niimi T, Tomita H, Sato S, Kawaguchi H, Akita K, Maeda H, Sugiura Y, Ueda R 1999 Vitamin D receptor gene polymorphisms in patients with sarcoidosis. Am J Respir Crit Care Med **160**:1107–1109.
219. Guleva I, Seitzer U 2000 Vitamin D receptor gene polymorphism in patients with sarcoidosis. Am J Respir Crit Care Med **162**:760–761.
220. Niimi T, Tomita H, Sato S, Akita K, Maeda H, Kawaguchi H, Mori T, Sugiura Y, Yoshinouchi T, Ueda R 2000 Vitamin D receptor gene polymorphism and calcium metabolism in sarcoidosis patients. Sarcoidosis Vasc Diffuse Ling Dis **17**:266–269.
221. Fukazawa T, Yabe I, Kikuchi S, Sasaki H, Hamada T, Miyasaka K, Tashiro K 1999 Association of vitamin D receptor gene polymorphism with multiple sclerosis in Japanese. J Neurol Sci **166**:47–52.
222. Niino M, Fukazawa T, Yabe I, Kikuchi S, Sasaki H, Tashiro K 2000 Vitamin D receptor gene polymorphism in multiple sclerosis and the association with HLA class II alleles. J Neurol Sci **177**:65–71.

222a. Steckley JL, Dyment DA, Sadovnick AD, Risch N, Hayes C, Ebers GC, and the Canadian Collaborative Study Group 2000 Genetic analysis of vitamin D related genes in Canadian multiple sclerosis patients. Neurology **54**:729–732.

222b. Garcia-Lozano JR, Gonzalez-Escribano MF, Valenzuela A, Garcia A, Nunez-Roldan A 2001 Association of vitamin D receptor genotypes with early onset rheumatoid arthritis. Eur J Immunogenet **28**:89–93.

222c. Lee CK, Hong JS, Cho YS, Yoo B, Kim GS, Moon HB 2001 Lack of relationship between vitamin D receptor polymorphism and bone erosion in rheumatoid arthritis. J Korean Med Sci **16**:188–192.

222d. Goertz B, Fassbender WJ, Williams JC, Marzeion AM, Bretzel RG, Stracke H, Berliner MN 2003 Vitamin D receptor genotypes are not associated with rheumatoid arthritis or biochemical parameters of bone turnover in German RA patients. Clin Exp Rheumatol **21**:333–339.

223. Simmons JD, Mullighan C, Welsh KI, Jewell DP 2000 Vitamin D receptor gene polymorphism: association with Crohns' disease susceptibility. Gut **47**:211–214.
224. Martin K, Radlmayr M, Borchers R, Heinzlmann M, Folwaczny C 2002 Candidate genes co-localized to linkage regions in inflammatory bowel disease. Digestion **66**:121–126.
225. Ban Y, Taniyama M, Ban Y 2000 Vitamin D receptor gene polymorphism is associated with Graves' disease in the Japanese population. J Clin Endocrinol Metab **85**:4639–4643.
226. Ban Y, Ban Y, Taniyama M, Katagiri T 2000 Vitamin D receptor initiation codon polymorphism in Japanese patients with Graves' disease. Thyroid **10**:475–480.
227. Pani MA, Seissler J, Usadel KH, Badenhoop K 2002 Vitamin D receptor genotype is associated with Adison's disease. Eur J Endocrinol **147**:635–640.
228. Liu W, Zhang CY, Wu XM, Tian L, Li CZ, Zhao QM, Zhang PH, Yang SM, Yang H, Zhang XT, Cao WC 2003 A case-control study on the vitamin D receptor gene polymorphisms and susceptibility to pulmonary tuberculosis. Zhonghua Liu Xing Bing Xue Za Zhi **24**:389–392.
229. Delgado JC, Baena A, Thim S, Goldfeld AE 2002 Ethnic-specific genetic associations with pulmonary tuberculosis. J Infect Dis **186**:1463–1468.
230. Roy S, Frodsham A, Saha B, Hazra SK, Mascie-Taylor CGN, Hill AVS 1999 Association of vitamin D receptor genotype with Leprosy type. J Infect Dis **179**:187–191.
231. Gelder CM, Hart KW, Williams OM, Lyons E, Welsh KI, Campbell IA, Marshall SE 2000 Vitamin D receptor gene polymorphisms and susceptibility to *Mycobacterium malmoense* pulmonary disease. J Infect Dis **181**:2099–2102.
232. Bellamy R, Ruwende C, Corrah T, McAdam KPWJ, Thursz M, Whittle HC, Hill AVS 1999 Tuberculosis and chronic hepatitis B virus infection in Africans and variation in the vitamin D receptor gene. J Infect Dis **179**:721–724.
233. Wilkinson RJ, Llewelyn M, Toosi Z, Patel P, Pasvol G, Lalvani A, Wright D, Latif M, Davidson RN 2000 Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. Lancet **355**:618–621.
234. Barber Y, Rubio C, Fernandez E, Rubio M, Fibla J 2001 Host genetic background at CCR5 Chemkine Recptor and vitamin D receptor loci and human immunodeficiency and (HIV) type 1 disease progression and HIV-seropositive injection drug users. J Infect Dis **184**:1279–1288.
235. Henning BJ, Parkhill JM, Chapple IL, Heasman PA, Taylor JJ 1999 Association of vitamin D receptor gene polymorphisms with localized early-onset periodontal diseases. J Perdiodontol **70**:1032–1038.
236. Tachi Y, Shimpuku H, Nosaka Y, Kawamura T, Shinohara M, Ueda M, Imai H, Ohura K 2003 Vitamin D receptor gene polymorphisms is associated with chronic periodontitis. Life Sci **73**:3313–3321.
237. Muray S, Parisi E, Cardus A, Craver L, Fernandez E 2003 Influence of vitamin D receptor gene polymorphisms and 25-hydroxyvitamin D on blood pressure in apparently healthy subjects. J Hypertens **21**:2069–2075.
238. Lee BK, Lee GS, Stewart WF, Ahn KD, Simon D, Kelsey KT, Todd AC, Schwartz BS 2001 Associations of blood pressure and hypertension with lead dose measures and polymorphisms in the vitamin D receptor and delta-aminovulinic acid dehydratase genes. Environ Health Perspect **109**: 383–389.
239. Nakano Y, Oshima T, Sasaki S, Yamaoka K, Matsumoto T, Hirao H, Ozono R, Matsuura H, Kajiyama G, Kambe M 2000 Vitamin D receptor gene polymorphism is associated with serum total and ionized calcium concentration. J Mol Med **78**:575–579.
240. Van Schooten FJ, Hirvonen A, Maas LM, de Mol BA, Kleinjans JC, Bell DA, Durrer JD 1998 Putative susceptibility markers of coronary artery disease: association between VDR genotype, smoking, and aromatic DNA adduct levels in human right atrial tissue. FASEBJ **12**:1409–1417.
241. Ortlepp JR, Hoffman R, Ohme F, Lauscher J, Bleckmann F, Hanrath P 2001 The vitamin D receptor genotype predisposes to the development of calcific aortic valve stenosis. Heart **85**:635–638.
242. Ortlepp JR, von Korff A, Hanrath P, Zerres K, Hoffman R 2003 Vitamin D receptor gene polymorphism BsmI is not associated with the prevalence and severity of CAD in a large-scale angiographic cohort of 3441 patients. Eur J Clin Invest **33**:106–109.
243. Zittermann A, Schulze Schleithoff S, Tenderich G, Berthold HK, Korfer R, Stehle P 2003 Low vitamin D status: a contributing factor in the pathogenesis of congestive heart failure JACC **41**:105–112.
244. Uitterlinden AG, Burger H, Witteman JCM, van Leeuwen JPTM, Pols HAP 1998 Genetic relation between osteoporosis and cardiovascular disease: vitamin D receptor polymorphism predicts myocardial infarction. Osteop Int **8**: (abstract OR12).

CHAPTER 69

Clinical Disorders of Phosphate Homeostasis

MARC K. DREZNER Department of Medicine, University of Wisconsin and Geriatrics Research Education and Clinical Center, William H. Middleton Veterans Administration Medical Center, Madison, Wisconsin

I. Introduction

Extensive studies over the past several decades have established that phosphate homeostasis and vitamin D metabolism are reciprocally regulated. As discussed in Chapters 26 and 29, calcitriol promotes phosphate absorption from the intestine, mobilization from bone, and reabsorption in the renal tubule [1]. In turn, phosphate depletion or hypophosphatemia stimulates renal production of $1,25(OH)_2D$ (calcitriol), while phosphate overload or hyperphosphatemia inhibits renal 25(OH)D-1α-hydroxylase activity [2].

Since phosphorus is one of the most abundant constituents of all tissues, disturbances in phosphate homeostasis can affect almost any organ system [3]. Indeed, a deficiency or excess of this mineral can have profound effects on a variety of tissues, which include consequences of hypophosphatemia, such as osteomalacia, rickets, red cell dysfunction, rhabdomyolysis, metabolic acidosis and cardiomyopathy, and of hyperphosphatemia, such as soft tissue calcification, hypocalcemia, tetany, and secondary hyperparathyroidism. In many cases, the inter-relationship between phosphate homeostasis and vitamin D metabolism precludes establishing whether the consequences of hypo- or hyperphosphatemia are singularly related to this abnormality or are modified by changes in calcitriol production. Occasionally, however, discrimination between these possibilities has been achieved by evaluation of the therapeutic response to phosphate supplementation or depletion. Such studies indicate that few of the phosphate homeostatic disorders respond adequately to therapeutically induced alterations in phosphate alone, but do regress upon coincident modification of the vitamin D status. The detailed control mechanisms that regulate phosphate homeostasis and vitamin D metabolism in intestine and kidney are reviewed in Chapters 24 and 29 and overall physiology in Chapter 26. Following is a summary of important elements of the phosphate homeostatic schema and the regulation of vitamin D metabolism that pertain to an understanding of the diseases described in the remainder of this chapter and in Chapter 70.

A. Regulation of Phosphate Homeostasis

The kidney is the major arbiter of extracellular phosphate (Pi) homeostasis and plays a key role in bone mineralization and growth. Most of the filtered phosphorus is reabsorbed in the proximal tubule, with approximately 60% of the filtered load reclaimed in the proximal convoluted tubule and 15–20% in the proximal straight tubule [4]. In addition, a small but variable portion (<10%) of filtered phosphorus is reabsorbed in more distal segments of the nephron.

Transepithelial phosphorus transport is effectively unidirectional and includes uptake at the brush border membrane of the renal tubule cell, translocation across the cell and efflux at the basolateral membrane [5]. Apical sodium-dependent phosphate (Na/Pi) cotransport across the luminal (brush border) membrane is rate limiting and the target for physiological/pathophysiological alterations. The uptake at this site is mediated by Na^+-dependent phosphate transporters that reside in the brush border membrane and depend on the Na^+,K^+-ATPase to maintain the Na^+ gradient that drives the transport system [6]. In contrast, basolateral Pi-transport systems are not well defined. Efflux of Pi across the basolateral membrane may involve an anion exchange mechanism and/or a "Pi leak" to complete transcellular reabsorptive flux, and a Na^+-dependent Pi uptake mechanism to guarantee Pi uptake from the interstitium if apical influx is insufficient to maintain cellular metabolism.

Three distinct and unrelated families of mammalian Na/Pi-cotransporters have been identified, types I, II, and III. All three types are expressed in proximal tubular cells and have the capacity to induce an increase in Na-dependent Pi uptake in heterologous expression

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systems [7]. Although the type I Na/Pi cotransporter is localized in the brush border membrane of proximal tubular cells [8], this transporter does not contribute significantly to regulation of proximal tubular Pi flux. Indeed heterologous expression studies indicate that the type I-mediated Na/Pi cotransport does not have the characteristics and regulatory features of brush border membrane Na/Pi cotransport. Rather, these studies suggest that the type I cotransporter has a channel function, mediating the flux of chloride and organic anions [7,9,10].

In contrast, the type II cotransporter, located predominantly in the apical membrane of the cells in the S_1 segments of the proximal tubule in kidneys of young (IIc) and adult animals (IIa) [11], plays a key role in renal Pi handling. Indeed, studies in adult animals support the vital function of the type IIa receptor in several ways: 1) exclusive proximal tubular brush border membrane localization of this receptor [12]; 2) loss of 70–80% of brush border membrane Na/Pi cotransport upon disruption of the gene encoding the type IIa cotransporter [13]; and 3) correlation of type IIa protein abundance in brush border membranes with Na/Pi cotransport activity under a variety of physiologic/pathophysiological conditions [7]. The gene for the type II cotransporter has been mapped to chromosome 5q35.

The type III Na/Pi cotransporters are cell-surface viral receptors [14], and they appear to exhibit ubiquitous renal and extrarenal expression. Although type III mRNA expression is detected in all nephron segments [6], studies to localize the type III protein in apical or basolateral cell membranes are lacking. However, the type III Na/Pi cotransporters may be responsible for basolateral Pi influx in all tubular cells to maintain cell metabolism, as well as in proximal tubular cells under conditions of limited apical influx.

Proximal tubular Na/Pi cotransport is regulated by a variety of hormones/metabolic factors, which elicit an increase or decrease in Pi reabsorption through alteration of the type IIa cotransporter (NPT2a) protein availability (Fig. 1). Thus, parathyroid hormone (PTH)-dependent inhibition of Na^+-Pi cotransport depends upon internalization of the cell surface NPT2a protein [15]. Likewise, the acute phase of renal adaptation to phosphate restriction is associated with an increase in NPT2a protein, which is rapidly reversed by a high Pi diet [16]. Not surprisingly, NPT2a protein availability is also central to modulation of the abnormal renal phosphate transport underlying a large number of phosphate homeostatic disorders. These changes in Na/Pi cotransport and NPT2a protein expression occur predominantly in the absence of variations in NPT2a mRNA levels. Thus, rapid increments or decrements in Na/Pi cotransport follow either membrane insertion of NPT2a protein, which may be preceded by *de novo* synthesis of the protein, or to membrane retrieval of NPT2a protein, followed by lysosomal degradation [7]. Although such membrane trafficking of NPT2a protein is an unequivocally central element in the regulation of Na/Pi cotransport, changes in NPT2a mRNA levels do occur after prolonged treatment with $1,25(OH)_2D$, PTH or thyroid hormone, following chronic changes in dietary Pi and in disorders of Pi homeostasis, such as

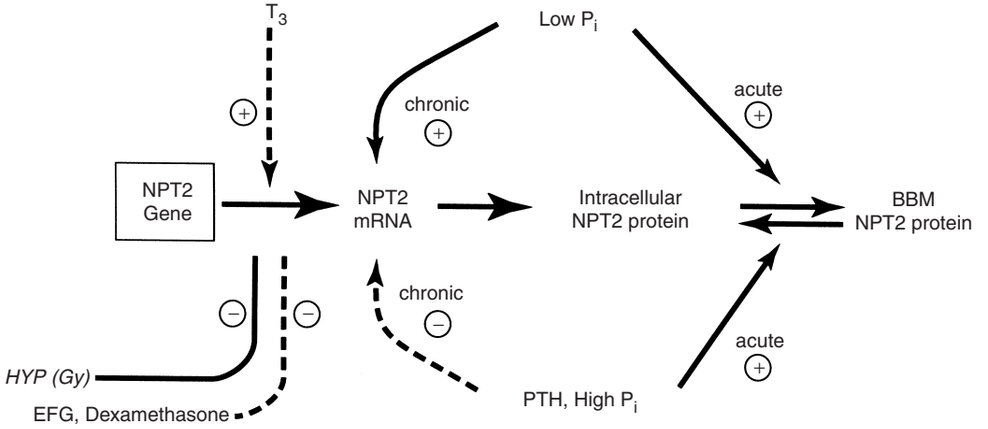

FIGURE 1 Hormonal and metabolic regulation of NPT2a gene expression and protein production in the kidney. Solid lines represent pathways of regulation that are supported by available data, while dashed lines depict instances where direct evidence for a regulatory mechanism is not available. BBM, brush border membrane; EGF, epidermal growth factor; NPT2a, Na^+-dependent phosphate cotransporter; *Hyp (Gy),* murine homologues of X-linked hypophosphatemic rickets (XLH); T_3, triiodothyronine. Adapted from Tenenhouse [4].

X-linked hypophosphatemia (XLH). Thus, a secondary mechanism operates under select conditions to regulate Na/Pi cotransport (Fig. 1).

The intracellular signaling mechanisms involved in insertion/retrieval of NPT2a protein remain incompletely understood. However, several studies have identified numerous signals for NPT2a internalization. PTH effects on NPT2a protein are initiated by binding to receptors that activate protein kinase C on apical membranes and protein kinase A and C on basolateral membranes [17]. In contrast, atrial natriuretic protein induced internalization of NPT2a protein is mediated by protein kinase G activation [18]. Recent investigations suggest that these different signaling pathways converge on the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway to internalize the NPT2a protein [19]. In concert with these observations, additional reports indicate that FGF-23, a putative phosphatonin, which may contribute to renal Pi wasting in a variety of genetic and acquired human disorders (see below), inhibits NPT2a-mediated Na/Pi cotransport via activation of MAPK [20]. However, the downstream targets for ERK/MAPK remain unknown, as changes in the phosphorylation state of the NPT2a transporter have not been demonstrated in response to ERK/MAPK modulated hormonal/metabolic regulation of renal Pi transport [21].

In any case, the signaling process regulating internalization/retrieval of NPT2a is insufficient to explain the integrated regulation of Pi homeostasis at the kidney. Rather it appears that protein/protein interactions may also participate in regulation of Pi reabsorption. In this regard, NaPi-Cap 1 and NHERF-1, brush border membrane proteins, may interact with NPT2a via one of multiple PDZ-domains, resulting in release of the protein from an apical scaffold, thereby permitting internalization [22,23]. Clearly, further studies are necessary to determine the interrelated mechanisms regulating the many processes that affect the abundance of NPT2a in brush border membranes.

B. Phosphate Dependent Modulation of Vitamin D Metabolism

The production of $1,25(OH)_2D$, the active metabolite of vitamin D, is under stringent control (see Chapter 5). Indeed, 1α-hydroxylation represents the most important regulatory mechanism in the metabolism of vitamin D [24]. In normal adults, serum $1,25(OH)_2D$ concentrations change little in response to repeated dosing with vitamin D, and remain normal, or even decline, in vitamin D intoxication. Phosphorus is one of the three major factors that regulate the activity of the enzyme [25]. In this regard, in rats, mice, and humans, phosphate depletion and resultant hypophosphatemia stimulate 25-hydroxyvitamin D-1α-hydroxylase activity and increase the serum $1,25(OH)_2D$ concentration, whereas phosphate loading and consequent hyperphosphatemia inhibit formation of this metabolite. The mechanism whereby phosphorus modulates this adaptive effect remains unknown. Fukase *et al.* [26] reported that phosphorus may have a direct effect on the kidney and several studies indicate that the effect is, in fact, independent of PTH. In contrast, alternative evidence suggests that phosphorus regulation of $1,25(OH)_2D$ production may depend on growth hormone [27] or insulin-like growth factor I [28].

Regardless of the mechanism, the potential role of phosphorus in regulating vitamin D metabolism is central to understanding and appropriately treating many of the clinical disorders of phosphate homeostasis. As will become evident in the remainder of this chapter, however, the aberrant regulation of vitamin D metabolism encountered in many of these diseases is considered paradoxical. Thus, in virtually all disorders secondary to abnormal renal phosphate transport, hypophosphatemia or hyperphosphatemia is perplexingly associated with decreased or increased serum $1,25(OH)_2D$ levels, respectively. These observations suggest that the effect of phosphorus on 25-hydroxyvitamin D-1α-hydroxylase activity may, in fact, occur indirectly and secondary to alterations in renal phosphate transport systems. In this regard, phosphate depletion and hypophosphatemia and phosphate loading and hyperphosphatemia are associated with compensatory changes in renal phosphate transport that may mediate changes in $1,25(OH)_2D$ production. In accord, the prevailing serum calcitriol levels in normals and patients with disorders of renal phosphate transport, XLH, tumor-induced osteomalacia (TIO) and tumoral calcinosis (TC), display a highly significant positive correlation with the renal TmP/GFR [29], supporting the possibility that renal tubular reabsorption of phosphate may be a major determinant of renal $1,25(OH)_2D$ production (Fig. 2). Of course, these data do not establish that alterations in 25-hydroxyvitamin D-1α-hydroxylase activity, in response to phosphate depletion or loading, are dependent upon renal phosphate transport. However, the recent observations that phosphate depletion does not increase $1,25(OH)_2D$ production in mice subjected to treatment with phosphonoformic acid, which precludes a compensatory alteration in TmP/GFR, suggests an important role for the renal phosphate transport system in modulating phosphorus mediated effects on 1α-hydroxylase activity under all conditions (Fig. 3). Additionally, preliminary studies have documented that mice with targeted disruption of the NHERF-1

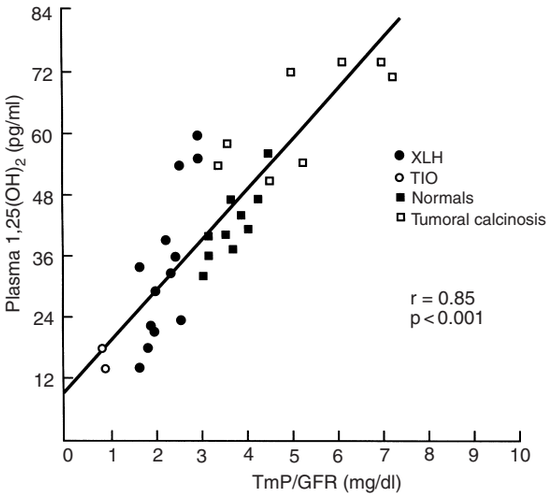

FIGURE 2 Correlation between renal TmP/GFR and plasma $1,25(OH)_2D$ levels in normals and patients with XLH, TIO, and tumoral calcinosis. Significant linear correlation is evident, suggesting a potential relationship between renal phosphate transport and the prevailing plasma levels of active vitamin D. TIO, tumor-induced osteomalacia. From Drezner [29].

gene, which singularly promotes internalization of proximal tubule Npt2a, manifest ~50% decreased apical membrane localized Npt2a and consequent renal P wasting and hypophosphatemia [22], and paradoxically exhibit inappropriately normal serum $1,25(OH)_2D$ levels, similar to *hyp*-mice with comparable Npt2a deficiency. In contrast, Tenenhouse *et al.* [30] concluded from studies in the *Npt2a*$^{-/-}$-mouse that Npt2a and renal P transport do not influence 25(OH)D-1α-hydroxylase activity. However, these data may have limited applicability to understanding the role of renal phosphate transport on vitamin D metabolism, since it is well known that compensatory developmental changes often confound studies in knockout mice with complete absence of a protein function. In this regard, several investigators [11,31] recently reported that the sodium-dependent P cotransporter, Npt2c, normally expressed in murine kidneys of young animals, has sustained activity in adult *Npt2a*$^{-/-}$-mice, which is likely responsible for the "inappropriate" residual renal P transport in these mutants and the resultant limited hypophosphatemia. The absence of similar Npt2c expression in the kidneys of adult normal and NHERF knockout mice [32] substantiates that enhanced Npt2c expression in adult *Npt2a*$^{-/-}$-mice may be a unique compensatory mechanism, which limits the applicability of studies in this model to conclusions regarding regulation of vitamin D metabolism.

While these data indicate that the renal production of $1,25(OH)_2D$ and the renal tubular reabsorption of phosphate are linked, the precise mechanism(s) underlying this association remain uncertain. However, it is clear that understanding the pathophysiology of, and defining appropriate treatment regimens for, many clinical disorders of phosphate homeostasis require investigation of the vitamin D regulatory system.

II. DISORDERS OF PHOSPHATE HOMEOSTASIS

The variety of diseases, therapeutic agents, and physiological states that affect phosphate homeostasis are numerous and reflect a diverse pathophysiology. Indeed, rational choice of an appropriate treatment for many of these disorders depends on determining the precise cause for the abnormality. In general, defects in phosphate homeostasis result from impaired renal tubular phosphate reabsorption and a consequent change in the TmP/GFR, an altered phosphate load due to varied intake, or abnormal gastrointestinal absorption or translocation of phosphorus between the extracellular fluid and tissues (Table I). The disorders of renal phosphate transport are the most common of these diseases and have been intensively studied. Indeed, investigation of these conditions has provided new insight into the reciprocal regulation of phosphate homeostasis and vitamin D metabolism. In the remainder of this chapter and in Chapter 70, several clinical states that represent disorders of phosphate homeostasis will be discussed and the potential role of the vitamin D endocrine system in their pathogenesis and phenotypic presentation highlighted.

A. Disorders of Renal Phosphate Transport: Hypophosphatemic Diseases

The disorders of renal phosphate transport, which lead to phosphate wasting and hypophosphatemia, are by far the most common disturbances of phosphate homeostasis. However, the pathophysiological basis of these disorders has remained elusive, largely because the hormonal/metabolic control of phosphate homeostasis at the kidneys and in bone is not completely understood. In this regard, the function of the PTH-vitamin D axis is not sufficient to explain the physiological complexity of systemic phosphate homeostasis. Nevertheless, the PTH-vitamin D independent mechanism(s) by which phosphate excess or phosphate depletion influence the net rate of proximal tubule phosphate reabsorption and bone mineralization remains unknown. Recently, significant new information has emerged from studies of the hypophosphatemic disorders, Tumor-induced

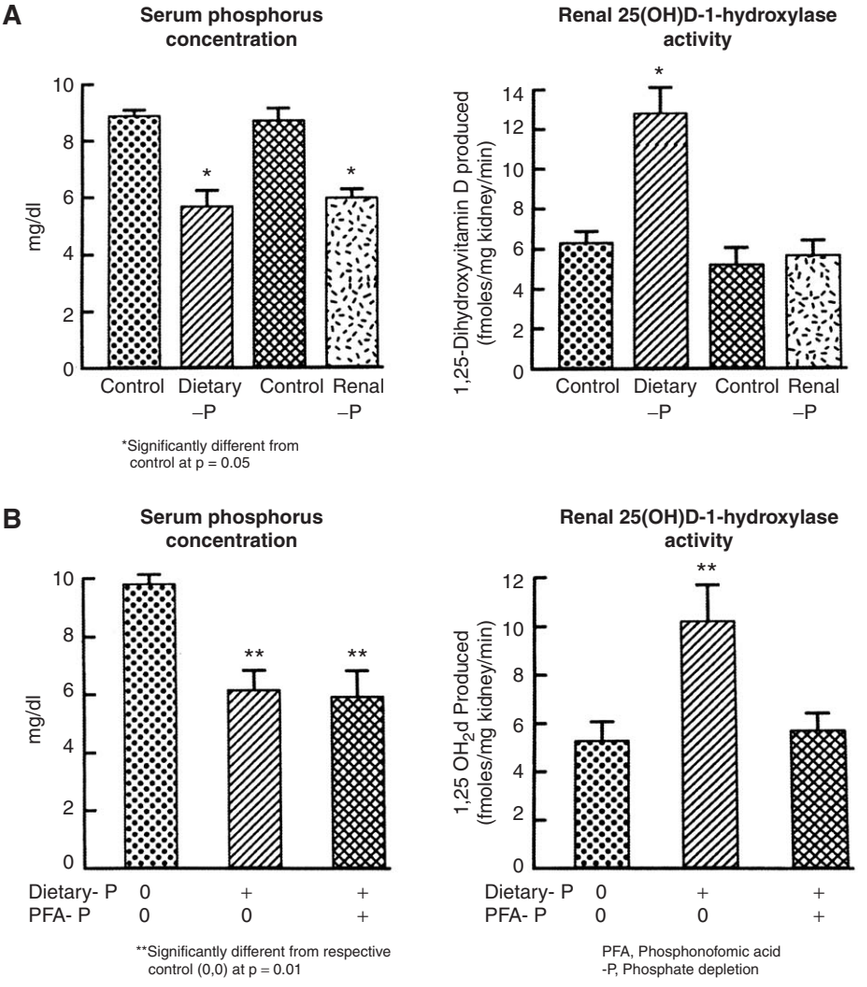

FIGURE 3 **A.** While hypophosphatemia resulting from phosphate restriction increases renal 25(OH)D-1α-hydroxylase activity, hypophosphatemia of similar magnitude secondary to phosphonoformic acid (PFA) mediated renal phosphate wasting has no effect on enzyme function. These data suggest that hypophosphatemia is not the proximal trigger mechanism for $1,25(OH)_2D$ production. **B.** In similar experiments the effects of phosphate depletion and consequent hypophosphatemia on renal 25(OH)D-1α-hydroxylase activity are blocked by the coincident administration of phosphonoformic acid, which prevents the expected compensatory change in renal phosphate flux attendant upon the depletion. These observations reaffirm the potentially important role of renal phosphate transport in modulating the effects of phosphate on for $1,25(OH)_2D$ production.

osteomalacia (TIO), X-linked hypophosphatemic rickets/osteomalacia (XLH) and Autosomal dominant hypophosphatemic rickets (ADHR), which brings new light to the mechanisms potentially regulating phosphate homeostasis and bone mineralization. These investigations have putatively identified circulating factors that alter renal tubular phosphate reabsorption and bone mineralization. Indeed, this work has lent credence to the possibility that a hormone or family of hormones, called *phosphatonin(s)* and/or *minhibin(s)* exist, which regulate renal phosphate transport and bone mineralization and participate in the pathophysiological cascade of events underlying many of the renal phosphate wasting disorders. In fact, current theories have emerged, which suggest that a common metabolic pathway underlies many of these hypophosphatemic diseases.

1. THE COMMON PATHWAY UNDERLYING THE PATHOGENESIS OF TIO, XLH, AND ADHR

As detailed below and in Chapter 70, XLH and ADHR are disorders characterized by hypophosphatemia, due to impaired renal tubular reabsorption

TABLE I Disorders of Phosphate Homeostasis

Abnormal Renal Phosphate Transport

Hypophosphatemic syndromes

- Genetic Diseases
  - X-Linked hypophosphatemia (XLH)
  - Hereditary hypophosphatemic rickets with hypercalciuria (HHRH)
  - Tumor-induced osteomalacia (TIO)
  - Autosomal dominant hypophosphatemic rickets (ADHR)
  - Autosomal recessive hypophosphatemic rickets (ARHR)
  - McCune Albright Syndrome (MAS)
  - Fanconi syndrome, type I (FS I)
    - Familial Idiopathic
    - Cystinosis (Lignac-Fanconi Disease)
    - Oculocerebrorenal (Lowe) syndrome
    - Glycogen storage disease
    - Wilson disease
    - Galactosemia
    - Tyrosinemia
    - Hereditary Fructose Intolerance
  - Neurofibromatosis
  - Linear nevus sebaceous syndrome
  - Fanconi syndrome, type II (FS II)
- Acquired Disorders
  - Tumor-Induced Osteomalacia
    - Mesenchymal, epidermal and endodermal tumors
  - Light chain nephropathy
  - Renal transplantation
  - Multiple Myeloma
  - Cadmium intoxication
  - Lead intoxication
  - Tetracycline (outdated) administration

Hyperphosphatemic syndromes

- Tumoral calcinosis

Altered Phosphate Load

Hypophosphatemic syndromes

- Decreased Phosphate Availability
  - Phosphate deprivation
  - Gastrointestinal malabsorption
- Transcellular Shift of Phosphate
  - Alkalosis
  - Glucose Administration
- Combined Mechanisms
  - Alcoholism
  - Burns
  - Nutritional recovery syndrome
  - Diabetic ketoacidosis

Hyperphosphatemic syndromes

- Vitamin D intoxication
- Rhabdomyolysis
- Cytotoxic therapy
- Malignant hyperthermia

of phosphate, inappropriately normal or decreased production and serum levels of $1,25(OH)_2D$, and defective cartilage and bone mineralization. In contrast, TIO and McCune-Albright syndrome (MAS) (caused by activating mutations of $G_s\alpha$) are hypophosphatemic disorders caused by production of a factor(s) by tumors or fibrous dysplastic bone cells, respectively, which leads to phenotypic features similar to those of the hereditary phosphate wasting diseases. Based on the shared phenotype of these disorders, several groups have postulated the existence of a unique circulating protein(s), common to each of these diseases, which inhibits sodium-dependent phosphate reabsorption by the renal proximal tubule by PTH distinct mechanisms, impairs bone and cartilage mineralization, and counters hypophosphatemia-mediated increments in the renal production of $1,25(OH)_2D$.

Initial evidence lending credence to the possibility that there is such a common metabolic pathway underlying these diseases derived from studies of patients with TIO. By culturing a tumor associated with this disease, Cai *et al.* [33] demonstrated that supernatants of such tumor cells maintained in culture contained a factor(s) that inhibited sodium-dependent phosphate reabsorption in renal cultured epithelia. The effects of this factor(s) were specific, cyclic AMP independent and distinct from those of PTH. These data, as well as subsequent reports from other groups [34–36], indicated the existence of a novel substance, putatively named *phosphatonin* [37], that alters phosphate reabsorption in the renal tubule. Further investigation, utilizing serial analysis of gene expression and array profiling to identify overexpressed genes in tumors from patients with TIO, confirmed enhanced production of several genes, including those that encode fibroblast growth factor (FGF) 23, secreted frizzle-related protein (sFRP)-4, and matrix extracellular phosphoglycoprotein (MEPE) [38–42].

The possibility that these observations linked the pathogenesis of TIO to that of ADHR was suggested

by the seminal discovery that FGF 23 has a pathogenetic role in patients with ADHR [43,44]. Patients with this syndrome have missense mutations in the *FGF 23* gene at the 176-RXXR-179 site (R176Q, R179W, and R179Q), rendering the FGF 23 protein resistant to cleavage/hydrolysis and inactivation by proteolytic enzymes [43,45]. The resultant increase in circulating levels of cleavage-resistant FGF 23 was presumed responsible for phosphaturia and abnormal bone mineralization due to direct actions of this hormone on the renal proximal tubule and the osteoblasts. Since tumors in patients with TIO overexpress FGF 23 and mutations in the *FGF 23* gene underlie ADHR, the possibility that FGF 23 is the presumed phosphatonin operative in these diseases seemed plausible.

Indeed, this possibility was supported by a series of observations, which established the biological activity of FGF 23, an approximately 26-kDA circulating protein, consisting of an N-terminal FGF homology domain and a novel 71-amino acid C-terminus of uncertain function. In this regard, Shimada *et al.* [41] reported that administration of biosynthetically prepared full-length FGF 23 to mice resulted in phosphaturia but did not lead to upregulation of $1,25(OH)_2D$ production. Bai *et al.* [46] also discovered that intact FGF 23 has enhanced *in vivo* biological potency, and tumors derived from cells that overexpressed FGF 23 caused a more severe osteomalacia and rickets in nude mice than observed due to hypophosphatemia alone, suggesting a direct effect of FGF 23 on cartilage and bone. In concert, Bowe *et al.* [39] found that FGF 23 inhibited sodium-dependent phosphate transport in cultured renal epithelia by decreasing the expression of the NPT2a mRNA and protein. Conversely, FGF 23-null mice exhibit elevated serum phosphorus levels and increased $1,25(OH)_2D$ production, confirming an essential and nonredundant role of FGF 23 in regulation of phosphate homeostasis [47], while FGF 23 transgenic mice exhibit a phenotype consistent with a severe form of ADHR [48]. In addition, several groups have found that circulating FGF 23 levels are elevated in the majority of patients with TIO and those with ADHR, as well as those with MAS, who manifest hypophosphatemia [49–51].

Studies of XLH have further reinforced the possibility that FGF 23 is the phosphatonin, providing a common link to the pathogenesis of the phosphate wasting diseases. Early studies by Meyer *et al.* [52], testing parabiosis between *hyp*- and normal mice, suggested the presence of a circulating factor that causes hypophosphatemia in the mouse model of XLH. In concert, Lajeunesse *et al.* [53] discovered the presence of a serum factor in *hyp*-mice that inhibits phosphate transport in renal epithelia, and others reported that a similar factor is elaborated by *hyp*-mouse osteoblasts, which not only inhibits renal phosphate transport but impairs osteoblast mineralization [54,55]. Further, renal cross-transplantation studies confirmed the presence of a phosphate transport inhibitory factor in *hyp*-mice [56].

With the discovery that mutations of the PHEX gene underlie XLH (see below), several observations suggested the likely interrelated events linking the HYP phenotype to FGF 23. Most notably, the absence of PHEX in the kidneys of *hyp*-mice [57–63] indicated that the gene mutation must indirectly regulate the expression of NPT2/Npt2 in renal tubular cells. In addition, recognition that PHEX is one of six members of the M13 family of zinc-dependent type II cell surface membrane metalloproteinases [64] suggested that PHEX/Phex most likely inactivates a novel phosphaturic/minhibin hormone. Thus, the most plausible pathophysiological basis for XLH is: 1) an inactivating mutation of PHEX produces inadequate amounts of the PHEX endopeptidase; 2) a resultant ineffective or inadequate degradation/inactivation of phosphatonin/minhibin occurs, causing elevated circulating levels of bioactive protein; and 3) a consequent repressed expression of NPT2 manifests, resulting in renal P wasting and hypophosphatemia, and unknown hormone provoked changes in the osteoblast retard mineralization. This formulation has been supported, and the role of FGF 23 as the phosphatonin apparently confirmed by the observations that: 1) FGF 23 is possibly a substrate for PHEX [39]; 2) circulating FGF 23 levels are elevated in *hyp*-mice [65] and in a subset of patients with XLH [49,50]; 3) and antibody mediated neutralization of FGF 23 ameliorates hypophosphatemia and rickets in *hyp*-mice [65].

Collectively, the aforementioned observations have formed the basis for an enzyme/substrate model for the common pathogenesis of XLH, TIO, and ADHR. According to this model, FGF 23 is phosphatonin(/minhibin), the hormone that inhibits sodium-dependent phosphate uptake in the renal proximal tubule and impairs bone mineralization. This model presumes that only full-length FGF 23 is phosphaturic and impairs mineralization, and it postulates that the cell surface enzyme PHEX degrades FGF 23 into inactive fragments. Further, the model predicts that FGF 23 is increased in ADHR because of mutations in FGF 23 that render it resistant to PHEX-dependent cleavage, in XLH because inactivating mutations of PHEX prevent the normal degradation of FGF 23, and in TIO because overproduction of FGF 23 overwhelms the inactivation capacity of degradative mechanisms. Although this model has support, especially for ADHR and possibly TIO, there are a number of inconsistencies and unexplained observations, which raise concerns about whether this simple PHEX-FGF 23 hypothesis

is correct. First and foremost, several investigators have identified other potential phosphatonins/minhibins, which may be operative in TIO and XLH. Second, it has not been established that FGF 23 is unequivocally a substrate for PHEX. Third, the biololgical activites of FGF 23, which have been documented, are incomplete and, more importantly, do not explain the panorama of phenotypic abnormalities, common to the hypophosphatemic diseases.

a. The Family of Phosphatonins/Minhibins The most compelling observation, which challenges the PHEX-FGF 23 hypothesis, is the existence of additional phosphatonin/minhibin-like molecules. Thus, while it is tempting to speculate that TIO is due solely to excessive production of FGF 23, this is not likely the case since tumors overexpress other molecules, including sFRP-4 and MEPE [42] that exhibit the characteristics of a phosphatonin/minhibin. In this regard, although the previously conceived information about secreted frizzle related proteins, including their localization at the plasma membrane and/or in the ECM and their dependence on Wnt-dependent signaling, created an initial bias that sFRP-4 is an unlikely candidate for a phosphatonin, recent investigations by Berndt *et al.* [66] have required re-evaluation of this preconception. Indeed, they have provided compelling evidence that sFRP-4 has unmistakable characteristics of a phosphatonin. Thus, recombinant sFRP-4 inhibits sodium-dependent phosphate transport in cultured opossum renal epithelial cells, indicating a possible direct action of sFRP-4 on proximal tubular phosphate transport. Further, the systemic administration of recombinant sFRP-4 caused phosphaturia (and hypophosphatemia) in normal rats without stimulating renal 25-hydroxyvitamin D-1α-hydroxylase activity. Moreover, the effects on renal epithelia occurred through a mechanism that involved antagonism of Wnt-dependent β-catenin pathways. And finally, Berndt *et al.* [66] extended these observations by showing that sFRP-4 is present in normal human serum and in the serum of patients with TIO.

Similar experiments have established MEPE as a phosphatonin or more likely a minhibin. In this regard, extensive experiments have documented that MEPE is exclusively expressed in osteoblasts and osteocytes in rodents [67–72] and in abundance in human bone, as well as in human brain, albeit in lesser amounts [67,70–72]. Moreover, MEPE-expression occurs in all tumors from patients with TIO and is notably absent in nonphosphaturic tumors [38,39,41,67]. Further, Rowe *et al.* [73] have demonstrated that MEPE inhibits phosphate transport in renal epithelia *in vitro* and when administered *in vivo* to rats results in significant dose dependent phosphaturia and hypophosphatemia. However, a recent report of the MEPE null-mutant mouse phenotype showed that the MEPE-deficient mice did not have abnormalities of serum phosphorus, as might be expected if MEPE played an important role in phosphate homeostasis [72]. Moreover, Liu *et al.* [74] reported that transfer of MEPE deficiency onto the *hyp*-mouse background failed to rescue the hypophosphatemia in the mutants, suggesting that MEPE may not cause phosphaturia in the setting of inactivating *Phex* mutations. Thus, it is possible that MEPE may serve only as a minhibin and not a phosphatonin. In accord with this possibility, MEPE-deficient mice exhibit increased bone formation and mineralization, consistent with the possibility that MEPE, under physiological conditions, plays an inhibitory role in bone mineralization and formation. Moreover, Rowe *et al.* [73] documented that MEPE dose-dependently inhibited BMP2 mediated mineralization of a murine osteoblast cell-line (2T3) *in vitro.* This activity was localized to a cathepsin-B released carboxy-terminal MEPE-peptide, in accord with the multifunctional nature of MEPE, which harbors distinct domains. Further, MEPE transcripts are increased in poorly mineralizing bone derived from *hyp*-mice [70], consistent with a role for MEPE in the mineralization defect in XLH. In addition, the targeted overexpression of PHEX to osteoblasts is associated with reductions in MEPE expression and normalization of osteoblast-mediated mineralization *ex vivo* [69]. Finally, consistent with the enzyme/substrate model for the common pathogenesis of the hypophosphatemic disorders, studies suggest that Phex may modify the hydrolysis of MEPE by inhibiting cathepsin B-dependent cleavage of the protein, which may inplicate MEPE in the local regulation of mineralization through Phex-dependent mechanisms [68].

b. The Role of FGF 23 as a PHEX Substrate A variety of additional observations likewise challenge the PHEX-FGF 23 hypothesis. Most notably, recent investigations provide compelling evidence that FGF 23 is not a substrate for PHEX. The initial studies of Bowe *et al.* [39], which identified FGF 23 as a substrate for PHEX, provided fundamental justification of the enzyme/substrate model. However, documentation that recombinant PHEX may cleave FGF 23 at the RXXR motif or a nearby site has not been confirmed by other investigations [75,76]. Indeed, additional studies suggest that the initial report of PHEX-dependent metabolism of FGF 23 may have represented degradation secondary to furin-like proteolytic enzymes, contaminating the reticulolysate preparation used [40,41], an hypothesis confirmed by the observed degradation of recombinant FGF 23 after incubation with reticulolysates alone, as well as with reticulolysates expressing either the inactive 3′ truncated Phex or the full-length wild-type Phex [77].

Moreover, the inability to demonstrate Phex-dependent cleavage of intact FGF 23 or its N- and C-terminal fragments using active recombinant Phex and FGF 23 proteins expressed in mammalian cells or proteins synthesized *in vitro* confirmed that FGF 23 is not a substrate for Phex [77]. Such failure to demonstrate that FGF 23 is a substrate for Phex occurred despite the observations that full-length FGF 23 contains putative cleavage sites for Phex hydrolysis [76]. Thus, the mere presence of consensus cleavage sites for an enzyme in a protein does not necessarily indicate that such a site is available to the enzyme. Indeed, the inability of Phex to hydrolyze intact FGF 23 may indicate a constraint placed by incompatible three-dimensional structure that limits enzyme substrate interactions. Regardless, the available data do not substantiate the enzyme/substrate model of common pathogenesis for the hypophosphatemic disorders. In this regard, although the genetic mutation underlying ADHR undoubtedly limits proteolytic degradation of FGF 23, thereby increasing the circulating concentration of a biologically active molecule, the enzyme normally responsible for the degradation remains questionable. In addition, the failure of Phex to degrade FGF 23 discredits the hypothesis that failure of mutated Phex to degrade FGF 23 accounts for the increase of this phosphaturic hormone in XLH.

Nevertheless, the circulating concentration of FGF 23 is increased in *hyp*-mice and a subset of patients with XLH, suggesting that an alternative mechanism enhances hormone production or impairs hormone degradation in this disease state. In this regard, Liu *et al.* [77] recently reported that inactivating mutations of Phex resulted in increased expression of FGF 23 transcripts in the bone and cultured osteoblasts of the *hyp*-mouse, indicating that *Phex* may somehow regulate FGF 23 biosynthesis. Indeed, in *hyp*-mice lacking a functional *Phex* increased levels of *fgf 23* transcripts were observed in mandible, calvaria, and diaphysis of the long bone. Moreover, cultured osteoblasts from *hyp*-mice likewise exhibited increased *fgf 23* transcripts. In concert with these observations, recent investigations in patients with the McCune-Albright syndrome have identified FGF 23 production by fibrous displasia osteoprogenitors and normal bone forming cells *in vitro* [51,78,79]. While these data suggest that bone is the source of increased FGF 23 in XLH, several observations challenge this interpretation. First, the FGF 23 produced by the bone in *hyp*-mice, according to Liu *et al.* [77], is of relatively small magnitude and may have no physiological effect. Second, other studies have failed to confirm the presence of FGF 23 in normal bone and/or osteoblasts [80]. Third, no data exist providing a mechanism by which inactive Phex results in increased FGF 23 production. Fourth, the presence of increased protease activity in the *hyp*-mouse bone matrix, secondary to inactivated *Phex* [69], raises significant doubt that osteoblast-secreted full-length FGF 23 would reach the circulation. And finally, transgenic overexpression of *Phex* in the *hyp*-mouse, which should limit the increased production of *fgf 23* transcripts and the resultant circulating levels of this protein, according to the hypothesis advanced by Liu *et al.* [81], fails to normalize renal phosphate transport. As a consequence, it appears that the mechanism underlying the purported elevation of FGF 23 levels in XLH remains controversial, precluding progress towards ascertainment of a common pathogenesis for the hypophosphatemic disorders.

c. The Biological Activities of the Phosphatonins/Minhibins As related above, multiple studies have linked the biological activities of the putative phosphatonins/minhibins to the phenotype of the hypophosphatemic diseases. However, in each case, the testing is incomplete or the documented biological activity does not explain a phenotypic characteristic manifest as part of the ADHR, TIO, or XLH syndrome. For example, as shown in Table II, studies do not yet exist that demonstrate the *in vitro* inhibitory activity of either FGF 23 or sFRP-4 on osteoblast mineralization.

TABLE II Activity of Candidate Phosphatonin/Minhibin Proteins

	FGF 23	sFRP4	MEPE
Bone			
Inhibits mineralization			
In vitro	?	?	+
In vivo	+	?	?
Kidney			
Inhibits Na^+-Pi transport			
In vitro	+	+	+
In vivo	+	+	+
Decreases Npt2a transcription			
In vitro	+	+	–
In vivo	+	+	–
Vitamin D metabolism			
Alters 25(OH)D-1α-hydroxylase mRNA			
In vitro	⇓	–	–
In vivo	⇓	–	⇑
Alters 25(OH)D-1α-hydroxylase protein			
In vitro	?	?	?
In vivo	?	?	?

Yet, studies in *hyp*-mice and/or transgenic FGF 23 mice clearly indicate that the mineralization defect in the hypophosphatemic disorders exceeds that expected from hypophosphatemia alone and does not normalize with phosphate therapy. Hence, an effect of minhibin on osteoblast mineralization function seems certain. Interestingly, such an *in vitro* effect has been documented for MEPE but no *in vivo* data are available yet.

In contrast, testing of the putative phosphatonins on renal sodium-dependent phosphate transport is relatively complete and supports the possible role of each of these compounds in the pathogenesis of the hypophosphatemic diseases. However, as noted previously, various *in vivo* animal models do not uniformly substantiate such a role for either MEPE or FGF 23. Moreover, presumed restoration of normal FGF 23 activity in *hyp*-mice by a variety of techniques fails to normalize renal phosphate transport and hypophosphatemia [65,69,81,82].

Interestingly, the anticipated effects of the phosphatonins/minhibins on vitamin D metabolism are controversial. Although phosphate mediated enhancement of calcitriol production and serum levels is uniformly inhibited in ADHR, TIO, and XLH, the cause of this abnormality in each disorder is less certain. Since regulation of calcitriol production occurs primarily at the transcription level, several groups have anticipated that a phosphatonin/minhibin would inhibit renal 25(OH)D-1α-hydroxylase transcription. In accord, current studies indicate that FGF 23 fulfills this criterion. However, investigations in the *hyp*-mouse indicate that renal 25(OH)D-1α-hydroxylase mRNA is, in fact, elevated under basal conditions and following PTH stimulation [83]. Therefore, the inhibition of calcitriol production occurs at the translational level [84]. As shown in Table II, available data have not linked any of the phosphatonins/minhibins to this phenotypic characteristic of XLH. These observations clearly indicate that further studies are essential in order to identify which of the putative phosphatonin/minhibin proteins have biological properties consistent with those anticipated in the various hypophosphatemic diseases.

Although a common pathogenetic mechanism for these diseases has not been unequivocally defined, progress in our understanding of the various disorders of phosphate homeostasis, which have been identified, is truly remarkable. Consideration of these diseases in the remainder of this chapter and in Chapter 70 will highlight many of these advances.

2. X-LINKED HYPOPHOSPHATEMIA (XLH)

XLH is the prototypic renal phosphate wasting disorder, characterized in general by progressively severe skeletal abnormalities and growth retardation. The syndrome occurs as an X-linked dominant disorder with complete penetrance of a renal tubular abnormality resulting in phosphate wasting and consequent hypophosphatemia (Table III). The clinical expression of the disease is widely variable, ranging from a mild abnormality, the apparent isolated occurrence of hypophosphatemia, to severe rickets and/or osteomalacia [85]. In children, the most common clinically evident manifestations include short stature and limb deformities. This height deficiency is more evident in the lower extremities, since they represent the fastest growing segment before puberty. In contrast, upper segment growth is generally less affected. The majority of children with the disease exhibit enlargement of the wrists and/or knees secondary to rickets, as well as bowing of the lower extremities. Additional signs of the disease may include late dentition, tooth abscesses secondary to poor mineralization of the interglobular dentine, and premature cranial synostosis. Many of these features do not become apparent until age 6 to 12 months or older [86]. In spite of marked variability in the clinical presentation, bone biopsies in affected children and adults invariably reveal low turnover osteomalacia without osteopenia. The *severity* of the bone disorder has no relationship to sex, the extent of the biochemical abnormalities, or the degree of the clinical disability. In untreated youths and adults, the serum 25(OH)D levels are normal and the concentration of $1,25(OH)_2D$ is in the low-normal range [87–89]. The paradoxical occurrence of hypophosphatemia and normal serum calcitriol levels is due to aberrant regulation of renal 25(OH)D-1α-hydroxylase activity. Studies in *hyp*- and *gy*-mice, the murine homologues of the human disease, have established that defective regulation is confined to the enzyme localized in the proximal convoluted tubule, the site of the abnormal phosphate transport [90–93].

a. Pathophysiology Investigators generally agree that the primary inborn error in XLH results in an expressed abnormality of the renal proximal tubule that impairs Pi reabsorption. This defect has been indirectly identified in affected patients and directly demonstrated in the brush border membranes of the proximal nephron in *hyp*-mice. Until recently, whether this renal abnormality is primary or secondary to the elaboration of a humoral factor has been controversial. In this regard, demonstration that renal tubule cells from *hyp*-mice maintained in primary culture exhibit a persistent defect in renal Pi transport [94,95], likely due to decreased expression of NPT2a mRNA and immunoreactive protein [96–98], supported the presence of a primary renal abnormality. In contrast, transfer of the defect in renal Pi transport to normal and/or parathyroidectomized normal mice parabiosed to *hyp*-mice implicated a humoral factor in the pathogenesis of the

TABLE III. Biochemical/Genetic Characteristics of the Prototypic Phosphopenic Disorders in Man

	XLH	TIO	ADHR	ARHR	HHRH	FS		TC
						I	II	
P Homeostasis								
Serum P	⇓	⇓	⇓	⇓	⇓	⇓	⇓	⇑
Renal TmP/GFR	⇓	⇓	⇓	⇓	⇓	⇓	⇓	⇑
GI P Absorption	⇓	⇓	⇓	⇑	⇑	⇓	⇑	⇑
FGF-23	N or ⇑	⇑	⇑	?	?	?	?	?
Ca Homeostasis								
Serum Ca	N	N	N	N	N	N	N	N
Urine Ca	⇓	⇓	⇓	⇑	⇑	⇓	⇑	⇑
Nephrolithiasis	–	–	–	+	–	–	–	–
GI Ca Absorption	⇓	⇓	⇓	⇑	⇑	⇑	⇑	⇑
Serum PTH	N	N	N	N	N	N	N	N
Vitamin D Metabolism								
25(OH)D	N	N	N	N	N	N	N	N
$1,25(OH)_2D$	N/⇓	⇓	N/⇓	⇑	⇑	N/⇓	⇑	⇑
Bone Metabolism								
Serum Alk Phos	N/⇑	N/⇑	N/⇑	N/⇑	N/⇑	N/⇑	N/⇑	N
Serum NPT	N	N	N	N	N	N	N	?
Genetics								
Familial	+	–	+	+	+	Variable	+	+
Transmission	X-linked dominant	–	Autosomal dominant	Autosomal Recessive	Autosomal Recessive	Variable	?	Variable
Abnormal Gene	*PHEX*	–	FGF-23	CLCN5	?	Variable	?	?

XLH, X–linked hypophosphatemia; TIO, tumor-induced osteomalacia; HHRH, hereditary hypophosphatemic rickets with hypercalciuria; FS I, Fanconi syndrome, type I; FS II, Fanconi syndrome, type II; ADHR, autosomal dominant hypophosphatemic rickets; ARHR, autosomal recessive hypophosphatemic rickets; TC, tumoral calcinosis.

N, normal; ⇓, decreased; ⇑, increased.

Modified from Econs *et al.* [202].

disease [99,100]. Current studies, however, have provided compelling evidence that the defect in renal Pi transport in XLH is secondary to the effects of a circulating hormone or metabolic factor. In this regard, immortalized cell cultures from the renal tubules of *hyp*- and *gy*-mice exhibit normal Na^+-phosphate transport, suggesting that the paradoxical effects observed in primary cultures may represent the effects of impressed memory and not an intrinsic abnormality [101,102]. Moreover, the report that cross-transplantation of kidneys in normal and *hyp*-mice results in neither transfer of the mutant phenotype or its correction unequivocally established the humoral basis for XLH [56]. Subsequent efforts, which resulted in localization of the gene encoding the Na^+-phosphate co-transporter to chromosome 5, further substantiated the conclusion that the renal defect in brush-border membrane phosphate transport is not intrinsic to the kidney in XLH [103]. While these data establish the presence of a humoral abnormality in XLH, the identity of the putative factor and the spectrum of its activity have not been definitively elucidated. Nevertheless, several investigators have identified and characterized the biological activities of a variety of phosphaturic factors (inhibitors of Na^+-dependent phosphate transport) and mineralization inhibitory factors, which may play a role in the pathogenesis of XLH (see above). Moreover, several reports have documented production of phosphaturic and mineralization inhibitory factors by *hyp*-mouse osteoblasts maintained in culture [55,67,69,77,101].

Therefore, as noted above, these studies argue that a circulating factor(s), phosphatonin(s)/minhibin(s), plays an important role in the pathophysiological cascade responsible for X-linked hypophosphatemia.

b. Genetic defect Efforts to better understand XLH have led to identification of the genetic defect underlying this disease. In 1986 Read *et al*. [104] and Machler *et al*. [105] reported linkage of the DNA probes DXS41 and DXS43, which had been previously mapped to Xp22.31-p21.3, to the *HYP* gene locus. In subsequent studies Thakker *et al*. [106,107] reported linkage to the *HYP* locus of additional polymorphic DNA, DXS197, and DXS207 and, using multipoint mapping techniques, determined the most likely order of the markers as Xpter-DXS85-(DXS43/DXS197)-*HYP*-DXS41-Xcen and Xpter-DXS43-*HYP*-(DXS207/DXS41)-Xcen, respectively. The relatively small number of informative pedigrees available for these studies prevented definitive determination of the genetic map along the Xp22-p21 region of the X-chromosome and only allowed identification of flanking markers for the *HYP* locus 20 centimorgans (cM) apart. More recently, the independent and collaborative efforts of the HYP consortium resulted in the study of 13 multigenerational pedigrees and consequent refined mapping of the Xp22.1-p21 region of the X chromosome, identification of tightly linked flanking markers for the *HYP* locus, construction of a YAC contig spanning the *HYP* gene region, and eventual cloning and identification of the disease gene as *PHEX*, a **P**hosphate regulating gene with homologies to **E**ndopeptidases located on the **X**-chromosome. In brief, these studies ascertained a locus order on Xp22.1 of:

Xcen-DXS451-(DXS41/DXS92)-DXS274-DXS1052-DXS1683-*PHYP*-DXS7474-DXS365-(DXS443/DXS3424)-DXS257-(GLR/DXS43)-DXS315-Xtel

Moreover, the physical distance between the flanking markers, DXS1683 and DXS7474, was determined as 350kb and their location on a single YAC ascertained. Subsequently, a cosmid contig spanning the *HYP* gene region was constructed and efforts directed at discovery of deletions within the *HYP* region. Identification of several such deletions permitted characterization of cDNA clones that mapped to cosmid fragments in the vicinity of the deletions. Database searches with these cDNAs detected homologies at the peptide level to a family of endopeptidase genes that includes neutral endopeptidase (NEP), endothelin-converting enzyme-1 (ECE-1), and the Kell antigen. These efforts clearly established *PHEX* as the candidate gene responsible for XLH [64,108–112].

Identification of the gene associated with XLH as *PHEX* [64] has facilitated efforts to better understand this disease. The gene codes for a 749-amino acid protein, consisting of three domains: 1) a small aminoterminal intracellular tail; 2) a single, short transmembrane peptide; and 3) a large carboxyterminal extracellular peptide, which, typical of zinc metalloproteases [113], has 10 conserved cysteine residues and a HEXXH pentapeptide motif. The homology of *PHEX* with metalloproteases resulted in inclusion of this protein in the M13 family of membrane-bound metalloproteases, also known as *neutral endopeptidases* [114–116]. M13 family members, including neutral endopeptidase 24.11 (NEP), endothelin-converting enzymes 1 and 2, the Kell blood group antigen, neprilysin-like peptide (NL1), and endothelin converting enzyme-like 1 [113,115,117–123], degrade or activate a variety of peptide hormones. Preservation in the *PHEX* structure of catalytic glutamate and histidine residues (equivalent to Glu^{648} and His^{711} of NL1) argues for similar protease activity, as does alignment of *PHEX* mutations with regions required for peptidase activity in NL1 [124]. Further, like other neutral endopeptidases, immunofluorescent studies reveal a cell-surface location for PHEX in an orientation consistent with a type II integral membrane glycoprotein [124].

In any case, cloning the *PHEX* gene led relatively rapidly to cloning the homologous murine *Phex* gene and identification of the mutations in the murine homologues of XLH, the *hyp*- and *gy*-mice [58,125,126]. Unlike 97% of known genes, neither the human nor murine gene has a Kozak sequence, a purine at the –3 position before the ATG initiation sequence [125–127]. Since such genes are often post-transcriptionally regulated, this anomaly may impact understanding the hormonal and metabolic regulation of *PHEX/Phex*.

Many investigators [57–64,127–135] have used *Phex*/Phex localization and mutation detection to help formulate the pathogenetic scheme for XLH discussed above. Investigation of murine tissues and cell cultures revealed that *Phex* is predominantly expressed in bones and teeth [57,58,60–62,126], while mRNA, protein or both have also been found in lung, brain, muscle, gonads, skin, and parathyroid glands [124,136]. Experiments in neonatal and adult mice further documented that the cellular locations of Phex in bone and teeth are the osteoblast/osteocyte and the odontoblast/ameloblast, respectively, while subcellular locations are the cell surface membrane, the endoplasmic reticulum, and the Golgi compartment. Notably *Phex* expression is absent in the visceral abdominal organs, including the kidney, liver hepatocytes, and intestine, and in cardiac and skeletal muscle. In any case, the ontogeny of *Phex* expression reveals that the protein is expressed in osteoblasts at both primary and secondary ossification centers, suggesting a possible role in mineralization *in vivo*.

PHEX mutations consisting of deletions, insertions, and duplications, as well as splice site, nonsense and missense mutations, have been documented in >160 patients with XLH [64,127–134] and are scattered throughout exons 2–22, which encode the 749-amino acid extracellular protein domain. In addition, a single mutation within the 5′ untranslated region has been identified [130]. Although these mutations invariably cause loss of function, the mechanism by which such loss of activity occurs is unclear. However, preliminary data indicate that missense mutations interfere with protein trafficking, resulting in protein sequestration in the endoplasmic reticulum [133]. Until recently *PHEX* coding region mutations had not been detected in ~35% of patients. Accordingly, Christie *et al*. [134] explored whether such subjects have intronic mutations that result in mRNA splicing abnormalities. They found in one patient a unique mutation in intron 7 that created a novel donor splice site, which interacts with 3 naturally occurring acceptor splice sites, leading to the incorporation of 3 pseudoexons in *PHEX* transcripts. Translation of these pseudoexons results in the inclusion of missense amino acids into the PHEX protein or a truncated protein, lacking 5 of 10 conserved cysteine residues and the pentapeptide zinc-binding motif. These observations suggest that intron mutations may represent a proportion of the gene abnormalities undiscovered in one-third of patients with XLH.

In order to confirm the possibility that diminished *PHEX/Phex* expression in osteoblasts initiates the cascade of events responsible for the pathogenesis of XLH, several investigators have used targeted overexpression of *Phex* in attempts to normalize osteoblast mineralization, *in vitro,* and rescue the *Hyp* phenotype *in vivo.* Surprisingly, however, these studies [81,137] revealed that restoration of *Phex* expression and enzymatic activity to immortalized *hyp*-mouse osteoblasts, by retroviral mediated transduction, does not restore their capacity to mineralize extracellular matrix *in vitro,* under conditions supporting normal mineralization. Moreover, in complementary studies Liu *et al.* [81] and Bai *et al.* [69] found transgenic *hyp*-mice (*Osc-Phex-Hyp; pOb2.3[ColIaI]-Phex-Hyp*), despite expressing abundant Phex mRNA and enzyme activity in mature osteoblasts and osteocytes, exhibited hypophosphatemia and persistently abnormal vitamin D metabolism. In the setting of P depletion, although exhibiting a modest improvement in bone mineralization, the transgenic mice maintained histological evidence of osteomalacia, similar to that in nontransgenic *hyp*-mice. These observations are consistent with several possibilities, acknowledged by Liu *et al*. [81] and Bai *et al*. [69].

First, despite theoretical evidence to the contrary (see above), extraosseous *Phex* expression may play an important role in the modulation of phosphatonin activity. In support of this option, Miyamura *et al.* [138] using syngeneic bone marrow transplantation, were able to partly reverse the biochemical abnormalities in *hyp*-mice with an engraftment that was not restricted to cells of the osteoblast lineage, but included donor cells to alternate tissues, in many of which *PHEX* transcripts have been detected [58–61]. Second, the temporal and developmental expression of the *Osc* and *pOb2.3* promoter-driven *Phex* expression may not mimic the endogenous regulation of *Phex*. In this regard, the transgenic animals may experience PHEX expression later than, or in osteoblast-related cell subpopulations different from those in normal animals. In fact, neither promoter is expressed in the preosteoblast and the osteocalcin promoter appears at least four days later than PHEX in normally developing osteoblasts [69,81]. Thus, lack of *Phex* activity early in osteoblast development (in preosteoblasts or preceding osteocalcin expression in osteoblasts) may result in failure to alter an otherwise immutable osteoblast dysfunction, the continued presence of which contributes to the impairment of mineralization. In accord, later expression of PHEX may not rescue the phenotype, as the immutable change is refractory to endopeptidase activity. However, Erben *et al.* [82] recently reported that ubiquitous overexpression of *Phex* under the control of the β-actin promoter in two different mouse lines markedly improved BMD but did not completely resolve the bone mineralization defect and failed to alter the abnormal phosphate homeostasis, raising significant further questions regarding the interaction between the *PHEX* gene defect and phenotypic expression of the disease.

Regardless, in no way do these observations exclude a role in the mineralization process for *Phex* expression in the mature osteoblast. Indeed, recent studies support the possibility that abnormal mineralization in *hyp*-mouse osteoblasts is due to a combination of Phex inactivity in the mature cell and Phex refractory abnormal P transport in the osteoblast, possibly due to gene inactivity early in cell development [139]. In such circumstances, the rescue of the *Hyp* phenotype by Phex expression in mature osteoblasts may be limited, in part, by phosphate availability. Hence, hypophosphatemia per se may contribute to the severity of the mineralization defect in *hyp*-mice. The plausibility of this possibility is reinforced by the discovery that P deficiency results in rickets in developing animals (and humans) [140] and the recent observations, which document that *hyp*-mouse neonates, exposed to normal phosphorus levels during embryonic development, manifest at birth apparently normal endochondral mineralization and only mildly abnormal mineralization in metaphyseal bone [32]. In any case, the inconsistent results of

efforts to rescue the *HYP* phenotype clearly indicate that salient elements of the complex pathogenesis underlying XLH remain unknown.

c. Pathogenesis In spite of the remarkable advances that have been made in understanding the genetic abnormality and pathophysiology of XLH, the detailed pathogenetic mechanism underlying this disease remains unknown (see above). While as related previously, the identity of phosphatonin remains uncertain, it is still tempting to speculate that the PHEX gene product acts directly or indirectly on a phosphaturic factor that regulates renal phosphate handling. Moreover, precedent for such activity exists as neutral endopeptidases inactivate enkephalins and atrial natriuretic peptide [141,142]. However, the data from parabiotic studies of normal and *hyp*-mice argue strongly that extracellular degradation of the phosphaturic factor does not occur. Indeed, such activity would preclude transfer of the *hyp*-mouse phenotype to parabiosed normals. Alternatively, the PHEX gene product may function intracellularly to inactivate phosphatonin. In this regard, Jalal *et al*. [143] recently reported the internalization of NEP and a potential role for this enzyme in intracellular metabolism. Less likely, the PHEX gene product may enzymatically activate a protein, which suppresses production of phosphatonin. While this is consistent with all previous data, it is a complex process and requires production of PHEX, phosphatonin, and the suppressor protein in the same cell in order to accommodate the data from the parabiotic studies. Nevertheless, in accord with this possibility, Mari *et al.* [144] have reported that the NEP on human T-cells may be involved in the production of lymphokines through the processing of an activating factor at the surface of the lymphocyte. In any of these cases, a defect in the PHEX gene will result in overproduction and circulation of phosphatonin and consequent inhibition of renal Na^+-phosphate transport, the likely scenario in the pathogenesis of XLH. Although such overproduction of phosphatonin is a favored hypothesis, as noted previously, it is possible that XLH results from the inability of mutant PEX to activate a phosphate conserving hormone. However, the only known phosphate-conserving hormone, stanniocalcin, is synthesized in active form within the kidney and has little known bioactivity in man. These features strongly mitigate against a role for stanniocalcin in the pathogenesis of XLH.

The coexistence of osteoblast defects in XLH further confounds understanding the pathophysiology of this disorder. Elegant experiments, which documented the abnormal mineralization of periostea and osteoblasts of *hyp*-mice following transplantation into the muscle of normal mice, provide clear evidence for intrinsic defects in the bone of mutants [145]. Indeed, proof of specific osteoblast abnormalities has been provided by studies that show decreased phosphorylation of osteopontin and increased osteocalcin levels in cells from *hyp*-mice [146]. Based on these observations, it is tempting to speculate that co-ordinate PHEX expression and phosphatonin production in osteoblasts may impart innate functional abnormalities to these cells in X-linked hypophosphatemia [147]. Indeed, the phosphatonin, likely processed by the PHEX gene product, may have multiple activities beyond regulation of renal phosphate transport, which may include modulation of vitamin D metabolism and osteoid mineralization, defects that characterize XLH. In concert with this possibility, PHEX expression correlates temporally with osteoblast mediated mineralization *in vitro*.

In any case, it is evident that further information is requisite to enhance our understanding of the pathogenesis of XLH and, in turn, regulation of phosphate homeostasis, osteoblast function, vitamin D metabolism, and osteoid mineralization. Such data are not only critical to understanding the pathogenesis of XLH and the regulation of mineral homeostasis, but may have significant impact upon determination of optimal treatment strategies for many of the vitamin D–resistant diseases.

d. Treatment In past years, physicians employed pharmacologic doses of vitamin D as the cornerstone for treatment of XLH. However, long-term observations indicate that this therapy fails to cure the disease and poses the serious problem of recurrent vitamin D intoxication and renal damage. More recently, choice of therapy for this disease has been remarkably influenced by the increased understanding of the pathophysiological factors, which affect phenotypic expression of the disorder. Thus, current treatment strategies for children directly address the combined calcitriol and phosphorus deficiency characteristic of the disease. Generally, the regimen includes a period of titration to achieve a maximum dose of calcitriol, 1-3 μg/d in two divided doses and phosphorus, 1-4 g/d in 4-5 divided doses [148,149]. Such combined therapy often improves growth velocity, normalizes lower extremity deformities, and induces healing of the attendant bone disease. Refractoriness to the growth-promoting effects of treatment, however, is often encountered particularly in youths presenting at <5th percentile in height [150]. For that reason, the use of recombinant growth hormone as an additional treatment component has been advocated recently. Definite positive effects have been observed in young patients with XLH [151,152]. Of course, treatment involves a significant risk of toxicity that is generally expressed as abnormalities of calcium homeostasis, most notably secondary hyperparathyroidism that may become autonomous and require surgery.

Detrimental effects on renal function secondary to abnormalities such as nephrocalcinosis are also possible. Thus, frequent monitoring of the phosphate and calcitriol dosage during growth is mandatory. Therapy in adults is reserved for episodes of intractable bone pain and refractory nonunion bone fractures.

3. Hereditary Hypophosphatemic Rickets with Hypercalciuria (HHRH)

This rare genetic disease is marked by hypophosphatemic rickets with hypercalciuria [153]. The cardinal biochemical features of the disorder include hypophosphatemia due to increased renal phosphate clearance and normocalcemia. In contrast to other diseases in which renal phosphate transport is limited, patients with HHRH exhibit increased $1,25(OH)_2D$ production [153–157] (Table III). The resultant elevated serum calcitriol levels enhance the gastrointestinal calcium absorption, which in turn increases the filtered renal calcium load and inhibits parathyroid secretion. These events cause the characteristic hypercalciuria observed in affected patients.

The clinical expression of the disease is heterogeneous. In general, children become symptomatic between the ages of six months and seven years. Initial symptoms consist of bone pain or deformities of the lower limbs (or both), which progressively interferes with gait and physical activity. The bone deformities vary from genu varum or genu valgum to anterior external bowing of the femur and coxa vara. Additional features at presentation include short stature with disproportionately short lower limbs, muscle weakness, and radiological signs of rickets or osteomalacia (or both). These various symptoms and signs may exist separately or in combination and may be present in a mild or severe form.

A large number of apparently unaffected relatives of patients with HHRH exhibit an additional mode of disease expression [157]. These subjects, although without evidence of bone disease, manifest idiopathic hypercalciuria (IH), most evident in post-prandial periods, as well as a pattern of biochemical abnormalities similar to those of children with rickets and osteomalacia. Quantitatively, however, the abnormalities are milder, and the relevant biochemical values intermediate between those observed in family members with HHRH and those in normal relatives. The absence of bone disease in these patients may be explained by relatively mild hypophosphatemia compared to the severe phosphate depletion evidenced in patients with the full spectrum of the disorder [157].

Only four unrelated kindreds with HHRH have been described, including an extended family of Bedouin origin that includes 13 patients with HHRH and 42 with hypercalciuria; a smaller kindred of oriental Jewish origin with 5 affected members; and a family of Yemenite Jewish origin that includes 2 patients with HHRH and 2 with hypercalciuria [155]. However, a phenotypically similar disorder, childhood idiopathic hypercalciuria with bone lesions (rickets) and stunted linear growth has been independently recognized. The similarity of this syndrome to HHRH suggests that they may be one and the same pathologic condition. Moreover, several patients with a sporadic occurrence of HHRH have been recently recognized. Studies are generally incomplete, however, and the presence of hypercalciuria in relatives has not been excluded.

a. Pathophysiology Liberman, Tieder, and associates [153,157,158] have presented data that indicate that the primary inborn error underlying this disorder is an expressed abnormality in the renal proximal tubule, which impairs phosphate reabsorption. They propose that this pivotal defect in turn stimulates renal 25(OH)D-1α-hydroxylase, thus promoting the production of $1,25(OH)_2D$ and increasing its serum and tissue levels. As a result intestinal calcium and phosphorus absorption is augmented and the renal filtered calcium load consequently increased. The enhanced intestinal calcium absorption also suppresses parathyroid function. In addition, prolonged hypophosphatemia diminishes osteoid mineralization, resulting in rickets and/or osteomalacia.

The proposal that abnormal phosphate transport results in increased calcitriol production remains untested. Indeed, the elevation of $1,25(OH)_2D$ in patients with HHRH is a unique phenotypic manifestation of the disease that distinguishes it from other disorders in which abnormal phosphate transport is likewise manifest. Such heterogeneity in the phenotype of these disorders suggests that disease at variable anatomical sites along the proximal convoluted tubule or involving compensatory enhancement of sodium-dependent phosphate cotransporters, such as that in the *Npt2a*$^{-/-}$ mouse [11,31], while uniformly impairing phosphate transport, may not necessarily inhibit 25(OH)D-1α-hydroxylase activity.

b. Genetic Defect Autosomal recessive transmission of HHRH seems consistent with the inheritance pattern in the described kindreds. However, if HHRH is an autosomal recessive disease and individuals with IH are heterozygous for the mutant allele, IH must be an incompletely penetrant trait because not all obligate heterozygotes manifest hypercalciuria. Alternatively, it has been suggested that HHRH and IH could be the result of mutations in two different genes [157]. The strongest support against this hypothesis is that when individuals with HHRH are treated with oral Pi, both the hypophosphatemia and the hypercalciuria are corrected. Nevertheless, the variability in clinical

presentation in the various described kindred suggests that mutations in different genes may be involved. Regardless, the search for a specific gene defect underlying this disease has been unrewarding. Indeed, efforts to implicate an abnormality in the NPT2a gene in patients with HHRH have been unsuccessful [159].

c. Treatment In accord with the hypothesis that a singular defect in renal phosphate–transport underlies HHRH, patients have been treated successfully with high dose phosphorus (1–2.5 g/d in 5 divided doses) alone. Within several weeks after initiation of therapy, bone pain disappears and muscular strength improves substantially. Moreover, the majority of treated subjects exhibit accelerated linear growth and radiological signs of rickets are completely absent within 4–9 months. Concordantly, serum phosphorus values increase towards normal, the $1,25(OH)_2D$ concentration decreases, and alkaline phosphatase activity declines to normal. Despite this favorable response, limited investigation indicates that the osteomalacic component of the bone disease does not exhibit normalization. Further studies will be necessary, therefore, to determine if phosphorus alone will be sufficient treatment for this rare disorder.

In any case, administration of phosphorus in patients with this disease does not result in the same spectrum of complications encountered upon its use in other disorders. Most notably, nephrocalcinosis, a common complication in treated patients with XLH, occurs infrequently in subjects with HHRH. In fact, the rare occurrence of this complication is associated with a history of vitamin D intoxication prior to initiation of treatment with phosphorus. Similarly, the development of secondary hyperparathyroidism in treated patients with HHRH has not been reported, although expectation of this complication is high since oral administration of phosphate does diminish the circulating calcium concentration and, in turn, stimulates parathyroid function.

4. FANCONI SYNDROME (FS)

Rickets and osteomalacia are frequently associated with Fanconi syndrome, a disorder characterized by hyperphosphaturia and consequent hypophosphatemia, hyperaminoaciduria, renal glycosuria, albuminuria, and proximal renal tubular acidosis [160–163]. Damage to the renal proximal tubule, secondary to genetic disease or environmental toxins, represents the common underlying mechanism of this disease [164]. Resultant dysfunction causes renal wasting of those substances primarily reabsorbed at the proximal tubule. The inherited form may occur in isolation (in the absence of any other metabolic disease) or secondary to various primary Mendelian diseases. The associated bone disease in this disorder is likely secondary to hypophosphatemia and/or acidosis, abnormalities that occur in association with aberrantly (Fanconi syndrome, type I) or normally regulated (Fanconi syndrome, type II) vitamin D metabolism.

a. Fanconi Syndrome, Type I Renal phosphate wasting and hypophosphatemia are the hallmark abnormalities of this disease, which resembles in many respects the more common genetic disease, XLH. In this regard, occurrence of abnormal bone mineralization appears dependent upon the prevailing renal phosphate wasting and resultant hypophosphatemia. Indeed, disease subtypes in which isolated wasting of amino acids, glucose, or potassium occur, are not associated with rickets and/or osteomalacia. Further, in the majority of patients studied, affected subjects exhibit abnormal vitamin D metabolism, characterized by serum $1,25(OH)_2D$ levels that are overtly decreased or abnormally low relative to the prevailing serum phosphorus concentration [165–167]. Although the aberrantly regulated calcitriol biosynthesis may be due to the abnormal renal phosphate transport, as suspected in patients with XLH, proximal tubule damage and acidosis may play important roles.

A notable difference between this syndrome and XLH is a prevailing acidosis, which may contribute to the bone disease. In this regard, several studies indicate that acidosis may exert multiple deleterious effects on bone. Such negative sequellae are related to the loss of bone calcium that occurs secondary to calcium release for use in buffering [160,168]. Alternatively, several investigators [169,170] have reported that acidosis may impair bone mineralization secondary to direct inhibition of renal 25(OH)D-1α–hydroxylase activity. Others dispute these findings and claim that acidosis does not cause rickets or osteomalacia in the absence of hypophosphatemia. Indeed, Brenner *et al.* [171] reported that the rachitic/osteomalacic component of this disorder occurs only in patients with type 2 renal tubular acidosis and phosphate wasting. In contrast, those with type 1 and 4 renal tubular acidosis displayed no evidence of abnormal bone mineralization. Thus, the interplay of acidosis and phosphate depletion on bone mineralization in this disorder remains poorly understood. Most likely, however, hypophosphatemia and abnormally regulated vitamin D metabolism are the primary factors underlying rickets and osteomalacia in this form of Fanconi Syndrome.

b. Fanconi's Syndrome, Type II Tieder *et al.* [172] have described two siblings (from a consanguineous mating) who presented with classic characteristics of Fanconi syndrome, including renal phosphate wasting, glycosuria, generalized aminoaciduria, and increased urinary uric acid excretion. However, these patients had appropriately elevated (relative to the decreased serum phosphorus concentration) serum $1,25(OH)_2D$

levels and consequent hypercalciuria despite normal serum parathyroid hormone levels and cyclic AMP excretion. Moreover, treatment with phosphate reduced the serum calcitriol in these patients into the normal range and normalized the urinary calcium excretion. In many regards, this syndrome resembles HHRH and represents a variant of Fanconi syndrome, referred to as *type II disease*. The bone disease in affected subjects is likely due to the effects of hypophosphatemia. In any case, the existence of this variant form of disease is probably the result of renal damage to a unique segment of the proximal tubule or involvement of a different mechanism at the same site [172]. Further studies will be necessary to distinguish these possibilities.

c. Treatment Ideally, treatment of the bone disease in this disorder should offset the pathophysiological defect influencing proximal renal tubular function. In many cases, however, the primary abnormality remains unknown. Moreover, efforts to decrease tissue levels of causal toxic metabolites by dietary (such as in fructose intolerance) or pharmacological means (such as in cystinosis and Wilson syndrome) have met with variable success. Indeed, no evidence exists that indicates if the proximal tubule damage is reversible upon relief of an acute toxicity. Regardless, in instances when specific therapies are not available or do not lead to normalization of the primary defect, therapy must be directed at raising the serum phosphorus concentration, replacing calcitriol (in type I disease) and reversing the associated acidosis. However, use of phosphorus and calcitriol in this disease has been limited. In general, such replacement therapy leads to substantial improvement or resolution of the bone disease [173]. Unfortunately, growth and developmental abnormalities, more likely associated with the underlying genetic or acquired disease, remain substantially impaired [173]. More efficacious therapy, therefore, is dependent upon future research into the causes of the multiple disorders that underlie this syndrome.

5. X-LINKED RECESSIVE HYPOPHOSPHATEMIA (XLRH)

The initial description of X-linked recessive hypophosphatemic rickets involved a family in which males presented with rickets or osteomalacia, hypophosphatemia, and a reduced renal threshhold for phosphate reabsorption. In contrast to patients with XLH, affected subjects exhibited hypercalciuria, elevated serum $1,25(OH)_2D$ levels (Table I), and proteinuria of up to 3 g/day. Patients also developed nephrolithiasis and nephrocalcinosis with progressive renal failure in early adulthood. Female carriers in the family were not hypophosphatemic and lacked any biochemical abnormalities other than hypercalciuria. Three related syndromes have been reported independently: X-linked recessive nephrolithiasis with renal failure, Dent's disease, and low-molecular-weight proteinuria with hypercalciuria and nephrocalcinosis. These syndromes differ in degree from each other, but common themes include proximal tubular reabsorptive failure, nephrolithiasis, nephrocalcinosis, progressive renal insufficiency, and, in some cases, rickets or osteomalacia. Identification of mutations in the voltage-gated chloride-channel gene *CLCN5* in all four syndromes has established that they are phenotypic variants of a single disease and are not separate entities [174,175]. However, the varied manifestations that may be associated with mutations in this gene, particularly the presence of hypophosphatemia and rickets/osteomalacia, underscore that environmental differences, diet, and/or modifying genetic backgrounds may influence phenotypic expression of the disease.

B. Disorders of Renal Phosphate Transport: Hyperphosphatemic Diseases

1. (HYPERPHOSPHATEMIC) TUMORAL CALCINOSIS (TC)

Tumoral calcinosis is a rare genetic disease characterized by periarticular cystic and solid tumorous calcifications. Most patients in North America with this disorder are black and about one-third of the cases are familial. There is no gender preference. Biochemical markers of the disorder include hyperphosphatemia and an elevated serum $1,25(OH)_2D$ concentration in many patients. Using these criteria, evidence has been presented for autosomal recessive inheritance of this syndrome. However, an abnormality of dentition, marked by short bulbous roots, pulp stones, and radicular dentin deposited in swirls, is a phenotypic marker of the disease that is variably expressed [176]. Thus, this disorder may have multiple *formes frustes* that could complicate genetic analysis. Indeed, using the dental lesion as well as the more classic biochemical and clinical hallmarks of the disease, an autosomal dominant pattern of transmission has been documented [176].

The hyperphosphatemia characteristic of the disease results from an increase in capacity of renal tubular phosphate reabsorption secondary to an unknown defect [176,177]. Hypocalcemia is not a consequence of this abnormality, however, and the serum parathyroid hormone concentration is normal. Moreover, the phosphaturic and urinary cAMP responses to parathyroid hormone are not disturbed. Thus, the defect does not represent renal insensitivity to a hormone, or hypoparathyroidism. Rather, the basis of the disease is probably an innate or hormone/metabolic factor mediated abnormality of the renal tubule that enhances phosphate reabsorption. The identification of the phosphatonin family of

hormones and documentation of their biological activities suggest that an inactivating mutation of a phosphatonin may underlie tumoral calcinosis. Indeed, the presence of an elevated serum phosphorus concentration and increased $1,25(OH)_2D$ production in the FGF 23 knockout mouse supports this possibility [47].

Interestingly, affected patients manifest increased circulating $1,25(OH)_2D$ levels despite hyperphosphatemia, underlining the fact that it is TmP/GFR rather than the serum phosphorus concentration that controls 25(OH)D-1α-hydroxylase activity (Fig. 2). Undoubtedly, the calcific tumors result from the elevated calcium-phosphorus product. The observation that long-term phosphorus depletion alone [178] or in association with administration of aluminum hydroxide [179] or acetazolamide, a phosphaturic agent [180], leads to resolution of the tumor masses supports this possibility. Furthermore, reduction of phosphate levels in extracellular fluid helps prevent reformation of mineral deposits [178]. In addition, preliminary studies indicate that calcitonin therapy may also be efficacious by enhancing phosphaturia [181].

An acquired form of this disease is rarely seen in patients with end-stage renal failure. Affected patients manifest hyperphosphatemia in association with either: 1) an inappropriately elevated calcitriol level for the degree of renal failure, hyperparathyroidism or hyperphosphatemia; or 2) long-term treatment with calcium carbonate, calcitriol, or high calcium-content dialysates. Again, calcific tumors likely result from an elevated calcium-phosphorus product. Indeed, complete remission of the tumors occurs with treatment with vinpocetine, a mineral scavenger drug.

III. DISORDERS RELATED TO AN ALTERED PHOSPHATE LOAD

A. Decreased Phosphate Load

1. Phosphate Deprivation

Hypophosphatemia and phosphate depletion due to inadequate dietary intake are rare. With a decline in ingested phosphate, the renal TmP increases and urinary phosphate excretion decreases [182]. In addition, gastrointestinal phosphate secretion gradually lessens. However, severe dietary deprivation (less than 100 mg/d) leads to a prolonged period of negative phosphate balance and total body depletion. Affected females may display hypophosphatemia (1.4 to 2.5 mg/dL); interestingly, in contrast, males generally do not manifest a decreased serum phosphate concentration in response to dietary deprivation. Nevertheless, attempts to maintain phosphate homeostasis in both sexes include suppression of the serum PTH concentration and increased $1,25(OH)_2D$ production. Thus, hypercalciuria may be associated with the syndrome. Whether the efficiency of calcitriol responsiveness or differential effects on end organs, such as the gastrointestinal tract or bone, underlies the noted gender difference remains unclear.

Total starvation does not cause hypophosphatemia. The catabolic effects of total food deprivation result in the release of phosphate from intracellular stores, which compensates for the negative phosphorus balance. However, refeeding of the starved person will result in hypophosphatemia if phosphate deprivation is maintained.

2. Gastrointestinal Malabsorption

Gastrointestinal absorption of phosphorus may be decreased with the use of aluminum- or magnesium-containing antacids; prolonged use of these drugs in large amounts has been associated with hypophosphatemia and a negative phosphorus balance [183]. Long-term reduction of the serum phosphorus concentration owing to chronic, excessive use of antacids leads to frank osteomalacia and myopathy. The osteomalacia results as a direct consequence of the phosphate depletion and in spite of normal vitamin D stores and an increased serum $1,25(OH)_2D$ concentration, which occurs in response to the hypophosphatemia.

In contrast, the pathophysiology of variably occurring osteomalacia secondary to gastrointestinal diseases that cause steatorrhea or rapid transit time (e.g., Crohn's disease, postgastrectomy states, and intestinal fistulas) is significantly different [184]. In this case, mild to moderate hypophosphatemia occurs due to vitamin D malabsorption/deficiency and resultant secondary hyperparathyroidism and renal phosphate wasting. Further, the relation between the metabolic bone disease, vitamin D deficiency, and hypophosphatemia is complex and likely involves the influence of phosphopenia and vitamin D–dependent calciopenia on bone mineralization. However, the impact of vitamin D deficiency may be overriding since osteomalacia may occur in the absence of hypophosphatemia. Regardless, in the presence of hypophosphatemia (and/or phosphate depletion), an elevated serum calcitriol level is part of this syndrome and has no apparent effect on the evolution of the bone disease.

B. Transcellular Shift

In a large proportion of clinically important cases of hypophosphatemia, a sudden shift of phosphorus from the extracellular to the intracellular compartment is responsible for the decline of the serum phosphate concentration. This ion movement occurs in response

to naturally occurring disturbances and after the administration of certain compounds.

1. ALKALOSIS

Alkalosis secondary to intense hyperventilation may depress serum phosphate levels to less than 1 mg/dL [185]. A similar degree of alkalemia owing to excess bicarbonate also causes hypophosphatemia, but of a much lesser magnitude (2.5 to 3.5 mg/dL). The disparity between the effects of a respiratory and metabolic alkalosis is related to the more pronounced intracellular alkalosis that occurs during hyperventilation. The phosphate shift to the intracellular compartment results from the utilization attendant on glucose phosphorylation, a process stimulated by a pH-dependent activation of phosphofructokinase.

2. GLUCOSE ADMINISTRATION

The administration of glucose and insulin often results in moderate hypophosphatemia [186]. Endogenous or exogenous insulin increases the cellular uptake not only of glucose, but also of phosphorus. The most responsive cells are those of the liver and skeletal muscle. The decline of the serum phosphate concentration generally does not exceed 0.5 mg/dL. A lesser decrease is manifest in patients with type II diabetes mellitus and insulin resistance or those with a disease causing a diminished skeletal mass. The administration of fructose and glycerol similarly reduces the serum phosphorus concentration. In contrast to glucose, fructose administration may be associated with more pronounced hypophosphatemia; the more striking effect is due to unregulated uptake by the liver.

C. Combined Mechanisms

There are special clinical situations in which an altered phosphate and consequent hypophosphatemia result from both a transcellular shift of phosphorus and phosphate deprivation or renal phosphate wasting. These disorders represent some of the more common and profound causes of a decreased serum phosphorus concentration.

1. ALCOHOLISM

Alcoholic patients frequently enter the hospital with hypophosphatemia. However, many do not exhibit a decreased serum phosphate concentration until several days have elapsed and refeeding has begun. The multiple factors that underlie the hypophosphatemia include poor dietary intake, use of phosphate binders to treat gastritis, excessive urinary losses of phosphorus, and shift of phosphorus from the extracellular to the intracellular compartment, owing to glucose administration and/or hyperventilation that occurs in patients with cirrhosis or during alcohol withdrawal [186]. Moreover, many alcoholic patients are hypomagnesemic, which potentiates renal phosphate wasting by an unclear mechanism.

2. BURNS

Within several days after sustaining an extensive burn, patients often manifest severe hypophosphatemia. The initial insult induces a transient retention of salt and water. When the fluid is mobilized, significant urinary phosphorus loss ensues. Coupled with the shift of phosphorus to the intracellular compartment, which occurs secondary to hyperventilation, and the anabolic state, profound hypophosphatemia may result.

3. NUTRITIONAL RECOVERY SYNDROME

Refeeding of starved individuals or maintaining nutritional support by parenteral nutrition or by tube feeding, without adequate phosphorus supplementation, may also cause hypophosphatemia [187]. A prerequisite for the decreased serum phosphate concentration in affected patients is that their cells must be capable of an anabolic response. As new proteins are synthesized and glucose is transported intracellularly, phosphate demand depletes reserves. Several days are generally required after the initiation of refeeding in order to establish an anabolic condition. In patients receiving total parenteral nutrition, serum phosphate levels may be further depressed if sepsis supervenes and a respiratory alkalosis develops.

4. DIABETIC KETOACIDOSIS

Poor control of blood glucose and consequent glycosuria, polyuria, and ketoacidosis invariably cause renal phosphate wasting [186]. The concomitant volume contraction may yield a normal serum phosphate concentration. However, with insulin therapy, the administration of fluids, and correction of the acidosis, serum and urine phosphate fall precipitously. The resultant hypophosphatemia may contribute to insulin resistance and slow the resolution of the ketoacidosis. Hence, the administration of phosphate may improve the capacity to metabolize glucose and facilitate recovery.

D. Increased Phosphate Load

1. VITAMIN D INTOXICATION

An increase of the phosphate load from exogenous sources generally does not cause hyperphosphatemia because the excessive phosphorus is excreted by the kidney. However, an increased serum phosphate concentration may occur in vitamin D intoxication when the gastrointestinal absorption of phosphate is markedly

enhanced. Increased phosphate mobilization from bone and a reduction of GFR, secondary to hypercalcemia and/or nephrocalcinosis, may also contribute to the evolution of the hyperphosphatemia.

The chronic ingestion of large doses of vitamin D, in excess of 100,000 IU/day, is generally required to cause intoxication. Suspected hypervitaminosis D may be investigated using specific assays, which can document excessive amounts of vitamin D and its metabolites in the circulation (see Chapter 58).

2. RHABDOMYOLYSIS

Because muscle contains a large amount of phosphate, necrosis of muscle tissue may acutely increase the endogenous phosphate load and result in hyperphosphatemia. Such muscle necrosis (rhabdomyolysis) may complicate heat stroke, acute arterial occlusion, hyperosmolar nonketotic coma, trauma, toxic agents such as ethanol and heroin, and idiopathic paroxysmal myoglobinuria [188,189]. Muscle biopsy often reveals myolytic denervation, and as a consequence, acute renal failure caused by myoglobin excretion frequently complicates the clinical presentation and contributes to the hyperphosphatemia. However, an elevated serum phosphate concentration may precede evidence of renal failure, or occur in its complete absence when rhabdomyolysis is present. The diagnosis is confirmed by elevated serum creatine phosphokinase, uric acid, and lactate dehydrogenase concentrations, and the demonstration of heme-positive urine in the absence of red blood cells. Therapy is directed at the underlying disorder with maintenance of the extracellular volume to avoid volume depletion and alkalinization of the urine to prevent uric acid accumulation and consequent acute tubular necrosis.

3. CYTOTOXIC THERAPY

Cytotoxic therapy often causes cell destruction and liberation of phosphorus into the circulation [190]. The lysis of tumor cells begins within one to two days after initiating treatment, and is followed quickly by an elevation of the serum phosphate concentration. Hyperphosphatemia supervenes, however, only when the treated malignancies have a large tumor burden, rapid cell turnover, and substantial intracellular phosphorus content. Such malignancies include lymphoblastic leukemia, various types of lymphoma, and acute myeloproliferative syndromes. Hyperkalemia and hyperuricemia also occur. Indeed, uric acid nephropathy may cause renal insufficiency that predisposes to further phosphate retention and worsening of the hyperphosphatemia.

4. MALIGNANT HYPERTHERMIA

Malignant hyperthermia is a rare familial syndrome characterized by an abrupt rise in body temperature during the course of anesthesia [191]. The disease appears to be autosomal dominant in transmission, and an elevated serum creatine phosphokinase concentration is found in otherwise normal family members. Hyperphosphatemia results from shifts of phosphate from muscle cells to the extracellular pool. A high mortality rate accompanies the syndrome.

E. Clinical Signs and Symptoms of Abnormal Serum Phosphorus in Diseases Caused by an Altered Phosphate Load

As related above, a wide variety of diseases and syndromes with varying clinical manifestations have the characteristic biochemical abnormalities of hyperphosphatemia or hypophosphatemia. A unique complex of disturbances often is directly related to the abnormal phosphate homeostasis. The recognition of these signs and symptoms may lead to appropriate biochemical testing, the diagnosis of an unsuspected disease, and initiation of lifesaving or curative treatment.

1. HYPOPHOSPHATEMIA

A low serum phosphorus level is associated with symptoms only if there is concomitant phosphate depletion. The presence of phosphate deficiency, however, may cause widespread disturbances. This is not surprising, since severe hypophosphatemia causes two critical abnormalities that impact on virtually all organ systems. First, a deficiency of 2,3-diphosphoglycerate (2,3-DPG) occurs in red cells, which is associated with an increased affinity of hemoglobin for oxygen and, therefore, tissue hypoxia. Second, there is a decline of tissue ATP content and a concomitant decrease in the availability of energy-rich phosphate compounds that are essential for cell function [192,193]. The major clinical syndromes resulting from these abnormalities include nervous system dysfunction, anorexia, nausea, vomiting, ileus, muscle weakness, cardiomyopathy, respiratory insufficiency, hemolytic anemia, and impaired leukocyte and platelet function. Additionally, phosphate deficiency causes osteomalacia and bone pain, clinical sequelae that are probably independent of the aforementioned abnormalities.

Central nervous system dysfunction has been well characterized in severe hypophosphatemia, especially in patients receiving total parenteral nutrition for diseases causing severe weight loss. A sequence of symptoms compatible with a metabolic encephalopathy usually begins one or more weeks after the initiation of therapy with solutions that contain glucose and amino acids, but lack adequate phosphorus supplementation to prevent hypophosphatemia. The onset of dysfunction is marked by irritability, muscle weakness, numbness,

and paresthesias, with progression to dysarthria, confusion, obtundation, coma, and death [194]. These patients have a profoundly diminished red cell 2,3-DPG content. Both biochemical abnormalities and clinical symptoms improve as patients receive phosphorus supplementation. Peripheral neuropathies, Guillain-Barre-like paralysis, hyporeflexia, intention tremor, and ballismus have also been described with hypophosphatemia and phosphate depletion.

The effects of hypophosphatemia on muscle depend on the severity and chronicity of the deficiency. Chronic phosphorus deficiency results in a proximal myopathy with striking atrophy and weakness. Osteomalacia frequently accompanies the myopathy, so patients complain of pain in weight-bearing bones. Normal values for serum creatine phosphokinase and aldolase activities are characteristically present. Rhabdomyolysis does not occur with chronic phosphate depletion.

In contrast, acute hypophosphatemia can lead to rhabdomyolysis with muscle weakness and pain. Most cases occur in chronic alcoholics or patients receiving total parenteral nutrition. In both groups of patients, muscle pain, swelling, and stiffness occur three to eight days after the initiation of therapies that do not contain adequate amounts of phosphorus. Muscle paralysis and diaphragmatic failure may occur. Studies of muscle tissue from chronically phosphate-depleted dogs made acutely hypophosphatemic show a decrement in cellular content of phosphorus, ATP, and adenosine diphosphate (ADP). Rhabdomyolysis occurred in these muscle fibers. The laboratory findings in patients with hypophosphatemic myopathy and with rhabdomyolysis include elevated serum creatine phosphokinase levels; however, serum phosphate levels may become normal if enough necrosis has occurred with subsequent phosphorus release. Also, renal failure and hypocalcemia can be associated with the syndrome.

Myocardial performance can be abnormal at serum phosphate levels of 0.7 to 1.4 mg/dL. This occurs when ATP depletion causes impairment of the actin-myosin interaction, the calcium pump of the sarcoplasma, and the sodium-potassium pump of the cell membrane [195]. The net result is reduced stroke work and cardiac output, which may progress to congestive heart failure. These problems are reversible with phosphate replacement.

Respiratory failure can occur owing to failure of diaphragmatic contraction in hypophosphatemic patients. When serum phosphate levels are raised, diaphragmatic contractility improves. The postulated mechanism for the respiratory failure is muscle weakness secondary to inadequate levels of ATP and decreased glycolysis as the result of phosphate depletion [196].

Two disturbances of red cell function may occur secondary to phosphorus deficiency. First, as intraerythrocyte ATP production is decreased, the erythrocyte cell membrane becomes rigid, which can cause hemolysis [197]. This is rare and is usually seen in septic, uremic, acidotic, or alcoholic patients, when serum phosphate levels are less than 0.5 mg/dL. Second, the limited production of 2,3-DPG causes a leftward shift of the oxyhemoglobin curve and impairs the release of oxygen to peripheral tissues. Such a consequence of chronic hypophosphatemia has been documented in children with XLH and proposed as one factor underlying retarded statural growth [198].

Leukocyte dysfunction, which complicates phosphate deficiency, includes decreased chemotaxis, phagocytosis, and bactericidal activity [199]. These abnormalities increase the host susceptibility to infection. The mechanism by which hypophosphatemia impairs the various activities of the leukocyte probably is related to impairment of ATP synthesis. Decreased availability of energy impairs microtubules that regulate the mechanical properties of leukocytes and limit the rate of synthesis of organic phosphate compounds that are necessary for endocytosis.

Abnormal platelet survival, causing thrombocytopenia, profuse gastrointestinal bleeding, and cutaneous bleeding, has also been described in association with phosphate depletion in animal studies. Despite these abnormalities, there is little evidence that hypophosphatemia is a primary cause of hemorrhage in humans.

Perhaps the most consistent abnormalities associated with phosphate depletion are those on bone. Acute phosphate depletion induces dissolution of apatite crystal from the osseous matrix. This effect may be due to $1,25(OH)_2D_3$, which is increased in response to phosphate depletion in both animals and humans. More prolonged hypophosphatemia leads to rickets and osteomalacia. This complication is a common feature of phosphate depletion. However, the ultimate cause is variable. While simple phosphate depletion alone may underlie the genesis of the abnormal mineralization, in many disorders the defect is secondary to phosphate depletion and commensurate $1,25(OH)_2D_3$ deficiency. Thus, treatment of this complication may often require combination therapy, phosphate supplements, and calcitriol.

2. HYPERPHOSPHATEMIA

Hypocalcemia and consequent tetany are the most serious clinical sequelae of hyperphosphatemia [200]. The decreased serum calcium concentration results from the deposition of calcium phosphate salts in soft tissue, a process that may lead to symptomatic ectopic calcification. The dystrophic calcification is frequently seen in acute and chronic renal failure, hypoparathyroidism, pseudohypoparathyroidism, and tumoral calcinosis. Indeed, deposition of calcium-phosphate complexes in the kidney may predispose to acute

renal failure. When the calcium-phosphate product exceeds 70, the probability that soft tissue calcification will occur increases sharply. In addition, local factors, such as tissue pH and injury (e.g. necrotic or hypoxic tissue), may predispose to precipitation of the calcium-phosphate salts. In chronic renal failure, calcification occurs in arteries, muscle tissue, periarticular spaces, the myocardial conduction system, lungs, and the kidney. Affected patients may also have ocular calcification, causing the "red eye" syndrome of uremia and subcutaneous calcification, which also plays a role in uremic pruritus. On the other hand, a predisposition to calcification of periarticular surfaces of the hips, elbows, shoulders, and other large joints occurs in tumoral calcinosis.

In some disease states, hyperphosphatemia may also play an important role in the development of secondary hyperparathyroidism [201]. A decrement in the serum calcium concentration secondary to hyperphosphatemia stimulates the release of PTH. Furthermore, hyperphosphatemia decreases the activity of 25(OH)D-1α-hydroxylase. The consequent diminished production of $1,25(OH)_2D_3$ impairs the gastrointestinal absorption of calcium and induces skeletal resistance to PTH, influences that augment the development of hyperparathyroidism.

Thus, hyperphosphatemia triggers a cascade of events that impact on calcium homeostasis at multiple sites. The prevention of secondary hyperparathyroidism, metabolic bone disease, and soft tissue and vascular calcification in affected patients, therefore, depends on ultimately controlling the serum phosphate concentration.

F. Treatment of Abnormal Serum Phosphorus in Diseases Caused by an Abnormal Phosphate Load

Treatment of the myriad of diseases that characteristically display hyperphosphatemia or hypophosphatemia depends on determining the mechanism underlying their pathogenesis. The cause can almost always be ascertained by assessment of the clinical setting, determination of renal function, measurement of urinary phosphate excretion, and analysis of arterial carbon dioxide tension and pH. Therapy is aimed at correcting both the serum phosphate concentration and associated complications.

The treatment of phosphate depletion depends on replacing body phosphorus stores. Preventive measures, however, will preclude the onset of phosphate depletion. Thus, appropriate monitoring of patients taking large doses of aluminum-containing antacids and provision of phosphorus intravenously to patients with diabetic ketoacidosis will preserve phosphate stores. Alternatively, the treatment of established phosphate depletion may require 2.5 to 3.7 g of phosphate daily, preferably administered orally in four equally divided doses. Providing K-Phos Neutral tablets, which contain 250 mg of elemental phosphorus per tablet, will fulfill this goal. If oral therapy is not tolerated and the serum phosphate shows a downward trend approaching dangerous levels (<1.2 mg/dL), intravenous phosphate supplementation at a dose of 10 mg/kg body weight/day may be administered. Such therapy should be discontinued when the serum phosphate reaches values >2.0 mg/dL. However, therapy is not required for many of the conditions resulting in phosphate depletion. Only when the consequences of severe depletion are manifest should treatment be initiated.

Theoretically, decreasing the TmP, increasing the GFR, or diminishing the phosphate load (exogenous or endogenous) may decrease the elevated serum phosphate. There are no generally available pharmacologic means of acutely altering the GFR or reducing the TmP. However, chronic use of drugs, such as acetazolamide, which decreases TmP and induces phosphaturia, is effective as ancillary treatment of disorders such as tumoral calcinosis. Nevertheless, regulation of hyperphosphatemia is most often achieved by reducing the renal phosphate load. In tumoral calcinosis and chronic renal failure, such an effect is obtained by restricting the dietary phosphate intake or by administering phosphate binders such as calcium carbonate or aluminum hydroxide. Alternative strategies for management of load-dependent hyperphosphatemia include the administration of intravenous calcium or intravenous glucose and insulin. The consequence of such intervention is sequestration of phosphate in bone or soft tissues. Dialysis can also be used for the acute management of load-dependent disorders, or the chronic maintenance of phosphate overload such as those that complicate chronic renal failure.

References

1. Reichel H, Koeffler HP, Norman AW 1989 The role of the vitamin D endocrine system in health and disease. N Engl J Med **320**:980–991.
2. Fraser DR 1980 Regulation of metabolism of vitamin D. Physiol Rev **60**:551–613.
3. Yanagawa N, Nakhoul F, Kurokawa K, Lee DBN 1994 Physiology of phosphorus metabolism. In: Narins RG (ed.) Clinical Disorders of Fluid and Electrolyte Metabolism, 5th Edition, McGraw Hill, Inc., New York, pp. 307–371.
4. Tenenhouse HS 1997 Cellular and molecular mechanisms of renal P transport. J Bone Miner Res **12**:159–164.
5. Murer H, Biber J 1996 Molecular mechanisms of renal apical Na/phosphate cotransport. Annu Rev Physiol **58**:607–618.

6. Tenenhouse HS, Roy S, Martel J, Gauthier C 1998 Differential expression, abundance, and regulation of Na^+-phosphate cotransporter genes in murine kidney. Am J Physiol **275**(4 Pt 2):F527–F534.
7. Murer H, Hernanco N, Forster I, Biber J 2000 Proximal tubular P reabsorption: Molecular Mechanisms. Physiol Rev **80**(4):1373–1409.
8. Biber J, Custer M, Werner A, Kaissling B, Murer H 1993 Localization of Na-Pi, a Na/Pi cotransporter in rabbit kidney proximal tubules. II. Localization by immunohistochemistry. Pflugers Arch **424**:210–215.
9. Busch AE, Schuster A, Waldegger S, Wagner CA, Zempel G, Broer S, Biber J, Murer H, Lang F 1993 Expression of a renal type I sodium/phosphate transporter (NaPi-I) induces a conductance in *Zenopus* oocytes permeable for organic and inorganic anions. Proc Natl Acad Sci USA **93**:5347–5351.
10. Yabuuchi H, Tamai I, Morita K, Kouda T, Miyamoto K, Takeda E, Tsuji A 1998 Hepatic sinusoidal membrane transport of anionic drugs mediated by anion transporter NptI. Journal of Pharmacology and Experimental Therapeutics **286**:1391–1396.
11. Segawa H, Kaneko I, Takahashi A, Kuwahata M, Ito M, Ohkido I, Tatsumi S, Miyamoto K-i 2002 Growth-related renal type II Na/Pi cotransporter. J Biol Chem **277**(22): 19665–19672.
12. Custer M, Lotscher M, Biber J, Murer H, Kaissling B 1994 Expression of Na-Pi cotransport in rat kidney: Localization by RT-PCR and immunohistochemistry. Am J Physiol **266**:F767–F774.
13. Beck L, Karaplis AC, Amizuka N, Hewson AS, Ozawa H, Tenenhouse HS 1998 Targeted inactivation of Npt2 in mice leads to severe renal phosphate wasting, hypercalciuria, and skeletal abnormalities. Proc Natl Acad Sci USA **95**(9):5372–7.
14. Kavanaugh MP, Miller DG, Zhang W, Law W, Kozak SL, Kabat D, Miller AD 1994 Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent P symporters. Proc Natl Acad Sci USA **91**:7071–7075.
15. Kempson SA, Lotscher M, Kaissling B, Biber J, Murer H, Levi M 1995 Parathyroid hormone action on phosphate transporter mRNA and protein in rat renal proximal tubules. Am J Physiol **268**:F784–F791.
16. Levi M 2000 Npt2 is the major mediator of renal phosphate transport. Am J Dis Kidney **36**:1276–1280.
17. Traebert M, Volkl H, Biber J, Murer H, Kaissling B 2000 Luminal and contraluminal action of 1-34 and 3-34 PTH peptides on renal type IIa Na-Pi cotransporter. Am J Physiol **278**:F792–F798.
18. Bacic D, Hernando N, Traebert M, Lederer E, Volkl H, Biber J, Kaissling B, Murer H 2001 Regulation of the renal type IIa Na/Pi cotransporter by cGMP. Pflugers Arch **443**:306–313.
19. Bacic D, Schulz N, Biber J, Kaissling B, Murer H, Wagner CA 2003 Involvement of the MAPK-kinase pathway in the PTH-mediated regulation of the proximal tubule type IIa Na(+)/P(i) cotransporter in mouse kidney. Pflugers Arch **446**(1):52–60.
20. Yamashita T, Konishi M, Miyake A, Inui K, Itoh N 2002 Fibroblast growth factor (FGF)-23 inhibits renal phosphate reabsorption by activation of the mitogen-activated protein kinase pathway. J Biol Chem **277**(31):28265–70.
21. Jankowski M, Hilfiker H, Biber J, Murer H 2001 The opossum kidney cell type IIa Na/P(i) cotransporter is a phosphoprotein. Kidney Blood Press Res **24**:1–4.
22. Shenolikar S, Voltz JW, Minkoff CM, Wade JB, Weinman EJ 2002 Targeted disruption of the mouse NHERF-1 gene promotes internalization of proximal tubule sodium-phosphate cotransporter type IIa and renal phosphate wasting. Proc Natl Acad Sci USA **99**(17):11470–11475.
23. Hernando N, Deliot N, Gisler SM, Lederer E, Weinman EJ, Biber J, Murer H 2002 PDZ-domain interactions and apical expression of type IIa Na/Pi cotransporters. Proc Natl Acad Sci USA **99**:11957–11962.
24. Bell NH 1988 Vitamin D metabolism in health and disease. Henry Ford Hosp Med J **36**:40–52.
25. Tanaka Y, DeLuca HF 1973 The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. Arch Biochem Biophys **154**:566–574.
26. Fukase M, Birge S, Rifas L, Avioli LV, Chase LR 1982 Regulation of 25 hydroxyvitamin D3 1-hydroxylase in serum-free monolayer culture of mouse kidney. Endocrinology **110**:1073–1075.
27. Gray RW, Garthwaite TL 1985 Activation of renal 1,25-dihyroxyvitamin D3 synthesis by phosphate deprivation: Evidence for a role for growth hormone. Endocrinology **116**: 189–193.
28. Gray RW, Lemann JJ 1985 Vitamin D metabolism: the renal-pituitary axis. In: Norman AW, Schaefer K, Grigoleit H-G, Herrath Dv (eds.) Vitamin D—chemical, biochemical, and clinical update. de Gruyter: Berlin.
29. Drezner MK 1987 Understanding the pathogenesis of X-linked hypophosphatemic rickets: A requisite for successful therapy. In: Zackson DA (ed.) CPC series: Cases in metabolic bone disease. Triclinica Communications, New York, pp 1–11.
30. Tenenhouse HS, Martel J, Gauthier C, Zhang MY, Portale AA 2001 Renal expression of the sodium/phosphate cotransporter gene, Npt2, is not required for regulation of renal 1 alpha-hydroxylase by phosphate. Endocrinology **142**(3):1124–9.
31. Spitzer A, Barac-Nieto M 2001 Ontogeny of renal phosphate transport and the process of growth. Pediatr Nephrol **16**(9):763–71.
32. Drezner MK 2002 Unpublished Observations.
33. Cai Q, Hodgson SF, Kao PC, Lennon VA, Klee GG, Zinsmiester AR, Kumar R 1994 Brief report: inhibition of renal phosphate transport by a tumor product in a patient with oncogenic osteomalacia. N Engl J Med **330**:1645–1649.
34. Nelson AE 1996 Characteristics of tumor cell bioactivity in oncogenic osteomalacia. Mol Cell Biol **124**:17–23.
35. Wilkins GE, Granleese S, Hegele RG, Holden J, Anderson DW, Bondy GP 1995 Oncogenic osteomalacia: evidence for a humoral phosphaturic factor. J Clin Endocrinol Metab **80**(5):1628–1634.
36. Nelson AE, Mason RS, Hogan JJ, Diamond T, Robinson BG 1998 Tumor expression studies indicate that HEM-1 is unlikely to be the active factor in oncogenic osteomalacia. Bone **23**(6):549–553.
37. Econs MJ, Drezner MK 1994 Tumor-induced osteomalacia—unveiling a new hormone. N Engl J Med **330**(23):1679–1681.
38. Jan de Beur SM, Finnegan RB, Vassiliadis J, Cook B, Barberio D, Estes S, Manavalan P, Petroziello J, Madden S, Cho JY, Kumar R, Levine MA, Schiavi SC 2002 Tumors associated with oncogenic osteomalacia express markers of bone and mineral metabolism. J Bone Miner Res **17**:1102–1110.
39. Bowe AE, Finnegan R, Jan de Beur SM, Cho J, Levine MA, Kumar R, Schiavi SC 2001 FGF-23 inhibits renal tubular phosphate transport and is a PHEX substrate. Biochem Biophys Res Commun **284**(4):977–81.
40. White KE, Jonsson KB, Carn G, Hampson G, Spector TD, Mannstadt M, Lorenz-Depiereux B, Miyauchi A, Yang IM,

Ljunggren O, Meitinger T, Strom TM, Juppner H, Econs MJ 2001 The autosomal dominant hypophosphatemic rickets (ADHR) gene is a secreted polypeptide overexpressed by tumors that cause phosphate wasting. J Clin Endocrinol Metab **86**(2):497–500.
41. Shimada T, Mizutani S, Muto T, Yoneya T, Hino R, Takeda S, Takeuchi Y, Fujita T, Fukumoto S, Yamashita T 2001 Cloning and characterization of FGF23 as a causative factor of tumor-induced osteomalacia. Proc Natl Acad Sci USA **98**(11): 6500–6505.
42. De Beur SM, Finnegan RB, Vassiliadis J, Cook B, Barberio D, Estes S, Manavalan P, Petroziello J, Madden SL, Cho JY, Kumar R, Levine MA, Schiavi SC 2002 Tumors associated with oncogenic osteomalacia express genes important in bone and mineral metabolism. J Bone Miner Res **17**(6):1102–1110.
43. 2000 Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23. The ADHR Consortium. Nat Genet **26**(3):345–8.
44. Econs MJ, McEnery PT 1997 Autosomal dominant hypophosphatemic rickets/osteomalacia: clinical characterization of a novel renal phosphate-wasting disorder. J Clin Endocrinol Metab **82**(2):674–81.
45. White KE, Carn G, Lorenz-Depiereux B, Benet-Pages A, Strom TM, Econs MJ 2001 Autosomal-dominant hypophosphatemic rickets (ADHR) mutations stabilize FGF-23. Kidney International **60**(6):2079–86.
46. Bai XY, Miao D, Goltzman D, Karaplis AC 2003 The autosomal dominant hypophosphatemic rickets R176Q mutation in fibroblast growth factor 23 resists proteolytic cleavage and enhances *in vivo* biological potency. J Biol Chem **278**(11): 9843–9849.
47. Shimada T, Yoneya T, Hino R, Takeuchi Y, Fukumoto S, Tamashita T 2001 Transgenic mice expressing fibroblast growth factor 23 demonstrate hypophosphatemia with low serum 1,25-dihydroxyvitamin D and rickets/osteomalacia. J Bone Miner Res **16**(Suppl 1):S151.
48. Bai XY, Miao DS, Li JR, Goltzman D, Karaplis AC 2003 Transgenic mice overexpressing human FGF-23 (R176Q) recapitulate a severe form of the ADHR phenotype. J Bone Miner Res **18**:S24 (1089).
49. Jonsson KB, Zahradnik R, Larsson T, White KE, Hampson G, Miyauchi A, Econs M, Lavigne J, Jüppner H 2002 FGF-23 is a circulating factor that is elevated in oncogenic osteomalacia and X-linked hypophosphatemic rickets. N Engl J Med **348**: 1656–1663.
50. Yamazaki Y, Okazaki R, Shibata M, Hasegawa Y, Satoh K, Tajima T, Takeuchi Y, Fujita T, Nakahara K, Yamashita T, Fukumoto S 2002 Increased circulatory level of biologically active full-length FGF-23 in rickets/osteomalacia. J Clin Endocrinol Metab **87**:4957–4960.
51. Riminucci M, Collins MT, Fedarko NS, Cherman N, Corsi A, White KE, Waguespack S, Gupta A, Hannon T, Econs MJ, Bianco P, Gehron Robey P 2003 FGF-23 in fibrous dysplasia of bone and its relationship to renal phosphate wasting. J Clin Invest **112**(5):683–692.
52. Meyer RA, Jr., Meyer MH, Gray RW 1989 Parabiosis suggests a humoral factor is involved in X-linked hypophosphatemia in mice. J Bone Miner Res **4**(4):493–500.
53. Lajeunesse D, Meyer RA, Jr., Hamel L 1996 Direct demonstration of a humorally-mediated inhibition of renal phosphate transport in the Hyp mouse. Kidney Int **50**(5):1531–8.
54. Nesbitt T, Fujiwara I, Thomas R, Xiao ZS, Quarles LD, Drezner MK 1999 Coordinated maturational regulation of PHEX and renal phosphate transport inhibitory activity: evidence for the pathophysiological role of PHEX in X-linked hypophosphatemia. J Bone Miner Res **14**(12):2027–35.
55. Xiao ZS, Crenshaw M, Guo R, Nesbitt T, Drezner MK, Quarles LD 1998 Intrinsic mineralization defect in Hyp mouse osteoblasts. Am J Physiol **275**(4 Pt 1):E700–8.
56. Nesbitt T, Coffman TM, Griffiths R, Drezner MK 1992 Crosstransplantation of kidneys in normal and Hyp mice. Evidence that the Hyp mouse phenotype is unrelated to an intrinsic renal defect. J Clin Invest **89**(5):1453–9.
57. Thompson DL, Sabbagh Y, Tenenhouse HS, Roche PC, Drezner MK, Salisbury JL, Grande JP, Poeschla EM, Kumar R 2002 Ontogeny of Phex/PHEX protein expression in mouse embryo and subcellular localization in osteoblasts. J Bone Miner Res **17**(2):311–20.
58. Beck L, Soumounou Y, Martel J, Krishnamurthy G, Gauthier C, Goodyer CG, Tenenhouse HS 1997 Pex/PEX tissue distribution and evidence for a deletion in the 3′ region of the Pex gene in X-linked hypophosphatemic mice. J Clin Invest **99**(6):1200–9.
59. Meyer MH, Meyer RA, Jr. 2000 MRNA expression of Phex in mice and rats: the effect of low phosphate diet. Endocrine **13**(1):81–7.
60. Zoidis E, Zapf J, Schmid C 2000 Phex cDNA cloning from rat bone and studies on phex mRNA expression: tissue-specificity, age-dependency, and regulation by insulin-like growth factor (IGF) I in vivo. Mol Cell Endocrinol **168**(1–2):41–51.
61. Ruchon AF, Tenenhouse HS, Marcinkiewicz M, Siegfried G, Aubin JE, DesGroseillers L, Crine P, Boileau G 2000 Developmental expression and tissue distribution of Phex protein: effect of the Hyp mutation and relationship to bone markers. J Bone Miner Res **15**(8):1440–50.
62. Ruchon AF, Marcinkiewicz M, Siegfried G, Tenenhouse HS, DesGroseillers L, Crine P, Boileau G 1998 Pex mRNA is localized in developing mouse osteoblasts and odontoblasts. J Histochem Cytochem **46**(4):459–68.
63. Grieff M, Mumm S, Waeltz P, Mazzarella R, Whyte MP, Thakker RV, Schlessinger D 1997 Expression and cloning of the human X-linked hypophosphatemia gene cDNA. Biochem Biophys Res Commun **231**(3):635–9.
64. 1995 A gene (PEX) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. The HYP Consortium. Nat Genet **11**(2):130–6.
65. Aono Y, Shimada T, Yamazaki Y, Hino R, Takeuchi Y, Fujita T, Fukumoto S, Nagano N, Wada M, Yamashita T 2003 The neutralization of FGF-23 ameliorates hypophosphatemia and rickets in *hyp*-mice. J Bone Miner Res **18**(Suppl 2):S16 (1056).
66. Berndt T, Craig TA, Bowe AE, Vassiliadis J, Reczek D, Finnegan R, Jan de Beur SM, Schiavi SC, Kumar R 2003 Secreted frizzled-related protein 4 is a potent tumor-derived phosphaturic agent. J Clin Invest **112**:785–794.
67. Rowe PS, de Zoysa PA, Dong R, Wang HR, White KE, Econs MJ, Oudet CL 2000 MEPE, a new gene expressed in bone marrow and tumors causing osteomalacia. Genomics **67**:54–68.
68. Guo R, Rowe PSN, Liu S, Simpson LG, Xiao ZS, Quarles LD 2002 Inhibition of MEPE cleavage by *Phex*. Biochem Biophys Res Commun **297**:38–45.
69. Bai X, Miao D, Panda D, Grady S, McKee MD, Goltzman D, Karaplis AC 2002 Partial Rescue of the Hyp Phenotype by Osteoblast-Targeted PHEX (Phosphate-Regulating Gene with Homologies to Endopeptidases on the X Chromosome) Expression. Mol Endocrinol **16**(12):2913–2925.
70. Argiro L, Desbarats M, Glorieux FH, Ecarot B 2001 Mepe, the gene encoding a tumor-secreted protein in oncogenic hypophosphatemic osteomalacia, is expressed in bone. Genomics **74**(3):342–351.
71. Petersen DN, Tkalcevic GT, Mansolf AL, Rivera-Gonzalez R, Brown TA 2000 Identification of osteoblast/osteocyte factor 45 (OF45), a bone-specific cDNA encoding an RGD-containing

protein that is highly expressed in osteoblasts and osteocytes. J Biol Chem **275**:36172–36180.
72. Gowen LC, Petersen DN, Mansolf AL, Qi H, Stock JL, Tkalcevic GT, Simmons HA, Crawford DT, Chidsey-Frink KL, Ke HZ, McNeish JD, Brown TA 2003 Targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass. J Biol Chem **278**(3): 1998–2007.
73. Rowe PSN, Kumagai Y, Gutierrez G, Garrett IR, Blacher R, Rosen D, Chen D, Drezner MK, Quarles LD, Mundy GR 2003 MEPE regulates bone mineralization and phosphate transport: PHEX and the MEPE ASARM-peptide. Bone: In press.
74. Liu S, Brown TA, Xiao Z, Guo R, Quarles LD 2003 Deletion of *Mepe* in *hyp*-mice fails to correct hypophosphatemia but partially rescues abnormal mineralization. J Bone Miner Res **18**:In press.
75. Guo R, Liu S, Spurney RF, Quarles LD 2001 Analysis of recombinant Phex: an endopeptidase in search of a substrate. Am J Physiol Endocrinol Metab **281**(4):E837–E847.
76. Campos M, Couture C, Hirata IY, Juliano MA, Loisel TP, Crine P, Juliano L, Boileau G, Carmona AK 2003 Human recombinant endopeptidase PHEX has a strict S1′ specificity for acidic residues and cleaves peptides derived from fibroblast growth factor-23 and matrix extracellular phosphoglycoprotein. Biochem J **373(Pt 1)**:271–279.
77. Liu S, Guo R, Simpson LG, Xiao ZS, Burnham CE, Quarles LD 2003 Regulation of FGF23 expression but not degradation by phex. J Biol Chem **278**(39):37419–37426.
78. Collins MT, Chebli C, Jones J, Kushner H, Consugar M, Rinaldo P, Wientroub S, Bianco P, Robey PG 2001 Renal phosphate wasting in fibrous dysplasia of bone is part of a generalized renal tubular dysfunction similar to that seen in tumor-induced osteomalacia. Journal of Bone & Mineral Research **16**(5):806–13.
79. Yamamoto T, Miyamoto KI, Ozono K, Taketani Y, Katai K, Miyauchi A, Shima M, Yoshikawa H, Yoh K, Takeda E, Okada S 2001 Hypophosphatemic rickets accompanying McCune-Albright syndrome: evidence that a humoral factor causes hypophosphatemia. Journal of Bone & Mineral Metabolism **19**(5):287–95.
80. Guo R, Liu S, Spurney RF, Quarles LD 2001 Analysis of recombinant Phex: an endopeptidase in search of a substrate. Am J Physiol Endocrinol Metab **281**:E837-E847.
81. Liu S, Guo R, Tu Q, Quarles LD 2002 Overexpression of *Phex* in osteoblasts fails to rescue the Hyp mouse phenotype. Journal of Biological Chemistry **277**(5):3686–97.
82. Erben RG, Mayer D, Weber K, Johnson T, Nonsson K, Juppner H, Lanske B 2003 Ubiquitous overexpression of *Phex* does not fully rescue the *Hyp* mouse phenotype. J Bone Miner Res **18**:S22 (1080).
83. Fujiwara I, Thomas R, Horst RL, Aravindan RG, Drezner MK 2003 Aberrant post-transcriptional regulation of renal 25-hydroxyvitamin D-1a-hydroxylase activity in the hyp-mouse. J Bone Miner Res **18**:434–442.
84. Yuan B, Xing Y, Veber R, Drezner MK 2003 Abnormal translational regulation of renal 25-hydroxyvitamin D-1α-hydroxylase activity in the *hyp-mouse*. J Bone Miner Res **18**(Suppl 2): S282–S283 (SU427).
85. Lobaugh B, Burch WM, Jr, Drezner MK 1984 Abnormalities of vitamin D metabolism and action in the vitamin D resistant rachitic and osteomalacic diseases. In: Kumar R (ed.) Vitamin D. Martinus Nijhoff, Boston, pp 665–720.
86. Harrison HE, Harrison HC, Lifshitz F, Johnson AD 1966 Growth disturbance in hereditary hypophosphatemia. Am J Dis Child **112**:290–297.
87. Haddad JG, Chyu KJ, Hahn TJ, Stamp TCB 1973 Serum concentrations of 25-hydroxyvitamin D in sex-linked hypophosphatemic vitamin D–resistant rickets. J Lab Clin Med **81**:22–27.
88. Delvin EE, Glorieux FH 1981 Serum 1,25-dihydroxyvitamin D concentration in hypophosphatemic vitamin D–resistant rickets. Calcif Tissue Int **33**(173–175).
89. Lyles KW, Clark AG, Drezner MK 1982 Serum 1,25-dihydroxyvitamin D levels in subjects with X-linked hypophosphatemic rickets and osteomalacia. Calcif Tissue Int **34**(2): 125–30.
90. Lobaugh B, Drezner MK 1983 Abnormal regulation of renal 25-hydroxyvitamin D-1alpha-hydroxylase activity in the X-linked hypophosphatemic mouse. J Clin Invest **71**(2):400–3.
91. Nesbitt T, Drezner MK, Lobaugh B 1986 Abnormal parathyroid hormone stimulation of 25-hydroxyvitamin D-1 alpha-hydroxylase activity in the hypophosphatemic mouse. Evidence for a generalized defect of vitamin D metabolism. J Clin Invest **77**(1):181–7.
92. Nesbitt T, Lobaugh B, Drezner MK 1987 Calcitonin stimulation of renal 25-hydroxyvitamin D-1 alpha-hydroxylase activity in hypophosphatemic mice. Evidence that the regulation of calcitriol production is not universally abnormal in X-linked hypophosphatemia. J Clin Invest **79**(1):15–9.
93. Nesbitt T, Drezner MK 1990 Abnormal parathyroid hormone-related peptide stimulation of renal 25-hydroxyvitamin D-1-hydroxylase in Hyp mice: evidence for a generalized defect of enzyme activity in the proximal convoluted tubule. Endocrinology **127**(2):843–8.
94. Bell CL, Tenenhouse HS, Scriver CR 1988 Primary cultures of renal epithelial cells from X-linked hypophosphatemic (Hyp) mice express defects in phosphate transport and vitamin D metabolism. Am J Hum Genet **43**(3):293–303.
95. Dobre CV, Alvarez UM, Hruska KA 1990 Primary culture of hypophosphatemic proximal tubule cells express defective adaptation to P. J Bone Miner Res **5**(Suppl1):S205.
96. Collins JF, Ghishan FK 1994 Molecular cloning, functional expression, tissue distribution, and *in situ* hybridization of the renal sodium phosphate (Na+/P(i)) transporter in the control and hypophosphatemic mouse. Faseb J **8**(11): 862–8.
97. Tenenhouse HS, Werner A, Biber J, Ma S, Martel J, Roy S, Murer H 1994 Renal Na+-phosphate cotransport in murine X-linked hypophosphatemic rickets: molecular characterization. J Clin Invest **93**:671–676.
98. Tenenhouse HS, Martel J, Biber J, Murer H 1995 Effect of P(i) restriction on renal Na(+)-P(i) cotransporter mRNA and immunoreactive protein in X-linked Hyp mice. Am J Physiol **268**(6 Pt 2):F1062–F1069.
99. Meyer RA, Jr., Meyer MH, Gray RW 1989 Parabiosis suggests a humoral factor is involved in X-linked hypophosphatemia in mice. J Bone Miner Res **4**:493–500.
100. Meyer RA, Jr., Tenenhouse HS, Meyer MH, Klugerman AH 1989 The renal phosphate transport defect in normal mice parabiosed to X-linked hypophosphatemic mice persists after parathyroidectomy. J Bone Miner Res **4**:523–532.
101. Nesbitt T, Econs MJ, Byun JK, Martel J, Tenenhouse HS, Drezner MK 1995 Phosphate transport in immortalized cell cultures from the renal proximal tubule of normal and Hyp mice: evidence that the HYP gene locus product is an extrarenal factor. J Bone Miner Res **10**(9):1327–33.
102. Nesbitt T, Byun JK, Drezner MK 1996 Normal phosphate transport in cells from the S2 and S3 segments of Hyp-mouse proximal renal tubules. Endocrinology **137**(3):943–8.
103. Kos CH, Tihy F, Econs MJ, Murer H, Lemieux N, Tenenhouse HS 1994 Localization of a renal sodium P

cotransporter gene to human chromosome 5q35. Genomics **19**:176–177.
104. Read AP, Thakker RV, Davies KE, Mountford RC, Brenton DP, Davies M, Glorieux F, Harris R, Hendy GN, King A, McGlade S, Peacock CJ, Smith R, O'Riordan JLH 1986 Mapping of human X-linked hypophosphataemic rickets by multilocus linkage analysis. Hum Genet **73**(3):267–70.
105. Machler M, Frey D, Gal A, Orth U, Wienker TF, Fanconi A, Schmid W 1986 X-linked dominant hypophosphatemia is closely linked to DNA markers DXS41 and DXS43 at Xp22. Hum Genet **73**(3):271–5.
106. Thakker RV, Read AP, Davies KE, Whyte MP, Weksberg R, Glorieux F, Davies M, Mountford RC, Harris R, King A, *et al*. 1987 Bridging markers defining the map position of X-linked hypophosphataemic rickets. J Med Genet **24**(12): 756–60.
107. Thakker RV, Davies KE, Read AP, Tippett P, Wooding C, Flint T, Wood S, Kruse TA, Whyte MP, O'Riordan JLH 1990 Linkage analysis of two cloned DNA sequences, DXS197 and DXS207, in hypophosphatemic rickets families. Genomics **8**(2):189–193.
108. Econs MJ, Fain PR, Norman M, Speer MC, Pericak-Vance MA, Becker PA, Barker DF, Taylor A, Drezner MK 1993 Flanking markers define the X-linked hypophosphatemic rickets gene locus. J Bone Miner Res **8**(9):1149–52.
109. Econs MJ, Rowe PS, Francis F, Barker DF, Speer MC, Norman M, Fain PR, Weissenbach J, Read A, Davis KE, Becker PA, Lehrach H, O'Riordan J, Drezner MK 1994 Fine structure mapping of the human X-linked hypophosphatemic rickets gene locus. J Clin Endocrinol Metab **79**(5):1351–4.
110. Econs MJ, Francis F, Rowe PSN, Speer MC, Pericak-Vance MA, Becker PA, O'Riordan J, Lehrach H, Drezner MK 1994 Dinucleotide repeat polymorphism of the microsatellite repeat probe, A0563. Hum Mol Genet **3**:680.
111. Francis F, Rowe PS, Econs MJ, See CG, Benham F, O'Riordan JL, Drezner MK, Hamvas RM, Lehrach H 1994 A YAC contig spanning the hypophosphatemic rickets disease gene (HYP) candidate region. Genomics **21**(1):229–37.
112. Rowe PS, Goulding JN, Francis F, Oudet C, Econs MJ, Hanauer A, Lehrach H, Read AP, Mountford RC, Summerfield T, Weissenbach J, Fraser W, Drezner MK, Davies KE, O'Riordan JL 1996 The gene for X-linked hypophosphataemic rickets maps to a 200-300kb region in Xp22.1, and is located on a single YAC containing a putative vitamin D response element (VDRE). Hum Genet **97**(3):345–52.
113. Turner AJ, Tanzawa K 1997 Mammalian membrane metallopeptidases: NEP, ECE, KELL, and PEX. Faseb J **11**(5): 355–64.
114. Emoto N, Yanagisawa M 1995 Endothelian-converting enzyme-2 is a membrane-bound phosphramidon-sensitive metalloprotease with acidic pH optimum. J Biol Chem **270**:15262–15268.
115. Lee S, Zambas ED, Marsh WL, Redman CM 1991 Molecular cloning and primary structure of Kell blood group protein. Proc Natl Acad Sci USA **88**:6353–6357.
116. Kiryu-Seo S, Sasaki M, Yokohama H, Nakagomi S, Hirayama T, Aoki S, Wada K, Kiyama H 2000 Damage-induced neuronal endopeptidase (DINE) is a unique meallopeptidase expressed in response to neuronal damage and activates superoxide scavenge. Proc Natl Acad Sci USA **97**:4345–4350.
117. Shimada K, Takahashi M, Turner AJ, Tanzawa K 1996 Rat endothelin-converting enzyme-1 forms a dimer through Cys412 with a similar catalytic mechanism and a distinct substrate-binding mechanism compared with neutral endopeptidase-24.11. J Biochem **315**:863–867.
118. D'Adamio L, Shipp MA, Masteller EL, Reinherz EL 1989 Organization of the gene encoding common acute lymphoblastic leukemia antigen (neutral endopeptidase 24.11): multiple miniexons and separate 5′ untranslated regions. Proc Natl Acad Sci USA **86**:7103–7107.
119. Xu D, Emoto N, Giald A, Slaughter C, Kaw S, deWit D, Yanagisawa M 1994 ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. Cell **78**:473–485.
120. Russell F, Davenport A 1999 Evidence for intracellular endothelin-converting enzyme-2 expression in cultured human vascular endothelial cells. Circulation Research **84**:891–896.
121. Valdenaire O, Richards JG, Faull RLM, Schweizer A 1999 XCE, a new member of the endothelin-converting enzyme and neutral endopeptidase family, is preferentailly expressed in the CNS. Brain Res Mol Brain Res **64**:211–221.
122. Ghaddar G, Ruchon AF, Carpentier M, Marcinkiewicz M, Seidah NG, Crine P, Desgroseillers L, Boileau G 2000 Molecular cloning and biochemical characterization of a new mouse testis soluble-zinc-metallopeptidase of the neprilysin family. Biochem J **347**(Pt 2):419–29.
123. Valdenaire O, Rohrbacher E, Langeveld A, Schweizer A, Meijers C 2000 Organization and chromosomal localization of human ECEL1 (XCE) gene encoding a zinc metallopeptidase involved in the nervous control of respiration. Biochemistry Journal **346**:611–616.
124. Lipman ML, Panda D, Bennett HP, Henderson JE, Shane E, Shen Y, Goltzman D, Karaplis AC 1998 Cloning of human PEX cDNA. Expression, subcellular localization, and endopeptidase activity. J Biol Chem **273**(22):13729–37.
125. Strom TM, Francis F, Lorenz B, Boddrich A, Econs MJ, Lehrach H, Meitinger T 1997 Pex gene deletions in Gy and Hyp mice provide mouse models for X-linked hypophosphatemia. Hum Mol Genet **6**(2):165–71.
126. Du L, Desbarats M, Viel J, Glorieux FH, Cawthorn C, Ecarot B 1996 cDNA cloning of the murine Pex gene implicated in X-linked hypophosphatemia and evidence for expression in bone. Genomics **36**(1):22–8.
127. Francis F, Strom TM, Hennig S, Boddrich A, Lorenz B, Brandau O, Mohnike KL, Cagnoli M, Steffens C, Klages S, Borzym K, Pohl T, Oudet C, Econs MJ, Rowe PS, Reinhardt R, Meitinger T, Lehrach H 1997 Genomic organization of the human PEX gene mutated in X-linked dominant hypophosphatemic rickets. Genome Res **7**(6):573–85.
128. Rowe PS, Oudet CL, Francis F, Sinding C, Pannetier S, Econs MJ, Strom TM, Meitinger T, Garabedian M, David A, Macher MA, Questiaux E, Popowska E, Pronicka E, Read AP, Mokrzycki A, Glorieux FH, Drezner MK, Hanauer A, Lehrach H, Goulding JN, O'Riordan JL 1997 Distribution of mutations in the PEX gene in families with X-linked hypophosphataemic rickets (HYP). Hum Mol Genet **6**(4): 539–49.
129. Holm IA, Huang X, Kunkel LM 1997 Mutational analysis of the PEX gene in patients with X-linked hypophosphatemic rickets. Am J Hum Genet **60**(4):790–7.
130. Dixon PH, Christie PT, Wooding C, Trump D, Grieff M, Holm I, Gertner JM, Schmidtke J, Shah B, Shaw N, Smith C, Tau C, Schlessinger D, Whyte MP, Thakker RV 1998 Mutational analysis of PHEX gene in X-linked hypophosphatemia. J Clin Endocrinol Metab **83**(10):3615–23.

131. Filisetti D, Ostermann G, von Bredow M, Strom T, Filler G, Ehrich J, Pannetier S, Garnier JM, Rowe P, Francis F, Julienne A, Hanauer A, Econs MJ, Oudet C 1999 Nonrandom distribution of mutations in the PHEX gene, and underdetected missense mutations at nonconserved residues. Eur J Hum Genet **7**(5):615–9.
132. Tyynismaa H, Kaitila I, Nanto-Salonen K, Ala-Houhala M, Alitalo T 2000 Identification of fifteen novel PHEX gene mutations in Finnish patients with hypophosphatemic rickets. Hum Mutat **15**(4):383–4.
133. Sabbagh Y, Boileau G, DesGroseillers B, Tenenhouse HS 2001 Turnover and rescue of mutant PHEX proteins sequestered in the endoplasmic reticulum. J Bone Miner Res **16**(Suppl 1):S227.
134. Christie PT, Harding B, Nesbit MA, Whyte MP, Thakker RV 2001 X-linked hypophosphatemia attributable to pseudoexons of the PHEX gene. J Clin Endocrinol Metab **86**(8): 3840–4.
135. Econs MJ, Friedman NE, Rowe PS, Speer MC, Francis F, Strom TM, Oudet C, Smith JA, Ninomiya JT, Lee BE, Bergen H 1998 A PHEX gene mutation is responsible for adult-onset vitamin D–resistant hypophosphatemic osteomalacia: evidence that the disorder is not a distinct entity from X-linked hypophosphatemic rickets. J Clin Endocrinol Metab **83**(10):3459–62.
136. Blydt-Hansen TD, Tenenhouse HS, Goodyer P 1999 PHEX expression in parathyroid gland and parathyroid hormone dysregulation in X-linked hypophosphatemia. Pediatr Nephrol **13**(7):607–11.
137. Sabbagh Y, Londowski JM, Mathieson D, Gauthier C, Boileau G, Tenenhouse HS, Poeschia EM, Kumar R 2000 Stable expression of PHEX in hypophosphatemic (Hyp) mouse osteoblasts using a viral vector partially corrects the mutant cell phenotype: implications for gene therapy. Journal of the American Society of Nephrology **11**:413A.
138. Miyamura T, Tanaka H, Inoue M, Ichinose Y, Seino Y 2000 The effects of bone marrow transplantation on X-linked hypophosphatemic mice. J Bone Miner Res **15**(8): 1451–8.
139. Bhargava A, Xing Y, Drezner MK 2003 Abnormal phosphate transport in *hyp*-mouse osteoblasts: a possible factor in the bone mineralization defect. J Bone Miner Res In press.
140. Kumar R, Riggs BL 1980 Pathologic physiology of bone. In: Urist MR (ed.) Fundamental and Clinical Bone Physiology. J.B. Lippincott Company: Philadelphia, pp 394–405.
141. Florentin D, Sassi A, Roques BP 1984 A highly sensitive fluorimetric assay for "enkephalinase," a neutral metalloendopeptidase that releases Tyr-Gly-Gly from enkephalins. Anal Biochem **141**:62–69.
142. Koehn JA, Norman JA, Jones BN, LeSueur L, Sakane Y, Ghai RD 1987 Degradation of atrial natriuretic factor by kidney cortex membranes. J Biol Chem **262**:11623–11627.
143. Jalal F, Lemay G, Zollinger M, Berthelot A, Boileau G, Crine P 1991 Neutral endopeptidase, a major brush border protein of the kidney proximal nephron, is directly targeted to the apical domain when expressed in Madrin-Darbey kidney cells. J Biol Chem **266**:19826–19857.
144. Mari B, Checler F, Ponzio G, Peyron JF, Manie S, Farahifar D, Rossi B, Auberger P 1992 Jurkat T cells express a functional neutral endopeptidase activity (CALLA) involved in T cell activation. EMBO J **11**:3875–3885.
145. Marie PJ, Glorieux FH 1983 Relation between hypomineralized periosteocytic lesions and bone mineralization in vitamin D–resistant rickets. Calcif Tissue Int **35**:443–448.
146. Rifas L, Cheng S, Halstead LR, Gupta A, Hruska KA, Avioli LV 1997 Skeletal case in kinase activity defect in the HYP mouse. Calcif Tissue Int **61**(3):256–9.
147. Guo R, Quarles LD 1997 Cloning and sequencing of human PEX from a bone cDNA library: evidence for its developmental stage-specific regulation in osteoblasts. J Bone Miner Res **12**(7):1009–17.
148. Friedman NE, M.K. D 1991 Genetic Osteomalacia. In: Bardin CW (ed.) Current Therapy in Endocrinology and Metabolism, 4th Edition. BC Decker, Inc. Philadelphia, pp 421–428.
149. Glorieux FH, Marie PJ, Pettifor JM, Delvin EE 1980 Bone response to phosphate salts, ergocalciferol, and calcitrol in hypophosphatemic vitamin D–resistant rickets. N Engl J Med **303**:1023–1031.
150. Friedman NE, Lobaugh B, Drezner MK 1993 Effects of calcitriol and phosphorus therapy on the growth of patients with X-linked hypophosphatemia. J Clin Endocrinol Metab **76**(4):839–44.
151. Saggerve G, Baronelli G, Butelloni S, Perri G 1995 Long-term growth hormone treatment in children with renal hypophosphatemic rickets: effects on growth, mineral metabolism, and bone density. J Pediatr **127**:395–402.
152. Saggese G, Baroncelli GI, Barsanti S 1998 [Growth hormone treatment of familial hypophosphatemic rickets]. Arch Pediatr **5**(Suppl 4):360S–363S.
153. Tieder M, Modai D, Samuel R, Arie R, Halabi A, Bab I, Gabizon D, Liberman UA 1985 Hereditary hypophosphatemic rickets with hypercalciuria. N Engl J Med **312**:611–617.
154. Sermet-Gaudelus I, Garabedian M, Dechaux M, Lenoir G, Rey J, M. T 2001 Hereditary hypophosphatemic rickets with hypercalciuria: report of a new kindred. Nephron **88**:83–86.
155. Tieder M, Arie R, Bab I, Maor J, Liberman, UA 1992 A new kindred with hereditary hypophosphatemic rickets with hypercalciuria: implications for correct diagnosis and treatment. Nephron **62**:176–181.
156. Gazit D, Tieder M, Liberman UA, Passi-Even L, IA. B 1991 Osteomalacia in hereditary hypophosphatemic rickets with hypercalciuria: a correlative clinical-histomorphometric study. J Clin Endocrinol Metab **72**:229–235.
157. Tieder M, Modai D, Shaked U, Samuel R, Arie R, Halabe A, Maor J, Weissgarten J, Averbukh Z, Cohen N 1987 "Idiopathic" hypercalciuria and hereditary hypophosphatemic rickets. Two phenotypical expressions of a common genetic defect. N Engl J Med **316**:125–129.
158. Liberman UA 1988 Inborn errors in vitamin D metabolism—Their contribution to the understanding of vitamin D metabolism. In: Norman AW, Schaefer K, Grigoleit H-G, Herrath D (eds.) Vitamin D molecular, cellular, and clinical endocrinology. Walter de Gruyter: Berlin, pp 935–947.
159. Jones A, Tzenova J, Frappier D, Crumley M, Roslin N, Kos C, Tieder M, Langman C, Proesmans W, Carpenter T, Rice A, Anderson D, Morgan K, Fujiwara T, H. T 2001 Hereditary hypophosphatemic rickets with hypercalciuria is not caused by mutations in the Na/Pi cotransporter NPT2 gene. J Am Soc Nephrol **12**:507–514.
160. Chan JCM, Alon U 1985 Tubular disorders of acid-base and phosphate metabolism. Nephron **40**:257–279.
161. Chesney RW 1990 Fanconi syndrome and renal tubular acidosis. In: Favus MJ (ed.) Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism, 1st ed. Am Soc Bone Miner Res, Kelseyville, CA.
162. De Toni G 1933 Remarks on the relations between renal rickets (renal dwarfism) and renal diabetes. Acta Paediatr Scand **16**:479–484.

163. McCune DJ, Mason HH, Clarke HT 1943 Intractable hypophosphatemic rickets with renal glycosuria and acidosis (the Fanconi syndrome). Am J Dis Child **65**:81–146.
164. Bergeron M, Gougoux A, Vinay P 1995 The Renal Fanconi Syndrome. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds.) The metabolic and molecular bases of inherited disease, 7th ed. McGraw-Hill, New York, pp 3691–3704.
165. Chesney RW, Rosen JF, Hamstra AJ, DeLuca HF 1980 Serum 1,25-dihydroxyvitamin D levels in normal children and in vitamin D disorders. Am J Dis Child **134**:135–139.
166. Steinherz R, Chesney RW, Schulman JD, DeLuca HF, Phelps M 1983 Circulating vitamin D metabolites in nehphropathic cystinosis. J Pediatr **102**(592–294).
167. Chesney RW, Kaplan BS, Phelps M, DeLuca HF 1984 Renal tubular acidosis does not alter circulating values of calcitriol. J Pediatr **104**:51–55.
168. Chevalier RL 1983 Hypercalciuria in a child with primary Fanconi syndrome and hearing loss. Int J Pediatr Nephrol **4**:53–57.
169. Lee SW, Russell J, Avioli LV 1977 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol: conversion impaired by systemic metabolic acidosis. Science **195**:994–996.
170. Brewer ED, Tsai HC, Szeto KS, Morris RC 1977 Maleic acid induced impaired conversion of 25(OH)D3 to 1,25(OH)2D3: implications for Fanconi's syndrome. Kidney Int **12**: 244–252.
171. Brenner RJ, Spring DB, Sebastian A, McSherry EM, Genant HK, Palubinskas AJ, Morris RC 1982 Incidence of radiographically evident bone disease, nephrocalcinosis and nephrolithiasis in various types of renal tubular acidosis. N Engl J Med **307**:217–221.
172. Tieder M, Arie R, Modai D, Samuel R, Weissgarten J, Liberman UA 1988 Elevated serum 1,25-dihydroxyvitamin D concentrations in siblings with primary Fanconi's syndrome. N Engl J Med **319**:845–849.
173. Schneider JA, Schulman JD 1983 Cystinosis. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS (eds.) The Metabolic Basis of Inherited Disease, 5th ed. McGraw-Hill: New York, pp 1844–1866.
174. Scheinman SJ, Pook MA, Wooding C, Pang JT, Frymoyer PA, Thakker RV 1997 Mapping the gene causing X-linked recessive nephrolithiasis to Xp11.22 by linkage studies. J Clin Invest **91**:2351–2357.
175. Scheinman SJ 1998 X-linked hypercalciuric nephrolithiasis: clinical syndromes and chloride channel mutations. Kidney Int **53**:3–17.
176. Lyles KW, Burkes EJ, Ellis GJ, Lucas KJ, Dolan EA, Drezner MK 1985 Genetic transmission of tumoral calcinosis: Autosomal dominant with variable clinical expressivity. J Clin Endocrinol Metab **60**:1093–1096.
177. Prince MJ, Schaefer PC, Godsmith RS, Chausmer AB 1982 Hyperphosphatemic tumoral calcinosis: Association with elevation of serum 1,25, dihydroxycholecalciferol concentrations. Ann Inter Med **96**:586–591.
178. Mozaffarian G, Lafferty FW, Pearson OH 1972 Treatment of tumoral calcinosis with phosphorus deprivation. Ann Inter Med **77**:741–745.
179. Gregosiewicz A, Warda E 1989 Tumoral calcinosis: Successful medical treatment. J Bone Joint Surg Am **71A**:1244–1249.
180. Yamaguchi T, Sugimoto T, Imai Y, Fukase M, Fujita T, Chihara K 1995 Successful treatment of hyperphosphatemic tumoral calcinosis with long-term acetazolamide. Bone **16**:247S–250S.
181. Salvi A, Cerudelli B, Cimino A, Zuccato F, Giustina G 1983 Phosphaturic action of calcitonin in pseudotumoral calcinosis. Horm Metab Res **15**:260.
182. Levine BS, Ho LD, Pasiecznik K, Coburn JV 1986 Renal adaptation to phosphorus deprivation. J Bone Miner Res **1**:33–40.
183. Lotz M, Zisman E, Bartter FC 1968 Evidence for a phosphorus-depletion syndrome. N Engl J Med **278**:409–415.
184. Baker LRI, Ackrill P, Cattell WR, Stamp TC, Watson L 1974 Iatrogenic osteomalacia and myopathy due to phosphate depletion. British Medical Journal **3**:150–152.
185. Mostellar ME, Tuttle EP 1964 Effects of alkalosis on plasma concentration and urinary excretion of inorganic phosphate in man. J Clin Invest **43**:138–145.
186. Knochel JP 1977 The pathophysiology and clinical characteristics of severe hypophosphatemia. Arch Int Med **137**: 203–220.
187. Sheldon GF, Grzyb S 1975 Phosphate depletion and repletion: relation to parenteral nutrition and oxygen transport. Ann Surg **182**:683–689.
188. Grossman RA, Hamilton RW, Morse BM, Penn AS, Goldberg M 1974 Nontraumatic rhabdomyolysis and acute renal failure. N Engl J Med **291**:807–811.
189. Koffler A, Fnedler RM, Massry SG 1976 Acute renal failure due to nontraumatic rhabdomyolysis. Ann Inter Med **85**: 23–28.
190. Zusman J, Brown DM, Nesbitt ME 1973 Hyperphosphatemia, hyperphosphaturia, and hypocalcemia in acute Iymphoblastic leukemia. N Engl J Med **289**:1335–1337.
191. Denborough MA, Forster JFA, Hudson MC, Carter NG, Zapf P 1970 Biochemical changes in malignant hyperpyrexia. Lancet **1**:1137–1138.
192. Duhm J 1971 2,3-DPG-induced displacements of the oxyhemoglobin dissociation curve of blood: mechanisms and consequences. Adv Exp Med Biol **37A**:179–186.
193. Travis SF, Sugerman HJ, Ruberg RL, Dudrick SJ, Delivoria-Papadopoulos M, Miller LD, Oski FA 1977 Alterations of red cell glycolytic intermediates and oxygen transport as a consequence of hypophosphatemia in patients receiving intravenous hyperalimentation. N Engl J Med **297**:901–904.
194. Parfitt AM, Kleerekoper M 1980 Clinical disorders of calcium, phosphorus and magnesium metabolism. In: Maxwell MH, Kleeman CR (eds.) Clinical disorders of fluid and electrolyte metabolism. McGraw-Hill: New York, pp 947–1151.
195. O'Connor LR, Wheeler WS, Bethune JE 1977 Effect of hypophosphatemia on myocardial performance in man. N Engl J Med **297**:901–903.
196. Newman JH, Neff TA, Ziporen P 1977 Acute respiratory failure associated with hypophosphatemia. N Engl J Med **296**:1101–1103.
197. Klock JC, Williams HE, Mentzer WK 1974 Hemolytic anemia and somatic cell dysfunction in severe hypophosphatemia. Arch Intern Med **134**:360–364.
198. Glorieux FH, Scriver CR, Reade TM, Goldman H, Roseborough A 1972 Use of phosphate and vitamin D to prevent dwarfism and rickets in X-linked hypophosphatemia. N Engl J Med **287**(10):481–7.
199. Craddock PR, Yawota Y, Van Santen L, Gilberstadt S, Silivis S, Jacob HS 1974 Acquired phagocyte dysfunction: a complication of the hypophosphatemia of parenteral hyperalimentation. N Engl J Med **290**:1403–1407.
200. Herbert LA, Lemann J, Peterson JR, Lennon EJ 1966 Studies of the mechanism by which phosphate infusion lowers serum calcium concentration. J Clin Invest **45**:1886–1891.

201. Sinha TK, Allen DO, Queener SF, Bell NH, Larson S, McClintock R 1977 Effects of acetazolamide on the renal excretion of phosphate in hyperparathyroidism and pseudohypoparathyroidism. J Lab Clin Med **89**: 1188–1197.

202. Econs MJ, Drezner MK 1992 Bone Disease Resulting from Inherited Disorders of Renal Tubule Transport and Vitamin D Metabolism. In: Coe FL, Favus MJ (eds.) Disorders of Bone and Mineral Metabolism. Raven Press, Ltd: New York, pp 935–950.

CHAPTER 70

Disorders of Phosphate Metabolism: Autosomal Dominant Hypophosphatemic Rickets, Tumor Induced Osteomalacia, Fibrous Dysplasia, and the Pathophysiological Relevance of FGF23

MICHAEL J. ECONS Departments of Medicine and Medical and Molecular Genetics
Indiana University School of Medicine, Indianapolis, IN, 46202

I. INTRODUCTION

Disorders of phosphate homeostasis are not only clinically interesting, but recent data concerning these disorders sheds light on maintenance of normal phosphate homeostasis. Chapter 69 summarized recent work in X-linked hypophosphatemic rickets. This chapter will review the disorders autosomal dominant hypophosphatemic rickets (ADHR) and tumor induced osteomalacia (TIO) and the pathogenesis of hypophosphatemia in fibrous dysplasia. The relevance of FGF23 in the pathogenesis of these disorders, as well as XLH, will also be reviewed.

II. ADHR

A. Clinical Features

Autosomal dominant hypophosphatemic rickets (ADHR, MIM#193100) is characterized by isolated renal phosphate wasting and inappropriately normal concentrations of calcitriol. Patients frequently present with bone pain, rickets, and tooth abscesses. Bianchine *et al*. described a small ADHR family [1]. The father, who was markedly affected, had isolated renal phosphate wasting, short stature, and lower extremity deformity from rickets. He had two affected daughters and one affected son. These investigators reported that the father had a marked tendency towards fracture. Otherwise, the clinical course in these individuals appeared to be similar to that of XLH patients. However, the family was too small to fully appreciate the manifestations of the disorder and its incomplete penetrance.

We evaluated a large ADHR kindred with many affected individuals [2]. This kindred provided us with an opportunity to explore the phenotypic variability of this disease in a large number of individuals with the same mutation. There was no evidence of genetic anticipation or imprinting. In contrast to XLH (see Chapter 69), ADHR displays incomplete penetrance and delayed onset of penetrance. The family contains two subgroups of affected individuals. One subgroup consists of patients who presented during childhood with phosphate wasting, rickets, and lower extremity deformity in a pattern similar to the classic presentation of XLH. The second group consists of individuals who presented as adolescents or adults. These individuals complained of bone pain, fatigue, weakness, and insufficiency fractures, but did not have lower extremity deformities [2]. In some cases, patients were wheelchair or bed bound from bone pain and weakness. Their clinical presentations were essentially identical to patients who present

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with tumor induced osteomalacia (TIO, see below), although none of the ADHR patients were ever found to develop tumors. Of note, all of the individuals who presented with delayed onset of penetrance are female. In subsequent observations, all new patients that have presented with delayed onset of ADHR have also been women (unpublished observations). Furthermore, two male patients presented in childhood with renal phosphate wasting and radiographic evidence of rickets and were treated for several years. They were subsequently taken off treatment for several years. On subsequent reevaluation, they were found to be clinically asymptomatic and have normal serum phosphorus and TMP/GFR determinations [2]. In addition to the patients noted above, we found unaffected individuals who are carriers for the ADHR mutation [2]. Thus, the clinical manifestations of ADHR are even more variable than those observed in XLH.

B. Identification of the FGF23 Gene

To identify the gene responsible for ADHR, we performed a genome wide linkage scan in a large ADHR kindred. Analysis of these results demonstrated that the gene was located on chromosome 12p13 [3]. Further fine mapping experiments limited the critical region to approximately 1.5 Mb. Using a variety of exon prediction programs, other informatics based approaches, and RT-PCR, we identified several novel and known genes in the critical region. We found mutations in four ADHR families in a novel gene, subsequently named *FGF23* [4]. Mutation detection studies in the four ADHR kindreds revealed three missense changes affecting two arginines, residing three amino acids apart. Two unrelated families had the same change, R176Q (527G>A). One family had an R179W (535C>T) change and another had an R179Q (536G>A) substitution [4]. The mutations segregated with the disease in each family and were not found in normal individuals. In addition to the ADHR families, we performed mutation detection in index cases from a family with Hypophosphatemic Bone Disease [5], two families with tumoral calcinosis, a family with hypophosphatemia and multiple congenital abnormalities, as well as in 18 hypophosphatemia patients without PHEX mutations. Sequencing of the entire coding region, 880 bp upstream of the initiation codon, 1873 bp downstream of the coding sequence, and both of the predicted polyadenylation sites did not reveal mutations in any of these diseases except the ADHR patients.

FGF23 is a 251 amino acid protein and shares greatest similarity with FGF19 and FGF21. Although FGF receptor mutations are known to cause human diseases, until recently, when FGF14 were found to cause cerebral axatia [6], FGF23 was the only FGF implicated in human disease. The first 24 amino acids are the signal peptide and *in vitro* studies demonstrate that FGF23 is rapidly secreted [4,7].

To determine the mechanism of how missense mutations in FGF23 cause ADHR, we performed transient transfections with the native FGF23 cDNA, as well as cDNAs carrying the three disease causing mutations. Antibodies directed toward the C-terminal portion of FGF23 revealed that the native FGF23 protein resolved as 32 kD and 12 kD species in conditioned media; however, the three mutated proteins were detected only as the 32 kD band. An N-terminal FLAG-tagged native FGF23 resolved as two bands of 36 kD (32 kD+FLAG) and 26 kD when detected with a FLAG antibody, whereas the R176Q mutant resolved primarily as the 36 kD protein species (Fig. 1) [8]. This work has subsequently been confirmed by several investigators [9,10]. Cleavage of FGF23 was not enhanced by extracellular incubation of FGF23 with HEK293 cells, and native and mutant FGF23s bound heparin [8]. Therefore, FGF23 proteins containing the ADHR mutations are secreted and produce polypeptides less sensitive to protease cleavage than wild type FGF23. Thus, the ADHR mutations protect FGF23 from proteolysis, thereby potentially elevating circulating concentrations of intact FGF23 and leading to phosphate wasting in ADHR patients.

FGF23 is expressed at low levels in normal tissues. Originally, northern blots from multiple tissues failed to reveal expression. However, initial RT-PCR from RNA from a variety of tissues indicates that FGF23 is expressed in heart, liver, and thyroid/parathyroid [4], and additional reports indicate that the gene is also expressed in thymus [11]. Recent studies [12] indicate that FGF23 is expressed in bone in bone marrow stromal cells, osteoblasts, and osteocytes. Expression appears to be most prominent in active bone, such as during fracture repair.

III. TUMOR INDUCED OSTEOMALACIA

Tumor induced osteomalacia (TIO), also called *oncogenic hypophosphatemic osteomalacia*, is an uncommon disorder that results from tumor secretion of a substance or substances, often referred to as *phosphatonin*(s) [13]. The clinical picture is somewhat dependent on age of presentation, but patients frequently present with proximal muscle weakness, bone pain, and fatigue. In some cases the weakness can be quite profound as the disease progresses. Children who have open epiphyses will have rickets with resulting

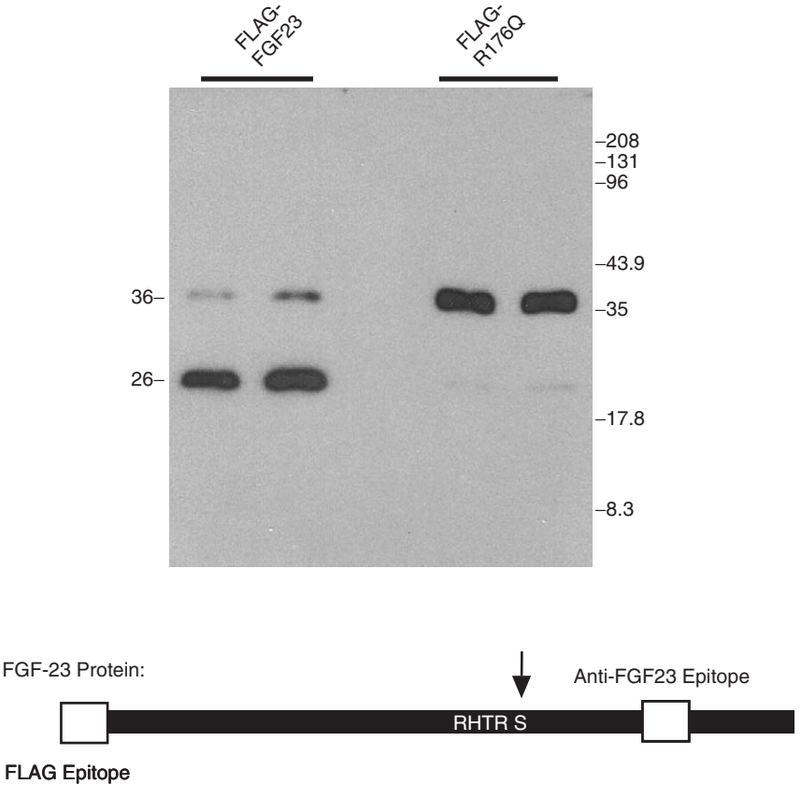

FIGURE 1 Western analysis demonstrating that an N-terminal FLAG-tagged native FGF23 with two bands (intact and cleaved fragment) while the R176Q mutant resolved primarily as the full length 36 kD protein species. A schematic showing the relative positions of the FLAG and FGF-23 antibody epitopes, as well as the cleavage site is shown below the Western analysis. From reference 8 with permission.

lower extremity deformities. Adults often develop fractures and pseudofractures. Of note, the clinical picture in adults is essentially identical to that seen in ADHR patients with adult onset of disease. Unfortunately, delayed diagnosis of TIO is common, and patients may become bedridden due to fractures and muscle weakness by the time the diagnosis is established [14]. Hypophosphatemia results from renal phosphate wasting as evidenced by a reduced TMP/GFR and calcitriol concentration is either inappropriately normal or low. Compared with XLH and ADHR, where calcitriol levels tend to be inappropriately normal, calcitriol levels in TIO are more often below the normal range. A wide variety of tumors have been implicated in causing the disease [15], although the tumors are frequently of mesenchymal origin. Recent studies [16] demonstrate that misclassification of these tumors is common. Upon reevaluation by a pathologist familiar with TIO, 24 of 29 tumors were classified as phosphaturic mesenchymal tumor (mixed connective tissue variant) (PMTMCT) [16]. Tumor removal, if the tumor can be found, results in complete remission of the phosphate wasting, vitamin D abnormalities, and osteomalacia. Unfortunately, even tumors that were originally classified as nonmalignant can reoccur, sometimes many years after successful removal of the original tumor [17]. Although there has been some success in locating these tumors with octreatide scans [18] and other functional imaging modalities, the tumors are frequently small and very difficult to locate. Many tumors are not found, and patients with presumed TIO frequently require treatment with high dose calcitriol and phosphate, using an identical protocol to that used for XLH patients (see Chapter 69).

In light of the clinical similarity between ADHR and tumor induced osteomalacia (TIO), we examined tumor tissue for FGF23 expression. These studies demonstrated that tumors that cause renal phosphate wasting markedly overexpress FGF23 [7]. The level of FGF23 expression in these tumors is several orders of

magnitude higher than in normal tissues. These results have been confirmed by other investigators [19] in subsequent studies. Shimada *et al*. [19] also found that FGF23 was highly expressed in tumors that cause phosphate wasting. They found that injection of FGF23 into mice resulted in renal phosphate wasting. Furthermore, mice implanted with FGF23 overexpressing cells manifested renal phosphate wasting, inappropriately low calcitriol concentrations, and rickets [19]. Essentially, these mice are models of tumor induced osteomalacia. In subsequent studies [20], these investigators made FGF23 transgenic mice. FGF23 transgenic mice manifest renal phosphate wasting with reduced expression of the sodium dependent phosphate cotransporter, Npt2a. Calcitriol concentrations were markedly reduced, and the transgenic mice had osteomalacia [20].

Although current data implicate FGF23 as a "phosphatonin," FGF23 is not the only gene that is overexpressed in these tumors. Jan de Beur *et al*. performed serial analysis of gene expression (SAGE) and found many genes that were differentially expressed between TIO tumors and other tumors, but matrix extracellular phosphoglycoprotein (MEPE), frizzled related protein 4 (FRP4), and FGF23 were prominently overexpressed in tumors that caused phosphate wasting [21]. Follow-up studies by several investigators are in progress, however, some data is currently available. MEPE is highly expressed in TIO tumors. However, the MEPE knockout mouse does not have a defect in phosphate homeostasis, but does have increased bone formation and increased mineralization, indicating that MEPE likely has a role in these processes [22]. Of note, implantation of MEPE overexpressing cells into nude mice did not result in defects in phosphate or vitamin D homeostasis [19]. However, MEPE is overexpressed in bone from the Hyp mouse compared to controls, indicating that it may have a role in defective bone mineralization in XLH [11]. Recently, Berndt *et al*. examined FRP4 as a candidate "phosphatonin"[23]. They reported that FRP4 inhibits phosphate transport in opossum kidney cells. Infusing FRP4 into rats results in phosphaturia without altering calcitriol concentrations. However, this group found that serum FRP4 concentrations in a TIO patient were normal. Thus, additional studies are necessary to fully elucidate the role of FRP4 in the pathogenesis of TIO.

IV. FIBROUS DYSPLASIA

Fibrous dysplasia can be a component of McCune-Albright syndrome (characterized by fibrous dysplasia, hyperpigmented café-au-lait skin lesions, and hyperfunctioning endocrinopathies), or it can occur alone [24]. Weinstein and coworkers have shown that fibrous dysplasia results from activating somatic mutations of GNAS, which codes for the alpha subunit of the stimulatory G protein, Gs [25]. These lesions result from excessive proliferation and abnormal differentiation of mesenchymal osteoprogenitor cells. Fibrous dysplasia, particularly when associated with the McCunne Albright syndrome, can be accompanied by renal phosphate wasting and inappropriately normal calcitriol concentrations. Studies by Collins *et al*. [26] demonstrated that phosphate wasting occurred in approximately 50% of their patient population. Analysis of patients with hypophosphatemia due to fibrous dysplasia demonstrated that FGF23 concentrations were markedly increased compared to patients with fibrous dysplasia without phosphate wasting and normal individuals [12]. Moreover, FGF23 concentrations correlated with disease burden. Thus, patients with extensive disease had higher concentrations of FGF23 and more phosphate wasting than patients with limited disease. In accord with this clinical finding, in situ hybridization data demonstrated that FGF23 is expressed in the lesions in both the fibrous component of the lesion (mostly made up of immature osteogenic cells) and in the bone cells associated with abnormal bone trabeculae [12]. To determine if FGF23 expression was limited to abnormal osteogenic cells Riminucci *et al*. performed in situ hybridization in normal bone [12]. FGF23 was weakly expressed in inactive osetoblasts, and osteocytes, but there was more robust expression in osteoprogenetor cells, osteoblasts, and osteocytes in a fracture callous. In light of the fact that mutant and nonmutant cells both made FGF23, we compared FGF23 expression in cell cultures of both mutant and normal bone marrow stromal cells. Under the conditions examined, mutant stromal cells in culture did not produce more FGF23 than normal cells under the same conditions [12]. In light of this observation, the reader may question why there is a correlation between FGF23 concentrations and disease burden in these patients. Perhaps the most tenable hypothesis is that normal bone cells are appropriately regulated by whatever homeostatic mechanisms regulate FGF23 concentrations. Cells in fibrous dysplasia lesions may not respond appropriately to signals to decrease production of FGF23. When disease burden rises to a critical level, production of FGF23 exceeds what is needed for normal phosphate and vitamin D homeostasis and phosphate wasting, inhibition of calcitriol production, and enhanced calcitriol degradation ensues. This hypothesis is particularly enticing when one considers preliminary data that suggests a role for FGF23 in normal phosphate and vitamin D homeostasis (see below).

V. THE ROLE OF FGF23 IN XLH

XLH is extensively covered in the previous chapter. Therefore, this chapter will briefly focus on the potential role of FGF23 in the pathogenesis of XLH. The potential roles of MEPE and FRP4 in the pathogenesis of XLH are also covered in Chapter 69 and will not be discussed further. As noted in the previous chapter, XLH is an X linked dominant disorder that results from inactivating mutations in the PHEX gene, which codes for a member of the M13 endopeptidase family [27]. Over 170 different mutations have been described that give rise to the disease (www.phexdb.mcgill.ca). XLH is characterized biochemically by renal phosphate wasting and inappropriately normal calcitriol concentrations. Parabiosis experiments [28] and renal cross-transplantation experiments [29] in the *Hyp* mouse implicate a circulating factor in the pathogenesis of the disease. In light of these findings, several investigators have measured FGF23 concentrations in XLH patients using assays that detect the C terminal portion of the molecule [30,31] or an assay with antibodies to both N and C termini [32]. Results indicate that most, but not all, XLH patients have increased FGF23 concentrations, compared to normal individuals. In those XLH patients with normal FGF23 levels, this concentration may be inappropriately normal given the low serum phosphorus concentrations. Of note, *Hyp* mice have an approximately tenfold increase in FGF23 concentrations compared to littermates [33]. Aono *et al*. studied the affects of anti FGF23 neutralizing antibodies in *Hyp* mice [33]. A single injection of neutralizing antibody caused dose dependent elevations of serum phosphate and calcitriol concentrations. Furthermore, a four-week trial of FGF23 neutralizing antibody led to a dose dependent improvement in growth and bone histomorphometric parameters, indicating that FGF23 may be at the center of the pathogenesis of XLH [33].

One issue that has not been adequately explained is why inactivating mutations in PHEX result in a dominant inheritance pattern. Most disorders that result from inactivating mutations in a gene that codes for an enzyme have a recessive inheritance pattern. Indeed, if FGF23 was a PHEX substrate one would expect XLH to be a recessive disorder and the parabiosis experiments performed by Meyer *et al*. [28] to result in rescue of the *Hyp* phenotype, rather than the normal mouse manifesting renal phosphate wasting, which is what was observed. Current data indicate that intact FGF23 is not a PHEX substrate [11,34]. Furthermore, recent data by Liu *et at*. [11] indicate that *Hyp* mice have markedly increased production of FGF23 mRNA in bone (MEPE is also overexpressed in Hyp mouse bone). Since PHEX and FGF23 are expressed by the same cells, the absence of functional PHEX protein in the cell likely leads to increased FGF23 production. These data provide a potential mechanism for why XLH is an X linked dominant disorder. X chromosome inactivation is random in female patients with XLH [35] and each cell in an affected female either has a normal or mutant copy of the PHEX gene. Thus, the possibility exists that those cells that have the mutant PHEX could secrete increased amounts of FGF23, resulting in increased circulating FGF23 concentrations. Clearly, this hypothesis must be adequately tested, but additional data may shed light on a question that has puzzled researchers since the PHEX gene was originally described.

VI. FGF23 IN HEALTH AND ITS POTENTIAL ROLE IN MAINTENANCE OF NORMAL PHOSPHATE AND VITAMIN D HOMEOSTASIS

Currently, there is inadequate data to definitively determine what role, if any, FGF23 plays in maintenance of normal serum phosphate and calcitriol concentrations. FGF23 concentrations do not vary with age, making it unlikely that FGF23 is the primary mechanism by which children maintain a higher serum phosphate concentration than adults [30] (and Econs unpublished results). However, this likely means that the set point for FGF23 is at a higher level of phosphate in children than adults. The first, and most striking, evidence that FGF23 may play a role in vitamin D and phosphate homeostasis is the phenotype of the FGF23 knock-out mouse [36]. Heterozygous FGF23 knock-out mice are reported to be indistinguishable from normal. The homozygous knock-out mice appear normal at birth, but manifest increased serum phosphorus and calcitriol concentration compared to controls by the tenth day of life. Additionally, these mice display growth retardation and shortened life span with none of the knock-outs surviving past 12 weeks [36]. No data is currently available to see if these mice could be rescued by a low phosphate diet. Further data supporting the potential role of FGF23 in normal phosphate homeostasis is provided by Yamashita *et al*. [37], who measured FGF23 concentrations in rats fed different amounts of dietary phosphate. As would be predicted if FGF23 played a role in phosphate homeostasis, rats fed a high phosphate diet had higher FGF23 concentrations than those fed normal diets. Moreover, rats fed low phosphate diets displayed suppressed FGF23 concentrations [37]. These studies are supported by a study by Ferrari and colleagues [38] who gave young

men normal, low, and high phosphate diets. Dietary phosphate restriction with addition of a phosphate binder decreased FGF23 concentrations by 29 ± 6%, and high phosphate diets increased FGF23 concentrations by 31 ± 9%. While the above data support the notion that FGF23 plays a role in normal phosphate homeostasis, there are no data regarding the rapidity of the response of FGF23 to changes in serum phosphate levels or dietary manipulations. Therefore, substantial work is needed to further investigate the role of FGF23 in normal phosphate homeostasis.

VII. SUMMARY

Over the past few years there has been tremendous progress in our understanding of a variety of phosphate wasting disorders. The field continues to evolve at a very rapid rate. The exact roles of FGF23 and other candidate "phosphatonins" are not completely elucidated. However, a few points should be emphasized: 1) XLH, ADHR, TIO, and fibrous dysplasia all manifest renal phosphate wasting and inappropriately normal or low calcitriol concentrations; 2) missense mutations that protect FGF23 from proteolytic degradation cause ADHR; 3) FGF23, MEPE, and FRP4 are overexpressed in tumors that cause TIO; 4) transgenic mice that overexpress FGF23 display phosphate wasting and abnormalities in vitamin D metabolism; 5) injection of FGF23 into mice results in phosphate wasting and abnormalities of vitamin D metabolism; 6) implantation of CHO cells that express FGF23 into nude mice results in a phenotype that replicates that seen in TIO; 7) the FGF23 knock-out mouse manifests hyperphosphatemia and increased calcitriol concentrations; 8) most XLH patients have increased concentrations of FGF23 and normal levels in the remaining patients may be inappropriately normal; and 9) injection of neutralizing antibody against FGF23 into *Hyp* mice results in correction of the biochemical defect in a dose dependent fashion. Thus, although much remains to be done, work over the past few years has identified new genes that play roles in the pathophysiology of a variety of disorders of phosphate and vitamin D metabolism. The exact role of these genes and their role in normal phosphate and vitamin D homeostasis will require additional study.

Acknowledgements

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References

1. Bianchine JW, Stambler AA, Harrison HE 1971 Familial hypophosphatemic rickets showing autosomal dominant inheritance. Birth Defects: Original Article Series **7**:287–295.
2. Econs MJ, McEnery PT 1997 Autosomal dominant hypophosphatemic rickets/osteomalacia: clinical characterization of a novel renal phosphate wasting disorder. J Clin Endocrinol Metab **82**:674–681.
3. Econs MJ, McEnery PT, Lennon F, Speer MC 1997 Autosomal dominant hypophosphatemic rickets is linked to chromosome 12p13. J Clin Invest **100**:2653–2657.
4. The ADHR Consortium 2000 Autosomal dominant hypophosphatemic rickets is associated with mutations in *FGF23*. Nature Genetics **26**:345–348.
5. Scriver CR, MacDonald W, Reade T, Glorieux RH, Nogrady B 1977 Hypophosphatemic nonrachitic bone disease: an entity distinct from X-linked hypophosphatemia in the renal defect, bone involvement, and inheritance. American Journal of Medical Genetics **1**:101–117.
6. Van Swieten JC, Brusse E, De Graaf BM, *et al*. 2003 A mutation in the fibroblast growth factor 14 gene is associated with autosomal dominant cerebral ataxia. American Journal of Human Genetics **72**:191–199.
7. White KE, Jonsson KB, Carn G, *et al*. 2001 The autosomal dominant hypophosphatemic rickets (ADHR) gene is a secreted polypeptide overexpressed by tumors that cause phosphate wasting. J Clin Endocrinol Metab **86**(2):497–500.
8. White KE, Carn G, Lorenz-Depiereux B, Benet-Pages A, Strom TM, Econs MJ 2001 Autosomal dominant hypophosphatemic rickets (ADHR) mutations stabilize FGF-23. Kidney International **60**:2079–2086.
9. Bai XY, Miao D, Goltzman D, Karaplis AC 2003 The autosomal dominant hypophosphatemic rickets R176Q mutation in fibroblast growth factor 23 resists proteolytic cleavage and enhances *in vivo* biological potency. J Bio Chem **278**:9843–9849.
10. Shimada T, Muto T, Urakawa I, *et al*. 2002 Mutant FGF-23, responsible for autosomal dominant hypophosphatemic rickets, is resistant to proteolytic cleavage and causes hypophosphatemia *in vivo*. Endocrinology **143**:3179–3182.
11. Liu SGR, Simpson JG, Ziao ZS, Burnham CE, Quarles LD 2003 Regulation of fibroblastic growth factor 23 expression but not degradation by PHEX. J Bio Chem **278**:37419–37426.
12. Riminucci M, Collins MT, Fedarko NS, *et al*. 2003 FGF-23 in fibrous dysplasia of bone and its relationship to renal phosphate wasting. J Clin Invest **112**:683–692.
13. Econs MJ, Drezner MK 1994 Tumor-induced osteomalacia—unveiling a new hormone. N Engl J Med **330**:1679–1681.
14. Ryan EA, Reiss E 1984 Oncogenous osteomalacia: Review of the world literature of 42 cases and report of two new cases. American Journal of Medicine **77**:501–512.
15. Drezner MK 1996) Tumor-induced rickets and osteomalacia. In: Favus MJ (ed) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, 3 ed. Lippincott-Raven, Philadelphia, pp. 319–325.
16. Folpe A, Fanburg-Smith JC., Billings SD, Bisceglia M, Bertoni F, Cho JY, Econs MJ, Inwards CY, JandeBeur SM, Mentzel T, Montgomery E, Michael M, Miettinen M, Reith JD, O'Connell JX, Rosenberg AE, Rubin BP, Sweet DE, Vinh TN, World LE, Wehrli BM, White KE, Zaino RJ, Weiss SW 2003 Most osteomalacia-associated mesencyhmal tumors are a single histopathologic entity: an analysis of 32 cases and a comprehensive review of the literature. Am J Surg Path. In Press.

17. Cai Q, Hodgson SF, Kao PC, *et al*. 1994 Brief report: Inhibition of renal phosphate transport by a tumor product in a patient with oncogenic osteomalacia. N Engl J Med **330**:1645–1649.
18. Seufert J, Ebert K, Muller J, *et al*. 2001 Octreotide therapy for tumor-induced osteomalacia. N Engl J Med **345**:1883–1888.
19. Shimada T, Mizutani S, Muto T, *et al*. 2001 Cloning and characterization of FGF23 as a causative factor of tumor-induced osteomalacia. Proceedings of the National Academy of Sciences 98:6494–6499.
20. Shimada T, Yoneya T, Hino R, Takeuchi Y, Fukumoto S, Yamashita T 2001 Transgenic mice expressing fibroblast growth factor 23 (FGF23) demonstrate hypophosphatemia with low serum 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] and rickets/osteomalacia. Journal of Bone and Mineral Research **16** Suppl.1:S151.
21. Jan De Beur SM, Finnegan RB, Vassiliadis J, *et al*. 2002 Tumors associated with oncogenic osteomalacia express genes important in bone and mineral metabolism. Journal of Bone & Mineral Research **17**:1102–1110.
22. Gowen LC, Petersen DN, Mansolf AL, *et al*. 2003 targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass. Journal of Biological Chemistry **278**:1998–2007.
23. Berndt T, Craig T, Bowe A, *et al*. 2003 Secreted frizzled related protein 4 is a potent tumor-derived phosphaturic agent. J Clin Invest **112**:642–646.
24. Weinstein LS 2000 Fibrous dysplasia and the McCune-Albright syndrome. Humana Press: Totowa NJ.
25. Weinstein LS, Shenker A, Gejman PV, Merino MJ, Friedman E, Spiegel AM 1991 Activating mutations of the stimulatory G protein in the McCune-Albright syndrome. [Comment]. N Eng J Med **325**:1688–1695.
26. Collins MT, Chebli C, Jones J, *et al*. 2001 Renal phosphate wasting in fibrous dysplasia of bone is part of a generalized renal tubular dysfunction similar to that seen in tumor-induced osteomalacia. J Bone Miner Res **16**:806–813.
27. The Hyp Consortium 1995 A gene (PEX) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. Nature Genetics **11**:130–136.
28. Meyer RA, Jr., Meyer MH, Gray RW 1989 Parabiosis suggests a humoral factor is involved in X-linked hypophosphatemia in mice. J Bone Miner Res **4**:493–500.
29. Nesbitt T, Coffman TM, Griffiths R, Drezner MK 1992 Crosstransplantation of kidneys in normal and Hyp mice. Evidence that the Hyp mouse phenotype is unrelated to an intrinsic renal defect. Journal of Clinical Investigation **89**:1453–1459.
30. Jonsson KBZR, Larsson T, White KE, Toshitsugu S, Imanishi Y, Tamamoto T, Hampson G, Miyauchi A, Econs MJ, Lavigne J, Juppner H 2003 Fibroblast growth factor 23 in oncogenic osteomalacia and X-linked hypophosphatemia. New England Journal of Medicine **348**(17):1656–1663.
31. Weber T, Liu S, Indridason OS, Quarles LD 2003 Serum FGF23 levels in normal and disordered phosphorous homeostasis. J Bone Miner Res **18**:1227–1234.
32. Yamazaki Y, Okazaki R, Shibata M, *et al*. 2002 Increased circulatory level of biologically active full-length FGF-23 in patients with hypophosphatemic rickets/osteomalacia. J Clin Endocrinol Metab **87**:4957–4960.
33. Aono Y, Shimada T, Yamazaki Y, Hino R, Takeuchi Y, Fujita T, Fukumoto S, Nagano N, Wada M, Yamashita T 2003 The neutralization of FGF-23 ameliorates hypophosphatemia and rickets in *Hyp* mice. American Society for Bone and Mineral Research: Minneapolis, MN, p. S16.
34. Campos MCC, Hirata IY, Juliano MA, Loisel TP, Crine P, Juliano L, Boileau G, Carmona CK 2003 Human recombinant endopeptidase PHEX has a strict S1, specificity for acidic residues and cleaves peptides derived from fibroblast growth factor-23 and matrix extracellular phosphoglycoprotein. Biochem J. **373**:271–279.
35. Orstavik KH, Orstavik RE, Halse J, Knudtzon J 1996 X chromosome inactivation pattern in female carriers of X linked hypophosphataemic rickets. J Medical Genetics **33**:700–703.
36. Shimada TKM, Hasegawa H, Yamazaki T, Ohguma A, Takeuchi Y, Fujita T, Fukumoto S, Tomzuka K, Yamashita T 2002 Targeted ablation of FGF-23 causes hyperphosphatemia, increased 1,25-dihydroxyvitamin D level and severe growth retardation. J Bone and Miner Res: S168.
37. Yamashita T, Hasegawa H, Yamazaki Y, Kawata T, Urakawa I, Shimada T, Takeuchi Y, Fujita T, Fukumoto S, Nagano N 2002 Involvement of FGF-23 in abnormal vitamin D and mineral metabolism associated with renal insufficiency. Journal of American Society of Nephrology **13**:577A.
38. Ferrari S, Bonjour J, Rizzoli R 2003 Evidence for a physiological role of FGF-23 in the regulation of renal phosphate reabsorption and plasma calcitriol in healthy humans. American Society of Bone and Mineral Research Annual Meeting: Minneapolis, MN, p. S24.

CHAPTER 71

Vitamin D Pseudodeficiency

FRANCIS H. GLORIEUX Genetics Unit, Shriners Hospital for Children, and Departments of Surgery, Pediatrics, and Human Genetics, McGill University, Montréal, Québec, Canada

RENÉ ST-ARNAUD Genetics Unit, Shriners Hospital for Children, and Departments of Surgery and Human Genetics, McGill University, Montréal, Québec, Canada

I. INTRODUCTION

Following the description by Albright *et al.* in 1937 [1] of "rickets resistant to vitamin D," a number of observations were published [2,3] that indicated that there was a variant of resistant rickets which differed from the classic hypophosphatemic type (X-linked hypophosphatemic rickets, or XLH; see Chapter 69) by its clinical and biological symptoms and response to therapy. It was indeed shown by Prader *et al.* [4] that this form of rickets had an early onset (within the first year of life), contrary to the XLH type. The disease symptoms also included the development of profound hypocalcemia, tooth enamel hypoplasia, and a response to daily administration of large amounts of vitamin D. In view of the latter, the term "vitamin D dependency" was proposed to describe the new syndrome [5]. In 1973, when calcitriol [1,25$(OH)_2D_3$] became available as a therapeutic agent, it was demonstrated that this rare form of rickets responded to physiological amounts of calcitriol [6]. It was then recognized that this disease was due to an inborn error of metabolism involving the defective conversion of calcidiol (25OHD_3) to calcitriol (Fig. 1). For this reason, we feel it more appropriate to return to the original terminology of Prader and use the term pseudovitamin D-deficiency rickets (PDDR) to describe this form of rickets.

In 1978, another inborn error of vitamin D metabolism was recognized in which a clinical picture of pseudovitamin D–deficiency developed despite high circulating concentrations of endogenously produced calcitriol [7]. In some pedigrees, the phenotype is compounded by the presence of complete alopecia [8]. This second variety of pseudodeficiency has been termed vitamin D–dependency type II, pseudovitamin D–deficiency type II, calcitriol-resistant rickets, hypocalcemic vitamin D–resistant rickets, and hereditary 1,25-dihydroxyvitamin D–resistant rickets (HVDRR). The latter term is favored by Malloy, Pike, and Feldman, who discuss it in detail in Chapter 72. HVDRR is caused by a spectrum of mutations affecting the vitamin D receptor (VDR) in target tissues causing true resistance to calcitriol action. The human VDR, a 50-kDa protein, belongs to the steroid-thyroid-retinoic acid receptor superfamily of genes [9]. It comprises at least two functional domains, a ligand binding domain and a DNA binding domain (Fig. 1). Mutations affecting both have been found in HVDRR families (see Chapter 72).

II. CLINICAL MANIFESTATIONS

The clinical course of PDDR is similar to that of nutritional rickets due to simple vitamin D deficiency. The patients are healthy at birth. The first symptoms usually appear within the first year of life. Hypotonia, muscle weakness (proximal myopathy), and growth retardation are common manifestations. Motor problems translate into regression in head control and the ability to stand. In some patients, the initial event is convulsions or tetany. Pathological fractures may occur. A history of adequate mineral and vitamin D intake, without evidence of intestinal malabsorption, is a constant finding. Infant death by hypocalcemia or pulmonary infections was not infrequent in the past when the diagnosis was either missed (confused with a neurological or respiratory condition) or made too late.

Physical examination reveals a small, hypotonic child with features similar to those found in patients with vitamin D–deficiency rickets. There is a wide anterior fontanel with frontal bossing and frequent

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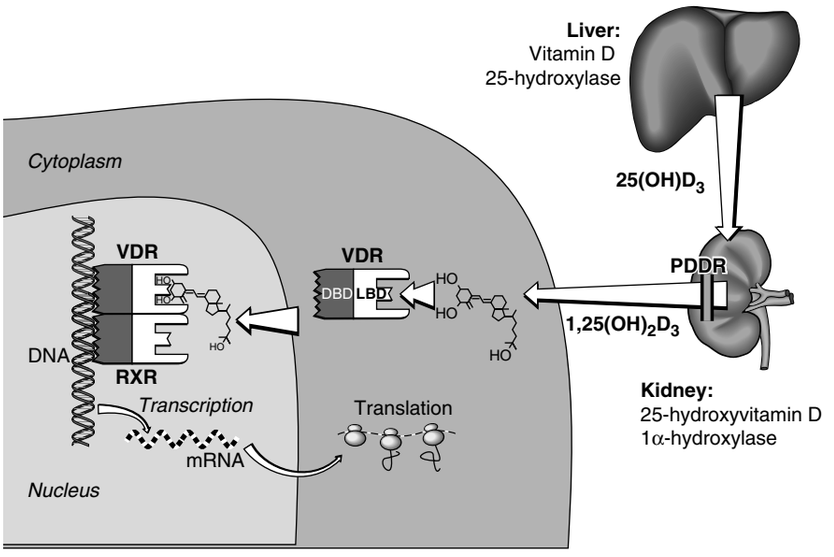

FIGURE 1 Schematic representation of the main steps of the vitamin D biosynthetic pathway, where genetic aberrations may lead to rickets and osteomalacia. The renal defect in PDDR is indicated by the break in the $1,25(OH)_2D_3$ arrow arising in the kidney. The mutation leads to insufficient synthesis of $1,25(OH)_2D_3$. The left part of the figure represents a target cell where schematic coupling of the ligand to its receptor (VDR) takes place in the cytosol or, more likely, in the nucleus. The VDR then heterodimerizes with the RXR receptor. For ease of presentation, the RXR ligand (9-cis retinoic acid) is not depicted. The complex then binds to DNA to regulate transcription. Various mutations affecting either one of the two VDR domains cause hereditary vitamin D–resistant rickets (HVDRR) (see Chapter 72).

craniotabes (easy depression of the softened parieto-occipital area). Tooth eruption is delayed, and erupted teeth show evidence of enamel hypoplasia. A rachitic rosary is either visible or palpable. In the appendicular skeleton, enlargement of the metaphyseal areas is more evident in the wrists and ankles, and there is a variable degree of deformity (bowing) of long bone diaphyses. The Chvostek sign (twitching of the upper lip on light finger tapping of the facial nerve) reflects nerve irritability, a consequence of a rapid drop in serum calcium.

Radiological examination of the skeleton reveals diffuse osteopenia and the classic metaphyseal changes of vitamin D deficiency. There is fraying, cupping, widening, and fuzziness of the zone of provisional calcification immediately under the growth plate. These changes are seen better and detected earlier in the most active growth plates, namely, the distal ulna and femur and the proximal and distal tibia. Changes in the diaphyses may not be evident when metaphyseal changes are first detected. However, they will appear a few weeks later as rarefaction, coarse trabeculation, cortical thinning, and subperiosteal erosion (see Chapter 60). The latter reflects the increased resorption induced by secondary hyperparathyroidism.

III. BIOCHEMICAL FINDINGS

Hypocalcemia is the cardinal feature in PDDR. Serum calcium concentration will drop below 2 mmol/liter (8 mg/dl). This, particularly if the decrease is rapid, may give rise to tetany and convulsions, which may occur prior to any radiological evidence of rickets. Persistent hypocalcemia triggers secondary hyperparathyroidism and hyperaminoaciduria [10]. Urinary calcium content is low, whereas fecal calcium is high, reflecting impaired intestinal calcium absorption. Increased urinary cAMP is not a consistent finding, and normal values have been observed in PDDR patients with high circulating parathyroid hormone (PTH) levels [11].

Serum phosphate concentration may be normal or low. Hypophosphatemia, when present, is usually of a lesser degree than in XLH. It is the result of both impairment of intestinal absorption and increased urinary loss induced by secondary hyperparathyroidism. Serum alkaline phosphatase activity is consistently elevated (over 300 IU/liter). Its increase often precedes the appearance of clinical symptoms. The calcemic response to PTH is usually but not necessarily absent [12].

Studies of circulating vitamin D metabolites have provided a key insight into the pathogenesis of PDDR.

Serum levels of 25OHD are normal in untreated patients and elevated in patients receiving large daily amounts of vitamin D [11]. These results indicate that intestinal absorption of vitamin D and its hydroxylation in the liver are not impaired in PDDR. Circulating levels of $1,25(OH)_2D$ are low in untreated patients [11–13]. This is evident immediately after birth, months before any clinical evidence of rickets develops. Even when patients are treated with large doses of vitamin D, causing major increases in the circulating levels of calcidiol, calcitriol levels do not reach the normal range (Fig. 2). This clearly identifies defective activity of the 25OHD 1α-hydroxylase enzyme as the basic abnormality in PDDR and differentiates it from HVDRR (see Chapter 72). Although $1,25(OH)_2D$ serum levels are low, they are not undetectable. This finding, coupled with the observation that serum levels of $1,25(OH)_2D$ are positively correlated to the serum concentrations of 25OHD in PDDR patients (either untreated or treated with large amounts of vitamin D) suggests that the renal 1α-hydroxylase is not totally absent in PDDR. Thus, the mutation probably affects the structural integrity of the enzyme, resulting in a modification of its kinetics [11]. Balsan and associates [14] have reported on normal calcitriol levels in untreated PDDR patients. Such values, however, should be considered as inappropriate in the face of rickets, hypocalcemia, and secondary hyperparathyroidism. These differences may also reflect genetic heterogeneity among PDDR pedigrees that will only be resolved at the molecular level.

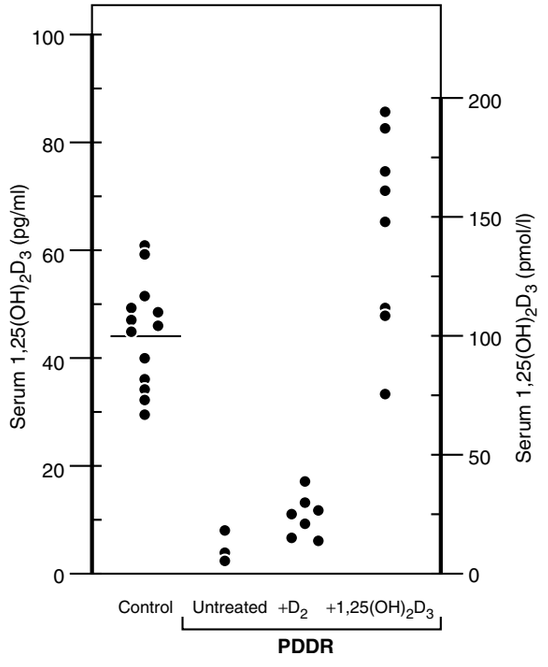

FIGURE 2 Serum calcitriol concentrations in control children and in PDDR patients either untreated or treated with high doses of vitamin D ($+D_2$) or calcitriol [$1,25(OH)_2D_3$]. The data scatter in the latter group reflects both dosage and the variable length of time between drug administration and blood sampling.

Circulating levels of $24,25(OH)_2D$ are normal in PDDR patients and are highly correlated with those of 25OHD, indicating a fully functional 24-hydroxylase enzyme [15,16]. These findings, as well as the observation that modulation of the expression of the 25OHD 24-hydroxylase is regulated independently from that of the 1α-hydroxylase [17], strongly suggest that the two renal hydroxylases are distinct gene products (see Chapters 5 and 6).

IV. PLACENTA STUDIES

In 1979, Weisman *et al.* [18] demonstrated that, besides the mammalian kidney, human placenta decidua was a major site of $1,25(OH)_2D$ synthesis. This was further substantiated by Delvin *et al.* [19], who also demonstrated that the involved enzyme was regulated by feedback mechanisms [20]. Over the years, in the cohort of our patients successfully treated with replacement therapy (see below), several have reached adulthood and became pregnant. At delivery, decidual cells were harvested from the placentas of these PDDR patients and were studied to evaluate their ability to hydroxylate 25OHD at the lα position. As shown in Fig. 3, we demonstrated that decidual cells from women with PDDR lack that function, making them likely targets for the mutation [21]. The physiological importance of this defect particularly with regard to fetal development is unclear. Replacement therapy with calcitriol was maintained throughout pregnancy, and patients had uneventful pregnancies and delivered healthy normocalcemic babies. The placenta thus represents a unique, albeit rare, source of mutant cells for further characterization of the PDDR mutation.

V. GENETIC STUDIES

Pseudovitamin D–deficiency rickets is inherited as a simple autosomal recessive trait [5]. No phenotypic abnormalities have been observed in presumed obligate heterozygotes [10]. Although quite rare, PDDR is present with unusual frequency in a subset of the French-Canadian population [22]. With the cooperation of the several large families under our care, we set out to map the PDDR locus by using DNA markers and linkage analysis to approach the primary defect in PDDR.

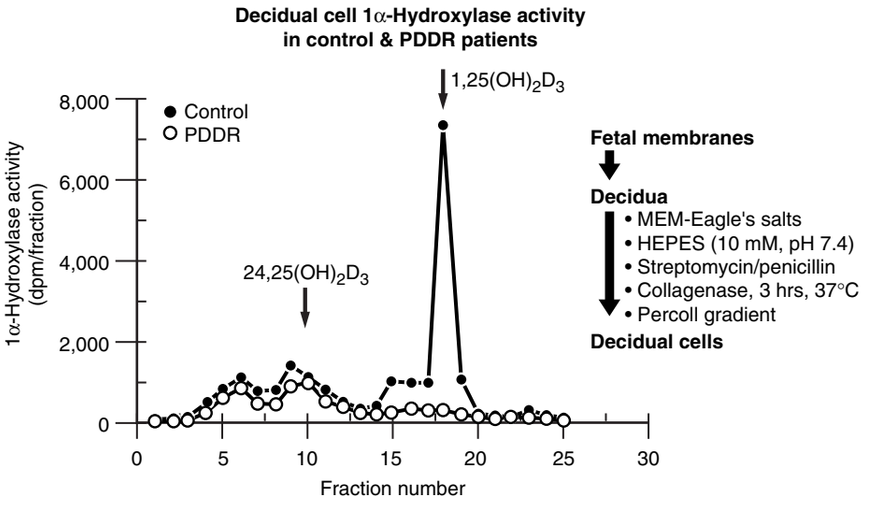

FIGURE 3 High-performance liquid chromatography elution patterns of the radioactivity extracted from medium and cells isolated from the decidua of one control and one PDDR patient. The elution positions of the two vitamin D dihydroxylated metabolites are indicated.

The studied kindreds included 17 affected individuals and 59 healthy relatives, of whom 17 were obligate heterozygotes (having affected progeny). It was found that the mutated gene was linked to polymorphic RFLP (restriction fragment length polymorphism) markers in the region of band 14 of the long arm of chromosome 12 [12ql4] [23]. Multipoint linkage analysis and studies of haplotypes (groups of tightly linked markers segregating together over the generations) and recombinants strongly suggest the localization of the PDDR locus between COL2A1 (coding for the α1 chain of type II collagen) and a cluster of three anonymous probes [D12S14, D12S17, and D12S6], which segregate as a three-marker haplotype. Linkage disequilibrium (i.e., combinations of closely linked genes occurring more often than expected with random distribution) has been observed between the PDDR locus and the three-marker haplotype in the group of kindreds studied [24]. The finding supports the notion of a founder effect that had taken place in the second half of the seventeenth century (about 12 generations ago). This is consistent with the present-day prevalence of 1 in 2400 births and carrier rate of 1 in 26 individuals in Northeastern Quebec [25].

The VDR gene has also been assigned to chromosome 12 by Southern blot analysis of a panel of human-Chinese hamster cell hybrid DNAs. Using *in situ* hybridization, the VDR was found to map to the same 12ql2-14 region where PDDR was localized [26]. Because the VDR cDNA exhibits an *Apa1* dimorphism [27], it was used as a RFLP marker in linkage analyses of samples from 21 of our PDDR families. The PDDR and VDR loci are located in close proximity to the markers COL2A1, ELA (elastase), and D12S15. It is likely that the genetic distance between the two genes involved in the control of vitamin D activity is in the range of a few centimorgans, which, in physical terms, may correspond to 1–10 megabases. We find, at present, no specific reason for this proximity, but its functional significance may be established in the future [26].

VI. MOLECULAR DEFECT

Remarkable progress was recently made in the understanding of the molecular etiology of PDDR through the cloning of the cDNA encoding the 1α-hydroxylase enzyme, from rat [28,29] and mouse [30] kidney, and human keratinocytes and kidney [31]. The human gene was also cloned, sequenced, and mapped to chromosome 12q13.1–13.3 by fluorescence *in situ* hybridization [30,32,33], consistent with the earlier mapping of the disease by linkage analysis. The definite proof that mutations in the 1α-hydroxylase gene are responsible for the PDDR phenotype comes from the identification of such mutations in PDDR patients and obligate carriers. The first report was by Fu *et al.* [34] in 1997; since then additional mutations in various ethnic groups have been published [32,35]. To date, 31 different mutations have been observed in PDDR patients and their parents [31–33,35–39]. They are dispersed throughout the 1α-hydroxylase sequence, affecting all exons and two intervening sequences (Fig 4). All patients have mutations on both alleles, but some

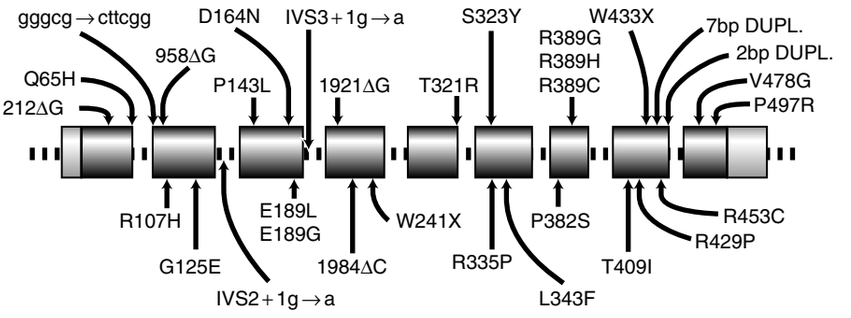

FIGURE 4 Mutations detected in PDDR patients and obligate heterozygotes. A schematic representation of the 1α-hydroxylase gene is shown. The dark-shaded boxes correspond to the nine exons of the gene, and the light-shaded boxes at either ends represent the 5′- and 3′-nontranslated regions. Mutations are presented above and below the gene map. Numbers refer to amino acid residues. Δ: deletion; gggcg → cttcg: deletion of gggcg and substitution of cttcg beginning at nucleotide 897 in exon 2; IVS2 or IVS3 + 1g → a: splice site mutation in intron (intervening sequence) 2 or 3; 7 or 2bp.DUPL.: 7 or 2 basepairs duplication.

harbor a compound heterozygosity (a different mutation on each allele). The mutations detected at the highest frequency are 958ΔG, common among French Canadian patients because of a founder effect [24,33,37], and a mutation in exon 8 that causes a 7 basepairs duplication. The latter alters the downstream reading frame [33,37,38].

An important aspect of the identification of mutations in the 1α-hydroxylase gene is to correlate genotype and phenotype, i.e. the severity of the disease and the circulating levels of $1,25(OH)_2D$. In most cases, although levels are low, they are detectable, suggesting some degree of residual 1α-hydroxylase activity. This could result from the mutations affecting the structural integrity of the enzyme and hence its kinetics. Such an effect could derive from missense mutations, but not from frameshift (deletions, inversions, and duplications) or nonsense mutations that would result in complete inactivity of the mutant 1α-hydroxylase.

An animal model of PDDR was engineered independently by two laboratories using targeted inactivation of the gene of interest in mice [39,40]. The engineered mutation is transmitted with the expected mendelian ratio. A detailed description of the model is presented in Chapter 7.

VII. TREATMENT

Vitamin D_2, given at an appropriate daily dose, can be used to treat PDDR. The biochemical and clinical abnormalities regress and normal linear growth is restored. The dose of vitamin D_2 to heal the bone disease may be as high as 2.5 mg (100,000 IU) per day. This dose can be reduced by half or more to a maintenance dose, probably for the lifetime of the patient (Table I). Under such treatment, circulating levels of 25OHD increase sharply, with only minor changes in the levels of $1,25(OH)_2D$ (Fig. 2). It is likely that massive concentrations of 25OHD are able to bind to VDR and induce the response of the target organs to normalize calcium homeostasis. However, because such therapy leads to progressive accumulation of vitamin D in fat and muscle tissues, adjustment in case of overdose is difficult and slow to come into effect. Furthermore, the therapeutic doses are close to the toxic doses and place the patient at risk for nephrocalcinosis and impaired renal function. There have been reports on the use of $25OHD_3$ as a therapeutic agent in PDDR [41]. The doses used are smaller than those of vitamin D (Table I) and induce a similar response. The action of $25OHD_3$ is likely to be similar to the one of vitamin D itself, by maintaining high serum concentrations of 25OHD. The low availability and high cost of such a preparation have discouraged its widespread use as a long-term therapy for PDDR.

TABLE I Vitamin D Dosage Requirements of PDDR Patients

	Dosage (μg/day)	
Compound	To heal rickets	Maintenance
Vitamin D	1000–2500	500–1250
$25OHD_3$	250–1000	100–500
$1\alpha OHD_3$	2–5	1–2
$1,25(OH)_2D_3$	1–3	0.25–1

The treatment of choice is replacement therapy with calcitriol. Before this compound became available from commercial sources, several investigators used the monohydroxylated analog $1\alpha OHD_3$, which requires only liver hydroxylation at the 25 position (a step not affected by the PDDR mutation) to fully mimic $1,25(OH)_2D_3$ [42]. The response is rapid with healing of rickets in 7–9 weeks, requiring a daily dosage of 2–5 μg. The maintenance dose is about half the initial dose (Table I). Withdrawal induces a reappearance of symptoms within 3 weeks. Thus, long-term compliance is a more important consideration than in the case of vitamin D treatment. On a weight basis, $1\alpha OHD_3$ is about half as potent as $1,25(OH)_2D$ [42], nullifying any possible economic advantage in favor of the monohydroxylated form. The reason for this difference in potency has not been investigated, but may be related to a difference in intestinal absorption or to a variable degree of 25-hydroxylation of $1\alpha OHD_3$.

Replacement therapy with calcitriol results in rapid and complete correction of the abnormal phenotype, eliminating hypocalcemia, secondary hyperparathyroidism, and radiographic evidence of rickets (Figs. 5 and 6). The restoration of bone mineral content is equally rapid (Fig. 5), and histological evidence of healing has been documented [43]. Severe tooth enamel hypoplasia is a common complication of PDDR, which is only partially corrected if treatment, as it is usually the case, is started around 12–15 months of age when permanent tooth enamel has already started to develop (Fig. 7).

The calcitriol regimen calls for an initial dose of 1–3 μg/day continued until bone is healed, and is followed by a maintenance dose of 0.25-1 μg/day (Table I) to be continued probably throughout life. An important component of treatment is to ensure adequate calcium intake during the bone healing phase. Dietary sources are supplemented to ensure a daily supply of around 1 g of elemental calcium. Needs are monitored by frequent (bimonthly) assessment of urinary calcium excretion. The latter can easily be assessed by measuring calcium and creatinine in an aliquot of the second

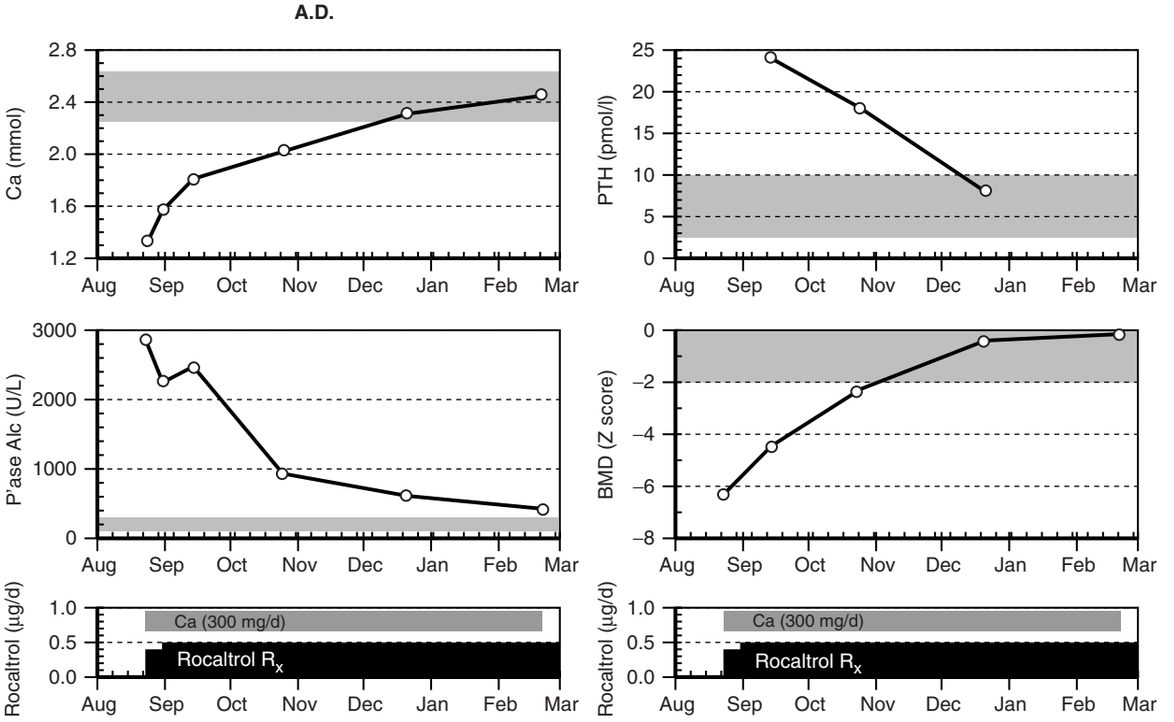

FIGURE 5 Biochemical response to treatment in a 16-month-old boy with PDDR treated with calcitriol (Rocaltrol) and calcium supplements. There was a progressive correction of the hypocalcemia (Ca) and secondary hyperparathyroidism (PTH) with concomittant decrease in alkaline phosphatase activity (P'ase Alc). Correction of the osteopenia followed the same pattern [BMD is bone mineral density of the lumbar spine by dual energy X-ray absorptiometry (DXA)]; the Z score is based on standard deviations from the mean BMD in age-matched controls.

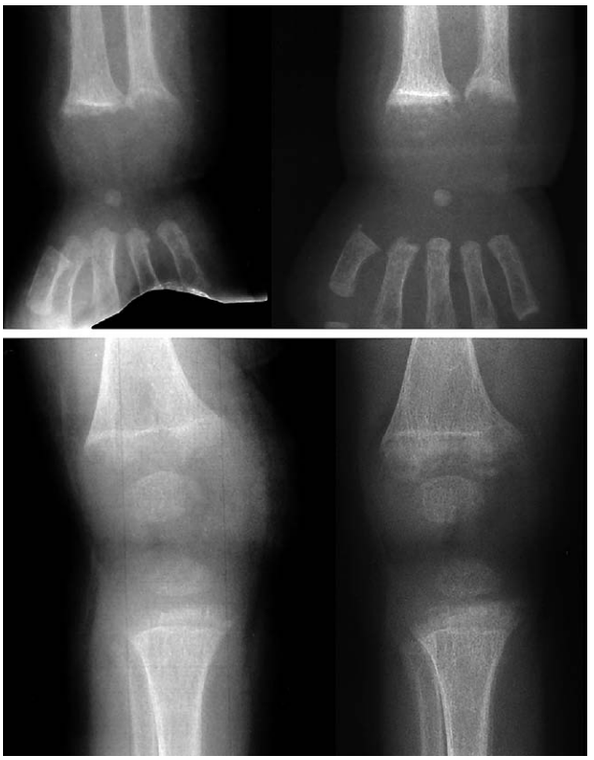

FIGURE 6 Radiographs of the right wrist (upper panel) and knee (lower panel) of a patient with PDDR (same as in Fig. 5). (Left) Before treatment; (right) after only 3 weeks of treatment, healing of rickets is well under way.

void of the morning by a fasting patient. Normal values for the calcium/creatinine ratio are <0.35 (mg/mg) or <1.1 (mmol/mmol).

In the untreated state, calciuria is very low. It will stay low as long as bone is rapidly remineralizing. An increase in calciuria is the most sensitive index of efficient therapy. Hypercalciuria (which precedes hypercalcemia by weeks) calls for reducing calcitriol progressively to the maintenance dosage. Once the latter is established, assessment of calciuria every 3 months is sufficient to keep control of the treatment. Requirements have been remarkably stable in our cohort of 32 patients treated for up to 21 years.

During normal pregnancy, calcitriol circulating levels steadily increase to about twice the control values [44]. This adaptation to the specific needs of pregnancy can be mimicked in pregnant patients with PDDR by increasing the daily calcitriol dose during the second half of pregnancy. In seven such patients, we increased the calcitriol dose by 50–100% of the maintenance dose. All women gave birth to normal

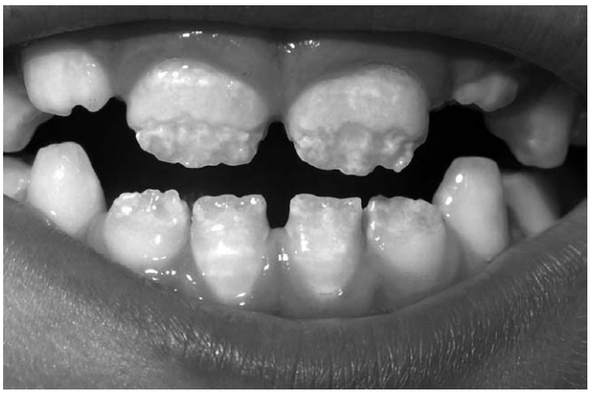

FIGURE 7 Permanent incisors of a 9-year-old patient with PDDR in whom calcitriol treatment was initiated at age 14 months. The part of the enamel that was formed before treatment remains hypoplastic. Subsequent to treatment, normal enamel was produced.

(obligate heterozygote) babies. The maintenance dose was progressively reestablished after delivery (F. H. Glorieux, unpublished data).

Hypercalciuria is not infrequent during treatment with calcitriol, particularly during the first year of administration, because changes in urinary calcium excretion are used to adjust the daily calcitriol dose. High levels of calcium excretion may amplify the pattern of calcium deposition in the normal kidney [45] and generate echodense images of the renal pyramids. This is referred to as nephrocalcinosis [46] and has been detected in several patients with PDDR treated with either vitamin D_2 or calcitriol [47]. As it may reflect a potential nephrotoxic effect of calcitriol, we have now included in our treatment protocol an annual renal ultrasonography study and evaluation of the creatinine clearance. A positive ultrasound was observed in 10 of 20 patients with PDDR treated for a mean of 8.1 years. The intensity of the images did not change with time. Two patients have shown a decrease in creatinine clearance. However, both had a history of vitamin D intoxication prior to calcitriol therapy [48]. Thus, duration of administration and dosage of the compounds used for treatment will influence the development of renal medullary changes. Frequent renal imaging and assessment of renal function are therefore essential.

VIII. CONCLUSION

Pseudovitamin D–deficiency rickets is a rare condition inherited as an autosomal recessive trait that results in an inadequate synthesis of calcitriol that

compromises intestinal calcium absorption and bone mineralization. The majority of the cases described are part of large kindreds from Northeastern Québec in Canada. Extensive genetic studies of those families have allowed an assignment of the PDDR locus to the long arm of chromosome 12 in close vicinity to the VDR gene. With the cloning of the 1α-hydroxylase gene came the characterization of an array of mutations that all lead to severe alteration of the 1α-hydroxylase activity. Replacement therapy with small daily doses of calcitriol is the treatment of choice in PDDR. It is highly efficient, removing this condition from the list of lethal mutations. It should probably be continued throughout life. Because of the potential nephrotoxicity of this treatment, regular monitoring of kidney function is mandatory. PDDR was the first described inborn error of vitamin D metabolism. Through the complete unraveling of its molecular defect, it has contributed in a major way to our understanding of vitamin D biology. Because of the easiness and efficacy of the replacement therapy, it is unlikely that any form of gene-based therapy will be considered anytime soon.

References

1. Albright F, Butler AM, Bloomberg E 1937 Rickets resistant to vitamin D therapy. Am J Dis Children **54**:529–547.
2. Royer P 1960. Etude sur les rachitismes vitamino-resistants hypophosphatemiques idiopathiques. Acta Clin Belg **15**:499–517.
3. Fraser D, Salter RB (1958) The diagnosis and management of various types of rickets. Pediatr Clin North Am **5**:417–441.
4. Prader A, Illig R, Heierli E 1961 Eine besondere form des primare vitamin D–resistenten rachitis mit hypocalcamie und autosomal-dominanten Erbgang: Die hereditare PseudoMangelrachitis. Helv Paediatr Acta **16**:452–468.
5. Scriver CR 1970 Vitamin D dependency. Pediatrics **45**:361–363.
6. Fraser D, Kooh SW, Kind HP, Rollick MF, Tanaka Y, DeLuca HF 1973 Pathogenesis of hereditary vitamin D–dependent rickets. An inborn error of vitamin D metabolism involving defective conversion of 25-hydroxyvitamin D to 1α,25-dihydroxyvitamin D. N Engl J Med **289**:817–822.
7. Marx SJ, Spiegel AM, Brown EM, Gardner DG, Downs RW, Attie M, Hamstra AJ, DeLuca HF 1978 A familial syndrome of decrease in sensitivity to 1,25-dihydroxyvitamin D, J Clin Endocrinol Metab **47**:1303–1310.
8. Rosen JF, Fleischman AR, Finberg L, Hamstra AJ, DeLuca HF 1979 Rickets with alopecia: An inborn error of vitamin D metabolism. J Pediatr **94**:729–735.
9. Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW 1988 Cloning and expression of full length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci USA **85**: 3294–3298.
10. Arnaud C, Maijer R, Reade TM, Scriver CR, Whelan DT 1970 Vitamin D dependency: An inherited postnatal syndrome with secondary hyperparathyroidism. Pediatrics **46**:871–880.
11. Delvin EE, Glorieux FH, Marie PJ, Pettifor JM 1981 Vitamin D–dependency: Replacement therapy with calcitriol. J Pediatr **99**:26–34.
12. Rosen JF, Finberg L 1972 Vitamin D–dependent rickets: Actions of parathyroid hormone and 25-hydroxycholecalciferol. Pediatr Res **6**:552–562.
13. Scriver CR, Reade TM, Hamstra AJ, DeLuca HF 1978 Serum 1,25-dihydroxyvitamin D levels in normal subjects and in patients with hereditary rickets or bone disease. N Engl J Med **299**:976–979.
14. Balsan S Garabedian M, Sorgniard R, Holick MF, DeLuca HF 1975 1,25-Dihydroxyvitamin D_3 and 1,α-hydroxyvitamin D_3 in children: Biologic and therapeutic effects in nutritional rickets and different types of vitamin D resistance. Pediatr Res **9**:593–599.
15. Glorieux FH, Delvin EE 1991 Pseudo-vitamin D–deficiency rickets. *In:* Vitamin D: Regulation, Structure-Function Analysis and Clinical Application, de Gruyter: Berlin and New York, pp. 238–245.
16. Mandla S, Jones G, Tenenhouse HS 1992 Normal 24-hydroxylation of vitamin D metabolites in patients with vitamin D–dependency rickets type I. Structural implications for vitamin D hydroxylases. J Clin Endocrinol Metab **74**:814–820.
17. Arabian A, Grover J, Barre MG, Delvin EE 1993 Rat kidney 25-hydroxyvitamin D_3 1α- and 24-hydroxylases: Evidence for two distinct gene products. J Steroid Biochem Mol Biol **45**:513–516.
18. Weisman Y, Harell A, Edelstein S, David M, Spirer Z, Golander A 1979 lα,25-Dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 *in vitro* synthesis by human decidua and placenta. Nature **281**:317–319.
19. Delvin EE, Arabian A, Glorieux FH, Mamer OA 1985 *In vitro* metabolism of 25-hydroxycholecalciferol by isolated cells from human decidua. J Clin Endocrinol Metab **601**:880–885.
20. Delvin EE, Arabian A 1987 Kinetics and regulation of 25-hydroxycholecalciferol 1α-hydroxylase from cells isolated from human term decidua. Eur J Biochem **163**:659–662.
21. Glorieux FH, Arabian A, Delvin EE 1995 Pseudo-vitamin D deficiency: Absence of 25-hydroxyvitamin D 1α-hydroxylase activity in human placenta decidual cells. J Clin Endocrinol Metab **80**:2255–2258.
22. Bouchard G, Laberge C, Scriver CR, Glorieux F, Declos M, Bergeron L, Larochelle J, Mortezai S 1984 Etude demographique et genealogique de deux maladies hereditaires au Saguenay. Cahiers Quebecois de Demographic **13**:117–137.
23. Labuda M, Morgan K, Glorieux FH 1990 Mapping autosomal recessive vitamin D–dependency type I to chromosome 12ql4 by linkage analysis. Am J Hum Genet **47**:28–36.
24. Labuda M, Labuda D, Korab-Laskowska M, Cole DEC, Zietkiewicz E, Weissenbach J, Popwska E, Pronicka E, Root AW Glorieux FH 1996 Linkage disequilibrium analysis in young populations: Pseudovitamin D–deficiency rickets (PDDR) and the founder effect in French Canadians. Am J Hum Genet **59**:633–643.
25. De Braekeleer M. 1991 Hereditary disorders in Saguenay-Lac-St-Jean (Quebec, Canada). Hum Hered **41**:141–146.
26. Labuda M, Fujiwara TM, Ross MV, Morgan K, Garcia-Heras J, Ledbetter DH, Hughes MR, Glorieux FH 1992 Two hereditary defects related to vitamin D metabolism map to the same region of human chromosome 12ql3-14. J Bone Miner Res **7**:1447–1453.
27. Faraco JH, Morrison NA, Baker A, Shine J, Frossard PM 1989 Apal dimorphism at the human vitamin D receptor gene locus Nucleic Acids Res **17**:2150.
28. St-Arnaud R, Messerlian S, Moir JM, Omdahl JL, Glorieux FH 1**997**: The 25-hydroxyvitamin D 1-alpha-hydroxylase gene maps to the pseudovitamin D–deficiency rickets (PDDR) disease locus. J Bone Miner Res **12**:1552–1559.

29. Shinki T, Shimada H, Wakino S, *et al*. 1997 Cloning and expression of rat 25-hydroxyvitamin D_3-1alpha-hydroxylase cDNA. Proc Natl Acad Sci USA **94**:12920–12925.
30. Takeyama K, Kitanaka S, Sato T, *et al*. 1997 25-Hydroxyvitamin D_3 1alpha-hydroxylase and vitamin D synthesis. Science **277**:1827–1830.
31. Fu GK, Lin D, Zhang MY, *et al.* 1997 Cloning of human 25-hydroxyvitamin D-1 alpha-hydroxylase and mutations causing vitamin D–dependent rickets type 1. Mol Endocrinol **11**:1961–1970.
32. Kitanaka S, Takeyama K, Murayama A, *et al.* 1998 Inactivating mutations in the 25-hydroxyvitamin D_3 1alpha-hydroxylase gene in patients with pseudovitamin D–deficiency rickets. N Engl J Med **338**:653–661.
33. Yoshida T, Monkawa T, Tenenhouse HS, *et al.* 1998 Two novel 1alpha-hydroxylase mutations in French-Canadians with vitamin D–dependency rickets type I. Kidney Int **54**:1437–1443.
34. Fu GK, Portale AA, Miller WL 1997 Complete structure of the human gene for the vitamin D 1alpha-hydroxylase, P450c1alpha. DNA Cell Biol **16**:1499–1507.
35. Kitanaka S, Murayama A, Sakaki T, *et al.* 1999 No enzyme activity of 25-hydroxyvitamin D3 1alpha-hydroxylase gene product in pseudovitamin D–deficiency rickets, including that with mild clinical manifestation. J Clin Endocrinol Metab **84**:4111–4117.
36. Wang X, Zhang MY, Miller WL, Portale AA 2002 Novel gene mutations in patients with 1alpha-hydroxylase deficiency that confer partial enzyme activity in vitro. J Clin Endocrinol Metab **87**:2424–2430.
37. Wang JT, Lin CJ, Burridge SM, *et al.* 1998 Genetics of vitamin D 1alpha-hydroxylase deficiency in 17 families. Am J Hum Genet **63**:1694–1702.
38. Smith SJ, Rucka AK, Berry JL, *et al.* 1999 Novel mutations in the 1alpha-hydroxylase (P450c1) gene in three families with pseudovitamin D–deficiency rickets resulting in loss of functional enzyme activity in blood-derived macrophages. J Bone Miner Res **14**:730–739.
39. Dardenne O, Prud'homme J, Arabian A, Glorieux FH, St-Arnaud R 2001 Targeted inactivation of the 25-hydroxyvitamin D(3)-1(alpha)-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D–deficiency rickets. Endocrinology **142**:3135–3141.
40. Panda DK, Miao D, Tremblay ML, *et al.* 2001 Targeted ablation of the 25-hydroxyvitamin D 1alpha-hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. Proc Natl Acad Sci USA **98**:7498–7503.
41. Balsan S, Garabedian M, Lieberherr M, Gueris J, Ulmann A 1979 Serum 1,25-dihydroxyvitamin D concentrations in two different types of pseudo-deficiency rickets. *In* Norman AW, Schaefer K, Herrath DV, Grigoleit H-G, Coburn JW, DeLuca HF, Mawer EB, Suda T (eds) Vitamin D: Basic Research and Its Clinical Application, de Gruyter: Berlin and New York, pp. 1143–1149.
42. Reade TM, Scriver CR, Glorieux FH, Nogrady B, Delvin E, Poirier R, Holick MF, DeLuca HF 1975 Response to crystalline la-hydroxyvitamin D_3 in vitamin D–dependency. Pediatr Res **9**:593–599.
43. Delvin EE, Glorieux FH, Marie PJ, Pettifor JM 1981 Vitamin D–dependency: Replacement therapy with calcitriol. J Pediatr **99**:26–34.
44. Delvin EE, Salle BL, Glorieux FH, Adeleine P, David LS 1986 Vitamin D supplementation during pregnancy: Effect on neonatal calcium homeostasis. J Pediatr **109**:328–334.
45. Anderson L, McDonald JR 1946 The origin, frequency, and significance of microscopic calculi in the kidney. Surg Gynecol Obstet **82**:275–282.
46. Alon U, Brewer WH, Chan JCM 1983 Nephrocalcinosis: Detection by ultrasonography. Pediatrics **71**:970–973.
47. Goodyear PR, Kronick JB, Jequier S, Reade TM, Seriver CR 1987 Nephrocalcinosis and its relationship to treatment of hereditary rickets. J Pediatr **11**:700–704.
48. Glorieux FH 1990 Calcitriol treatment in vitamin D–dependent and vitamin D–resistant rickets. Metabolism **39**:(Supp 1): 10–12.

CHAPTER 72

Hereditary 1,25-Dihydroxyvitamin D–Resistant Rickets

PETER J. MALLOY Division of Endocrinology, Gerontology, and Metabolism, Stanford University School of Medicine, Stanford, California

J. WESLEY PIKE Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin

DAVID FELDMAN Division of Endocrinology, Gerontology, and Metabolism, Stanford University School of Medicine, Stanford, California

I. INTRODUCTION

Vitamin D, the primary regulator of calcium homeostasis in the body, is particularly important in skeletal development and in bone mineralization. The active form of vitamin D, 1α,25-dihydroxyvitamin D, [$1,25(OH)_2D_3$], functions by binding with high affinity to specific vitamin D receptors (VDR). The VDR is a member of the steroid-thyroid-retinoid receptor gene superfamily of nuclear transcription factors that regulate the expression of specific target genes in response to hormone binding. Hereditary vitamin D–resistant rickets (HVDRR) is a rare genetic disease that is due to a generalized resistance to $1,25(OH)_2D_3$ [1–3]. HVDRR is caused by heterogeneous mutations in the VDR gene that alter the function of the receptor ultimately leading to complete or partial resistance to $1,25(OH)_2D_3$. In this chapter on HVDRR, we describe the clinical manifestations of the disease and discuss the genetic defects in the VDR underlying the molecular basis for HVDRR.

Over the years, a number of different names have been used to describe the condition caused by $1,25(OH)_2D$ resistance. In addition to HVDRR, the disease has been referred to as vitamin D–dependent rickets type II (VDDR-II), pseudovitamin D deficiency type II (PDDR II), calcitriol-resistant rickets (CRR), and hypocalcemic vitamin D–resistant rickets (HVDRR). We use the term hereditary 1,25-dihydroxyvitamin D–resistant rickets (HVDRR) since this disease is now known to be caused by genetic defects in the VDR that lead to resistance to the action of $1,25(OH)_2D_3$ [3]. The term HVDRR provides a better description of the disease as it is based on genetic defects that produce resistance; it is not dependent on $1,25(OH)_2D$ but is due to resistance to $1,25(OH)_2D_3$ action. In the Online Mendelian Inheritance in Man Web site (http://www.ncbi.nlm.nih.gov/omim/), this disease is referred to as vitamin D–resistant rickets with end-organ unresponsiveness to 1,25-dihydroxycholecalciferol, rickets-alopecia syndrome, VDDR II with alopecia, and hypocalcemic vitamin D–resistant rickets.

The notion that diseases could be due to hormone resistance emerged in 1937 when Albright *et al.* [4] described a patient with rickets who had normal serum calcium levels but low phosphate levels. The patient was treated with abnormally high doses of vitamin D and responded to the therapy. Keen observation of this case led the authors to suggest that the cause of the condition was due to end-organ resistance to vitamin D, and thus the concept of hormone resistance evolved. The patient they described appears to have had what is now known as X-linked hypophosphatemic rickets (XLH, described in Chapter 69). Twenty-four years later, Prader *et al.* [5] reported on two patients with rickets who were hypocalcemic and hypophosphatemic. These patients also responded to high doses of vitamin D and they referred to this condition as vitamin D–dependent rickets type I (VDDR-I). The cause of rickets in these individuals is due to an inborn error in the conversion of vitamin D to the hormonally active form $1,25(OH)_2D$. VDDR I arises from mutations in

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the gene encoding 25-hydroxy-1α-hydroxylase (1αOHase) [6], the enzyme that converts 25-hydroxyvitamin D_3 ($25(OH)D_3$) to $1,25(OH)_2D_3$ [7,8]. This disease is described in Chapter 71.

In 1978, the first cases of HVDRR were reported by Brooks *et al.* [9] and Marx *et al.* [10]. The patient in the Brooks *et al.* study exhibited hypocalcemia, hypophosphatemia, and secondary hyperparathyroidism. The clinical findings were similar to patients with VDDR I except that the patient had markedly increased serum levels of $1,25(OH)_2D$. Brooks *et al.* [9] postulated that the rickets was due to an end-organ resistance to $1,25(OH)2_2D_3$, and they named the syndrome vitamin D–dependent rickets, type II (VDDR II). Marx *et al.* [10] reported similar findings in two children and also suggested that the disease was due to end-organ resistance to $1,25(OH)_2D$. Since these initial studies there have been many reports of patients with apparent target organ resistance to $1,25(OH)_2D$ [3]. In this chapter, we will review the clinical features and the genetic basis underlying the disease.

II. THE CLINICAL FEATURES OF HVDRR

A. Clinical and Biochemical Findings

HVDRR is manifested by a constellation of signs and symptoms caused by a generalized resistance to $1,25(OH)_2D$. The major feature of HVDRR is rickets that is due to defective mineralization of newly forming bone and pre-osseous cartilage. In HVDRR the rickets is generally displayed early, usually within months of birth. The rickets is usually severe and affected children suffer from bone pain, muscle weakness, and hypotonia. In the worst cases, convulsions due to the hypocalcemia have occurred. Children are often growth-retarded, and they frequently develop severe dental caries or exhibit enamel hypoplasia of the teeth [11–17]. Some infants have died from pneumonia as a result of poor respiratory movement due to severe rickets of the chest wall [12,15,18]. In many cases, children with HVDRR have sparse body hair and some have total scalp and body alopecia including eyebrows and in some cases eyelashes (Fig. 1). Alopecia will be discussed in more detail below.

An example of the typical serum biochemistry levels found in HVDRR cases is shown in Table I. The abnormalities include low serum concentrations of calcium and phosphate and elevated serum alkaline phosphatase activity. The hypocalcemia leads to secondary hyperparathyroidism. The elevated parathyroid hormone (PTH) level then contributes to the hypophosphatemia. These clinical and biochemical findings are also common to patients with 1α-hydroxylase deficiency (Table II) as described in Chapter 71. On the other hand, in HVDRR patients the serum $25(OH)D$ values are normal and the $1,25(OH)_2D$ levels are elevated. This singular feature distinguishes HVDRR from 1α-hydroxylase deficiency (VDDR-I) where the serum $1,25(OH)_2D$ values are depressed or absent. When analyzed, the $24,25(OH)_2D$ levels have been normal or low [12,15,19–25]. Patients with 1α-hydroxylase deficiency (VDDR I) can be successfully treated with physiologic doses of calcitriol that circumvent the 1α-hydroxylase deficiency and restore the circulating $1,25(OH)_2D$ levels to normal. In contrast, patients with HVDRR do not respond to physiologic doses of calcitriol and most patients are resistant to even extreme supra-physiologic doses of all forms of vitamin D therapy (Table II).

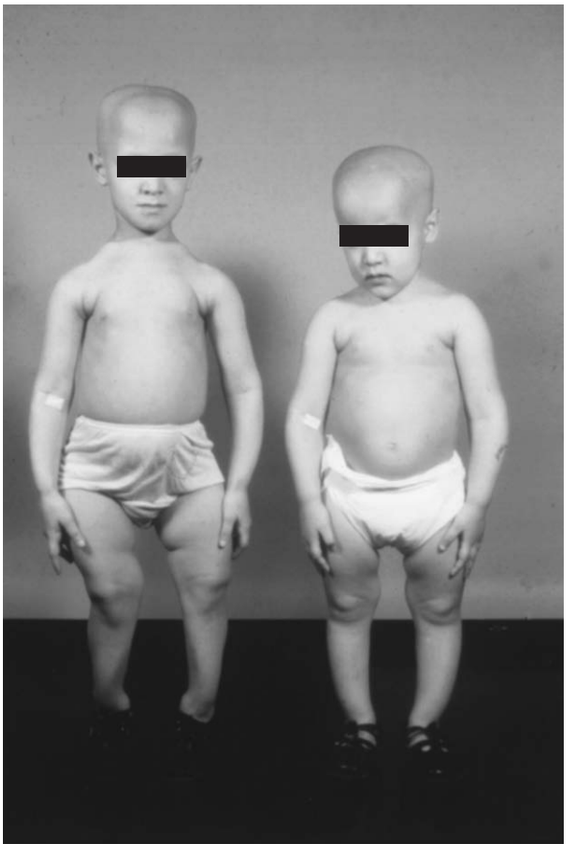

FIGURE 1 Children with HVDRR and alopecia. Reprinted with permission from *The Journal of Pediatrics*, JF Rosen, AR Fleischman, L Fineberg, A Hamstra, and HF DeLuca. Rickets with alopecia: An inborn error of vitamin D metabolism. 1979; **94**:729–735.

TABLE I Biochemical Profile of a Patient with HVDRR on Therapy

Biochemical marker	Normal values	Referral values	40 days[a]	80 days[b]	100 days
Calcium (mmol/liter)	2.2–2.6	1.86	1.77	1.80	1.71
Phosphate (mmol/liter)	1.4–2.2	1.0	1.0	1.0	0.9
ALP (IU/liter)	145–320	3056	3991	3800	3609
25(OH)D (nmol/liter)	25–85	30	37.4	250	211
$1,25(OH)_2D$ (pmol/liter)	40–105	521	953	1830	1560
PTH (pmol/liter)	<8	–	34.2	69.9	64.5

[a]Treatment: 250 mg elemental calcium 4 times per day and 0.5 mg calcitriol (Rocaltrol) twice per day and 20,000 IU vitamin D_3 daily.
[b]Treatment: 250 mg elemental calcium 4 times per day and 5 mg calcitriol twice per day. ALP, alkaline phosphatase. Adapted from Zhu *et al.*: J Bone Miner Res **13**:259–264, 1998 with permission of the American Society for Bone and Mineral Research.

HVDRR is inherited as autosomal recessive disease. The recessive nature of the disease is evident from the patient's parents and siblings who are heterozygous for the genetic trait, but show no symptoms of the disease and have normal bone development. In most cases, consanguinity in the family lineage can be found and intermarriage is highly associated with the disease. Males and females are equally affected and often a family has several affected children [26].

B. Pathophysiology

In HVDRR, the intestine, and other target organs, including bone, the parathyroid glands, and kidneys, are resistant to $1,25(OH)_2D$ action. Without vitamin D action, the intestine becomes less efficient in promoting calcium and phosphate absorption into the circulation. It is now well established that the biological actions of $1,25(OH)_2D$ are mediated by the VDR, a nuclear transcription factor that regulates gene expression in $1,25(OH)_2D$–responsive cells (see Chapter 13). As will be discussed in detail below, the hallmark of the HVDRR syndrome is resistance to $1,25(OH)_2D$ action. It is now clear that the usual cause of HVDRR is due to mutations in the VDR that render the receptor non–functional or less functional than the wild-type VDR.

TABLE II A Comparison of 1α-hydroxylase Deficiency and HVDRR

Feature	1α-Hydroxylase deficiency[a]	HVDRR
Gene mutated	CYP27B1	VDR
Autosomal recessive	Yes	Yes
Manifested at early age	Yes	Yes
Rickets	Yes	Yes
Hypocalcemia	Yes	Yes
Alopecia	No	Sometimes
PTH	Elevated	Elevated
25(OH)D levels	Normal	Normal
$1,25(OH)_2D$ levels	Low	Elevated
Response to physiological doses of $1,25(OH)_2D_3$	Yes	No

[a]1α-Hydroxylase deficiency is also known as VDDR I or PDDR.

The primary biological process attributed to vitamin D is maintenance of calcium and bone homeostasis. $1,25(OH)_2D$ is essential for promoting the transport of calcium and phosphate across the small intestine and into the circulation. Adequate delivery of calcium and phosphate to the bone is essential for the normal mineralization of bone. Approximately half of the total calcium absorption by the intestine is attributed to $1,25(OH)_2D$ action while passive absorption accounts for the remaining half [27,28]. Since vitamin D regulates the translocation of calcium, conditions that adversely affect the $1,25(OH)_2D$ action pathway cause a decrease in mineral transport leading to hypocalcemia. The hypocalcemia and the resistance of the parathyroid gland to suppression by $1,25(OH)_2D$ because of defective VDR within the gland, in turn, results in secondary hyperparathyroidism. The increase in circulating $1,25(OH)_2D$ levels are due to an increase in renal 1α-hydroxylase activity caused by both elevated PTH and hypophosphatemia to up-regulate 1α-hydroxylase gene expression, as well as failure of elevated $1,25(OH)_2D$ to suppress 1α-hydroxylase. The hypophosphatemia results from the elevated PTH down-regulating the Na/P cotransporter and/or by the loss of a functional VDR in the kidney, as well as decreased intestinal absorption (see Chapters 26, 29, and 69). The calcium and phosphate deficiencies compromise normal bone mineralization leading to rickets in children and osteomalacia in adults.

C. Alopecia

Alopecia totalis (sometimes called *atrichia*) is a clinical feature that is found in many patients with HVDRR (Fig. 1). Some patients have sparse body hair and some exhibit total scalp and body alopecia [21,29,30]. Children with extreme alopecia often lack eyebrows and in some cases eyelashes. Hair loss may be evident at birth or occurs during the first few months of life. An analysis of HVDRR patients shows that there is some correlation between the severity of rickets and the presence of alopecia [30]. Patients with alopecia are generally more resistant to calcitriol therapy than those without alopecia. In families with a prior history of the disease, the absence of scalp hair in newborns provides initial diagnostic evidence for HVDRR. The mechanism causing alopecia is unknown but VDRs are present in the hair follicle [31,32]. Skin biopsy has revealed apparently normal follicles with no hair. The lack of $1,25(OH)_2D$ action during a critical stage of hair follicle development is the suspected cause of alopecia. It is interesting to note that alopecia is not associated with other diseases of vitamin D deficiency. Alopecia is also discussed in Chapters 20 and 35.

D. Other Aspects of HVDRR

As mentioned above and discussed extensively in this volume, in addition to maintaining calcium homeostasis, $1,25(OH)_2D$ has been shown to regulate a number of biological processes in many tissues [33–39]. Although there are multiple pleiotropic tissue responses regulated by $1,25(OH)_2D$, children with HVDRR appear relatively normal except for the constellation of features that relate to their calcium deficiency, rickets, and alopecia. VDRs have been found in endocrine glands such as pituitary, pancreas, parathyroid, gonads, and placenta, and $1,25(OH)_2D_3$ regulates hormone synthesis and secretion from these glands [33–38]. Hochberg *et al.* [40] examined insulin, thyrotropin (TSH), prolactin (PRL), growth hormone (GH), and testosterone levels in sera from patients with HVDRR and found no abnormalities in hormone secretion. Furthermore, Even *et al.* [41] showed that urinary cAMP and excretion of potassium, phosphorus, and bicarbonate were normal in HVDRR patients following a PTH challenge. However, PTH failed to decrease urinary calcium and sodium excretion in these patients to the extent found in the control patients. This suggests that $1,25(OH)_2D$ may selectively modulate the renal response to PTH and facilitate the PTH-induced reabsorption of calcium and sodium [41].

VDRs have also been found in hematolymphopoietic cells and $1,25(OH)_2D$ has been shown to regulate cell differentiation and the production of interleukins and cytokines [42] (see Chapter 36). Neutrophils isolated from HVDRR patients exhibit only minor aberrations in their fungicidal activity [43], and HVDRR patients have no clinically apparent immunologic defects. In the light of the diverse actions of $1,25(OH)_2D$ demonstrated in many tissues, the absence of related findings in children with HVDRR suggests that the pleiotropic responses regulated by $1,25(OH)_2D$ in nonosteogenic tissues are redundant and that other factors or compensatory mechanisms subsume the role of vitamin D in such a way that abnormalities are not clinically manifested. This possibility does not necessarily minimize the contribution of $1,25(OH)_2D$ to these systems under normal physiologic conditions. Similarly, the VDR knockout mouse displays the same phenotypic and physiologic patterns as patients with HVDRR [44,45]. The VDR knockout mouse model can be used to analyze the abnormalities caused by the loss of VDR action in detail that is not possible in the HVDRR patients (see Chapter 20).

III. MECHANISM OF $1,25(OH)_2D$ ACTION

A. The Vitamin D Receptor

The VDR is a ligand-activated nuclear transcription factor and a member of the steroid-thyroid-retinoid receptor gene superfamily [46]. The VDR is similar in overall structure to the other members of the steroid-thyroid-retinoid receptor superfamily, having a highly conserved DNA-binding domain (DBD) and a more variable ligand-binding domain (LBD) (see Chapter 11). The VDR gene is located on chromosome 12 and is composed of 14 or more exons (Fig. 2). Exons 2 and 3 encode the DBD and exons 4 through 9 encode the LBD. The human VDR contains either 424 or 427 amino acids due a polymorphism in a transcription start site. Differential use of promoters and alternative splicing may lead to even longer forms (see Chapter 12).

1. DNA Binding Domain (DBD)

The DBD extends from amino acids residues 24–90 at the N-terminus of the VDR (Fig. 2). The DBD folds into two loops or modules of 12–13 amino acids each. Each module contains four cysteine residues that coordinate the binding of one zinc atom forming a "zinc-finger" structure. The two zinc modules of the VDR are not topologically equivalent and serve different functions within the protein [47]. In the first zinc finger

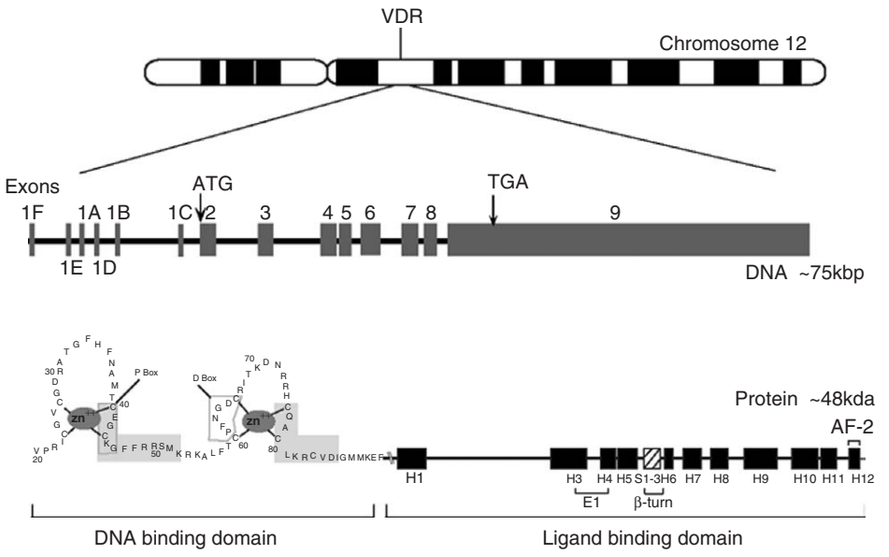

FIGURE 2 Arrangement of the chromosomal gene and domains of the VDR. The structural organization of the human VDR gene which spans approximately 75 kilobases of DNA is shown [187]. The location of the start (ATG) and termination (TGA) codons are indicated. The exons encoding the various domains and structural motifs in the VDR protein are shown. The VDR DNA binding domain is comprised of two zinc finger modules each of which contains 4 invariant cysteine residues that function to coordinate a single zinc atom. Two α-helices (helix A and B) shaded in the diagram are located on the carboxy terminal side of each zinc module. Amino acid residues essential to functional interaction of these α-helices with either DNA or with RXR are boxed and designated the P-box and D-box, respectively. In the ligand-binding domain the position of the α-helices (H1–H12) and β-turns (S1–3) are shown as shaded and hatched boxes respectively. The E1 and AF-2 regions are indicated.

module (the most N-terminal), an α-helix known as the *P-box* (residues 42–46), functions to direct specific DNA-binding in the major groove of the DNA-binding site. In the second zinc finger module, an α-helix known as the D-box (residues 61–65) serves as a dimerization interface for interaction with the retinoid X receptor (RXR) DNA-binding domain [48,49]. Immediately downstream of the second zinc finger (aa residues 90–101) lies an extended α-helix known as the T-box. This T-box region also likely interacts with RXR and makes minor groove contacts with nucleotides in the DNA half-sites of VDREs (see Chapters 13 and 14). As will be discussed below, mutations in critical amino acids within both zinc finger modules render the VDR nonfunctional and cause HVDRR, presumably by interfering with VDR binding to DNA.

2. LIGAND BINDING DOMAIN (LBD)

The structure of the VDR LBD that stretches over two-thirds of the protein from amino acids 123 to 427 has been determined by X-ray crystallography [50] (see Chapter 15). The VDR LBD is formed by 12 α-helices (H1–H12) and 3 β-sheets (S1–S3) (Fig. 2). The LBD also has a variable length region between helix H1 and helix H3 (loop 1–3). Conserved residues located in a 34 amino acid cluster from the C-terminus of helix H3 to the middle of helix H5 form the hydrophobic core. These residues hold together H3, H4, H5, H8, and H9, and the interhelical loops 3–4 and 8–9. Residues in H1, H3, H5, β-turn, loop 6–7, H11, loop 11–12 and H12 form the framework for a 3-dimensional ligand binding pocket. Helix H12 is thought to form a retractable lid that traps and holds the ligand in position. Once inside the binding pocket, the ligand makes contact with specific amino acid residues of the various α-helices that line the face of the pocket thereby transmitting its signal (see Chapter 15). Ligand binding causes a conformational change in the VDR that allows the receptor to form protein heterodimers with RXR. VDR elements involved in RXR heterodimerization are located within the LBD, and include H9, H10, and an E1 domain that overlaps H4 and H5 (Fig. 2). Two regions of the VDR LBD are essential for its transactivation function. These regions include the AF-2 domain that

encompasses H12 (residues 416–424) and the region between amino acids 232–272 encompassing H3 and H4 [50]. Repositioning of helix H12 after ligand binding is critical to the formation of a hydrophobic cleft that can accommodate the LxxLL motif of coactivators and allow them to bind to the receptor (see Chapter 16). Ligand binding also results in exposure of other regions of the VDR that act to recruit coactivator proteins or facilitate contact with proteins associated with the core transcriptional machinery, such as TFIIB or the TAFs [51,52]. The coactivators, such as SRC-1, are active in modifying chromatin [53–55] (see Chapter 19). Mutations in the VDR LBD may cause HVDRR through a number of mechanisms. They may completely prevent ligand binding or reduce its affinity for $1,25(OH)_2D_3$. Alternatively, mutations may alter VDR conformation compromising its ability to heterodimerize with RXR, bind to DNA, or interact with coactivators.

B. Regulation of Gene Expression by 1,25-Dihydroxyvitamin D

The mechanism of action of vitamin D is detailed in multiple chapters in Section II of this book. Thus, we will briefly review only those aspects of vitamin D action particularly relevant to understanding HVDRR and the mutations responsible for causing this syndrome.

The biological actions of $1,25(OH)_2D$ in tissues and cells are orchestrated through complex changes in gene expression [34,56–58]. These changes lead to cell-specific alterations in the level of proteins directly responsible for a myriad of differentiated cell functions, as well as in proteins that function as transcription factors or as signaling molecules to regulate secondary and tertiary levels of gene expression [59]. In the latter case, these molecules may function directly within the cell or indirectly via additional cellular signaling pathways in either autocrine or paracrine fashion. As indicated earlier, most, if not all, of the molecular actions of $1,25(OH)_2D$ in the nucleus are mediated by the VDR. A simplified model of $1,25(OH)_2D$ activated gene transcription is shown in Fig. 3. A more comprehensive analysis of $1,25(OH)_2D$ action is presented in Chapter 13.

In brief, after synthesis in the kidney, $1,25(OH)_2D$ circulates in the blood mostly bound to DBP and perhaps other carriers with a small fraction of hormone in the free state (see Chapter 8). The free, fat-soluble hormone is believed to enter target cells through the lipid bilayer of the cell membrane, although additional complex mechanisms may play a role (see Chapter 10). Once inside the cell, $1,25(OH)_2D_3$ activates the VDR, prompting translocation to the nucleus, whereupon a series of changes occur that enable the VDR to activate gene transcription [60]. The mechanism of action is described in great detail in Section II of this book. One event that happens following $1,25(OH)_2D$–binding is that the VDR is posttranslationally modified by phosphorylation [61]. Phosphorylation may increase transactivation, however, its precise role in the activation of the VDR is currently unclear.

Ligand binding promotes VDR-RXR heterodimerization and DNA binding. The VDR-RXR heterodimer complex binds with high affinity through their DBDs to vitamin D response elements (VDREs) located in the promoter region of target genes [57]. The typical VDRE contains two imperfect hexanucleotides arranged as direct repeats separated by a 3 nucleotide base spacer. In the LBD, $1,25(OH)_2D_3$ makes contact with specific amino acid residues lining the ligand-binding pocket [50]. $1,25(OH)_2D_3$ binding triggers helix H12 to fold back upon the LBD enclosing the ligand. A coactivator-binding cleft is formed by the repositioning of helix H12 together with helix H3, allowing the recruitment of coactivators and other transcription factors [62]. The VDR-complex together with the general transcription apparatus drives the transcription of $1,25(OH)_2D$-responsive genes that ultimately determine the cellular response to the hormone.

The proof that the cause of HVDRR was defective regulation of gene expression by VDR was initially developed through studies of HVDRR where natural mutations in the VDR gene prevented $1,25(OH)_2D_3$ induction of target genes, such as 24-hydroxylase [20,22,63]. Further studies demonstrated that $1,25(OH)_2D_3$ could regulate promoter activity and that specific DNA sequences within the promoter were required for recognition by the VDR-RXR heterodimer. Direct regulation of gene expression by $1,25(OH)_2D_3$ has been demonstrated using the human [64–66] and rat [67–69] osteocalcin gene, the osteopontin gene [70], the calbindin genes [71,72], and the 25-hydroxyvitamin D_3 24-hydroxylase genes [73–75]. HVDRR mutant VDRs were eventually shown to be incapable of activating such promoter constructs both supporting the critical role of functional VDR in transactivation as well as defining the defect causing HVDRR [76–78].

IV. CELLULAR BASIS OF HVDRR

A. Initial Studies Using Cultured Skin Fibroblasts

The syndrome of HVDRR was first recognized as an entity in 1978–79 [9–11,79]. At the present time, more than 100 patients with vitamin D resistance

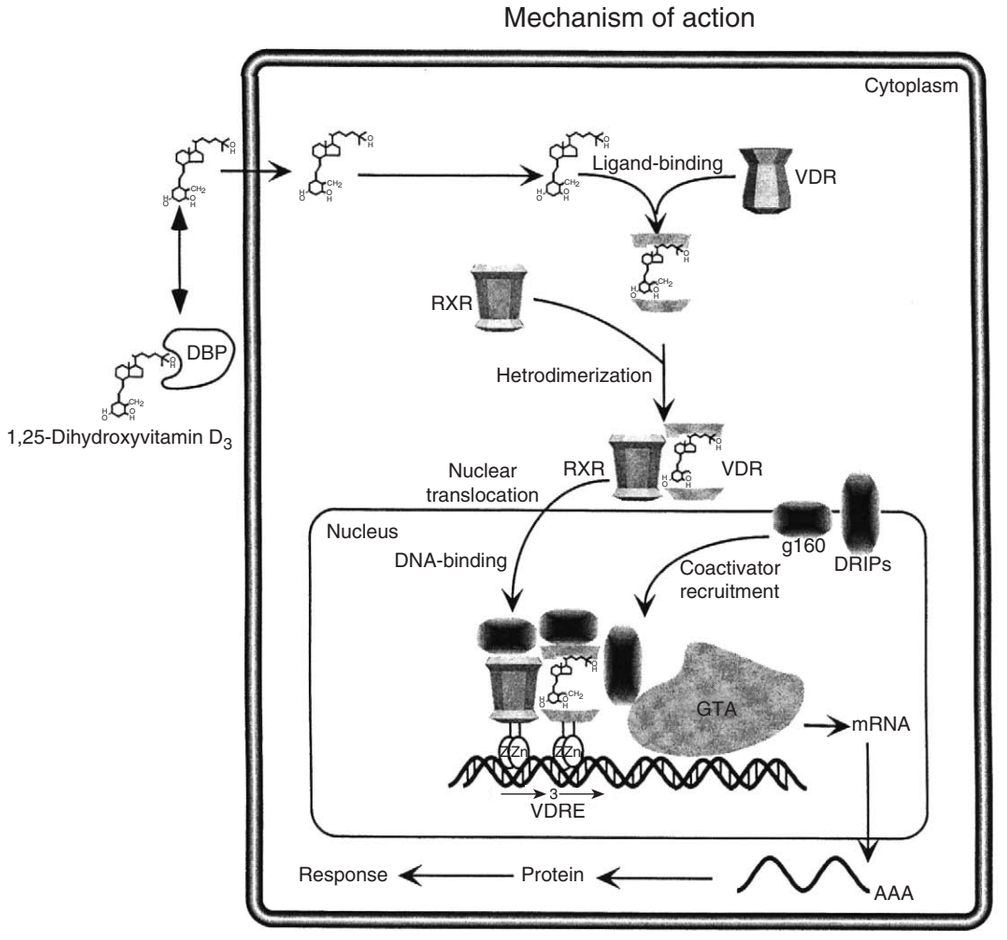

FIGURE 3 Mechanism of 1,25-dihydroxyvitamin D_3 action. 1,25-dihydroxyvitamin D_3 circulates in the blood mainly bound to the vitamin D–binding protein (DBP) with a small amount in equilibrium with the free or unbound state. The free lipophilic ligand diffuses through the lipid bilayer of the cell membrane and binds with high affinity to the VDR. In the nucleus the ligand-bound VDR heterodimerizes with RXR and binds to VDREs via the two zinc finger modules of the DNA–binding domain of the receptors. Ligand binding also induces changes in the VDR that allows it to recruit coactivators. The VDR-RXR–coactivator complex interacts with the general transcription apparatus (GTA) and initiates gene transcription. The physiologic response to the hormone is manifested by the newly synthesized proteins that then elicit intracellular or extracellular activities.

have been described [3]. A summary of these cases is shown in Table III. Throughout this chapter, the HVDRR cases are denoted by a family number, e.g. F1, F2, etc.

Studies to elucidate the nature of the defect in HVDRR cases began soon after receptors for $1,25(OH)_2D_3$ were found in skin [31,80–82]. Feldman *et al.* [81] demonstrated that the VDR was present in fresh and cultured human foreskin as well as in cultured keratinocytes and dermal fibroblasts grown from adult skin biopsies. The studies to unravel the cause of the disease began in earnest in 1981 when Eil *et al*. [83] showed in cultured skin fibroblasts that the cause of the cellular defect in patients (families F1 and F3) with HVDRR was due to the defective nuclear uptake of $1,25(OH)_2D_3$. The following year, Feldman *et al.* [20] analyzed the VDR in cultured skin fibroblasts from two siblings with HVDRR (F11). In this study, they demonstrated that cytosolic extracts of cultured fibroblasts from the HVDRR patients had undetectable levels of $[^3H]1,25(OH)_2D_3$ binding. Furthermore, when cultured fibroblasts from normal subjects were treated with $1,25(OH)_2D_3$, they were able to demonstrate an increase in 24-hydroxylase activity, a well-characterized

TABLE III Compilation of HVDRR Cases

Family[a]	Patient name/ description[b]	Ethnic origin	Consanguinity	Onset age	Male	Female	Total affected	Alopecia	1,25D binding	VDR mutation	References
F1	IIB, patient 1, 1a		No	20 months	1		2	No	+		[10,83,95]
	IIC, patient 2, 1b		No	5 months		1					[10,83]
F2	Patient		No	15 yr		1	1	No			[9]
F3	Patient 1, patient 2a, 2a		Yes	1 yr		1	2	Yes	+		[11,83,86,133]
	Patient 2, patient 2b, 2b		Yes	1 yr		1			+		[11,83,86,95]
F4	Patient		No	2 yr		1	1	No		Ile314Ser	[79,85,140]
F5	Patient, patient 3, 3, kindred 3, P3		Yes	10 months		1	1	Yes	+	Arg80Gln	[12,86,95,110]
F6	K.N.	Japanese	Yes	15 months		1	1	Yes	−		[96,160]
F7	Patient		No	18 months	1		1	Yes			[13]
F8	Patient		?	45 yr	1		1	?			[161]
F9	M.A., kindred 6, patient 6, 6	Arab	No	1 yr		1	1	Yes	−		[19,86]
F10	Patient		No	12 yr		1	1	No			[14]
F11	I.H., A1, patient 2, I.K., case 1	Arab	Yes	1 yr	1		2	Yes	−	Tyr295stop	[20,21,63,129]
	R.K., patient 1, A2, case 2		Yes	<1 yr		1	1	Yes	−	Tyr295stop	[21,29,63,129]
F12	Patient 4		Yes	?			1	Yes	±		[86,87]
F13	Patient 5		Yes	5 months			1	Yes	−		[86,87]
F14	Patient		Yes	2 yr	1		1	No			
F15	Patient A, patient 5		Yes	19 months	2	2	4	Yes	−		[15]
F16	Patient B, patient 4		Yes	8 months	1	1	2	Yes	±		[15]
F17	B patient		?				1	Yes	−		[21]
F18	S.H., patient 3, case 3, C1	Arab	No	6 months	1		2	Yes	−	Tyr295stop	[21,29,63,77]
	R.H., patient 4, case 4, C2		No	1 month	1			Yes	−	Tyr295stop	[21,29,63,77]
F19	D1	Haitian	Yes	?		3	3	Yes	+	Arg73Gln	[22,76]
	D2		Yes	?				Yes	+	Arg73Gln	[22,76]
F20	Kindred 7, patient 7, 7, P7						2	Yes	+	Arg 80Gln	[95,110,133]
F21	I.S., patient	Kuwait	Yes	1 yr	1		1	No	−	Arg274Leu	[18,132,139]
F22	Patient	Hispanic	No	1 yr	1		1	Yes	±		[96]
F23	Patient	Saudi	Yes		1		1	Yes	+	Gly46Asp	[117,118]
F24	Patient 1, N.D.	Arab	Yes	4 months	1		1	Yes	−		[97,100]
F25	Patient 1	Japanese	Yes	2 months		1	2	Yes			[23]
F26	Patient 2	Japanese	No	2 months		1	1	Yes	+	Arg50Gln	[23,111]
F27	Patient 3	Japanese	No	2 months	1		1	Yes			[23]

TABLE III Compilation of HVDRR Cases—**Cont'd**

Family[a]	Patient name/ description[b]	Ethnic origin	Consanguinity	Onset age	Male	Female	Total affected	Alopecia	1,25D binding	VDR mutation	References
F28	Patient 2, M.T.	Persian-Jewish	Yes	9 months	1		1	Yes	–		[97,100]
F29	Patient, line 10	Saudi	Yes	13 months	1		2	Yes	–	Tyr295stop	[17,130,133]
F30	Line 15	Saudi							–	Tyr295stop	[130]
F31	G1	Arab	Yes		1		2	Yes	+	Gly33Asp	[76,89]
	G2		Yes		1			Yes	+	Gly33Asp	[76,89]
F32	Line 11, patient 1	Turkish	Yes	5 weeks		1	2	Yes	–	Gln152stop	[132,133,162]
	Line 11b, patient 2		Yes		1			Yes	–	Gln152stop	[130,162]
F33	Patient 1, patient 1a, patient 4a	Japanese	Yes	16 months	1		2	Yes	+	Arg50Gln	[111,112]
	Patient 2, patient 1b, patient 4b		Yes	16 months		1		Yes	+	Arg50Gln	[111,112]
F34	E1	Arab	Yes		1		1	Yes	–	Tyr295stop	[26,77]
F35	F1	Arab	Yes			1	1	Yes	–	Tyr295stop	[26]
F36	H1	Arab	No			1	1	Yes	–	Tyr295stop	[26,77]
F37	J1	Arab	No		1		1	Yes	–	Tyr295stop	[26]
F38	K1	Arab	No		1		1	Yes	–	Tyr295stop	[26]
F39	L1	Arab	Yes			1	1	Yes			[26]
F40	Ro-VDR, brother				1		2	No			[101]
	Al-VDR, sister					1		No			[101]
F41	Ab-VDR				1		1	Yes			[101]
F42	Patient						1		–	Exon 7–9 deletion	[138]
F43	Child						1			Cys190Trp	[138]
F44	Patient II, case 2	Tunisian	Yes		1		1	Yes	+	Lys45Glu	[114,116]
F45	Propositus	Japanese-Brazilian	Yes		1		1	Yes	+	His35Gln	[24]
F46	Line 14	Moroccan	Yes				2	Yes	–	Arg73stop	[130]
F47	Patient I	Mauritius	No			1	1	Yes	+	Phe47Ile	[114,115]
F48	J.K.	English	No	16 months		1	1	Yes	+	None	[151,152]
F49	N1	Tunisian-Jewish	Yes	7 months	1		2	Yes	+	Arg80Gln	[25]
	N2		Yes	6 months		1		Yes	+	Arg80Gln	[25]
F50	Patient	Greek	No	9 months		1	1	Yes		Eliminate splice	[137]
F51	Patient	Turkish	Yes	3 months	1		2	No	+	His305Gln	[141,142]
	Sister		Yes			1		No	+	His305Gln	[141,142]

Continued

TABLE III Compilation of HVDRR Cases—**Cont'd**

Family[a]	Patient name/ description[b]	Ethnic origin	Consanguinity	Onset age	Male	Female	Total affected	Alopecia	1,25D binding	VDR mutation	References
F52	Patient 2					1	1	Yes	+	Arg391Cys	[140]
F53	Patient	French-Canadian			1		1	Yes	–	Arg30stop	[134]
F54	Patient	Brazilian	Yes		1	1	2	Yes	nd	Arg30stop	[135]
F55	Patient 1, B.G.	Greek	No	2 yr	1			Yes	–	Arg73stop	[131]
F56	Patient 2, A.H.	German	Yes	7 months	1			Yes	–	Create splice	[131]
F57	patient 3, A.J.	Indian	Yes	15 months	1		2	Yes	+	Gln259Pro	[131]
	patient 4, U.A.	Indian	Yes	2.5 yr		1		Yes	+	Gln259Pro	[131]
F58	patient	Hmong			1			Yes	+	Phe251Cys	[146]
F59	patient 1	Algerian	Yes	4 months	1		2	No	-	Trp286Arg	[144]
	patient 2	Algerian	Yes	5 months		1		No	-	Trp286Arg	[144]
F60	patient	Iranian	Yes		1			No	+	Glu420Lys	[149]
F61	patient					1		Yes	+	Gln317stop	[136]
F62	patient	Caucasian	No			1	1	Yes	nd	Glu329Lys/ 366delC	[150]
F63	patient	Saudi	Yes	20 months	1	2	3	No	+	Ile268Thr	[145]
F64	patient	Saudi	Yes					Yes	-	Arg30stop	Unpublished
F65	FC	Arab	Yes		1			Yes	-	Tyr295stop	Unpublished

[a]Number assigned to family for citation.
[b]Name or description of propositus used in references. In some cases multiple designations were used for the same patient.

marker of $1,25(OH)_2D_3$ responsiveness. However, the patients' fibroblasts failed to induce 24-hydroxylase activity following hormone treatment, demonstrating that they were resistant to high concentrations of $1,25(OH)_2D_3$. Subsequently, a number of other HVDRR cases were examined using cultured skin fibroblasts [15,84–86] or cells derived from bone [87]. Some patients' fibroblasts lacked specific $[^3H]1,25(OH)_2D_3$ binding [15,84–87], similar to the cases reported by Feldman *et al.* [20]. On the other hand, some fibroblasts exhibited normal $[^3H]1,25(OH)_2D_3$ binding but were unresponsive to $1,25(OH)_2D_3$ treatment [15,22, 85,87–89]. The conclusion reached was that the HVDRR syndrome was caused by cellular resistance to $1,25(OH)_2D_3$ action and was due to at least two types of defects in the VDR–one that impairs ligand binding, and one that retains ligand binding, but causes resistance to $1,25(OH)_2D_3$ by a defect downstream of binding. Griffin and Zerwekh [85] and Liberman *et al.* [86,87] also used 24-hydroxylase activity to demonstrate $1,25(OH)_2D_3$ resistance. Clemens *et al.* [84], on the other hand, showed that fibroblasts from HVDRR patients were not growth-arrested following hormone treatment in contrast to fibroblasts from healthy individuals that were growth-arrested. These early observations showed that cells from HVDRR patients were resistant to $1,25(OH)_2D_3$, and that there were a variety of abnormalities in the VDR.

As the number of reports on HVDRR increased, the heterogeneous nature of the defects in the VDR became more apparent. Hochberg *et al.* [21,29] reported the clinical findings in four patients from two unrelated families of Arab origin (F11,F18) who exhibited HVDRR and alopecia. A follow-up study by Chen *et al.* [63] showed that fibroblasts from three of these patients and a patient from an unrelated family from Germany (F17) had no $[^3H]1,25(OH)_2D_3$ binding and $1,25(OH)_2D_3$ treatment failed to induce 24-hydroxylase activity. Pike *et al.* [90] used a radioligand immunoassay [91] and a monoclonal antibody to the chick VDR [92–94], to demonstrate the presence of an immunoreactive protein in cell extracts from fibroblasts of HVDRR patients that exhibited no ligand binding. The authors speculated that the lack of $[^3H]1,25(OH)_2D_3$ binding in these patients was not due to defective synthesis of the VDR protein, but was due to defects in the VDR LBD that prevented ligand binding [90].

Liberman *et al.* [88] described four cases (F1,F3,F5,F20) with normal ligand binding and $1,25(OH)_2D_3$ resistance. Two of the cases (F5, F20) exhibited VDRs with a low affinity for DNA similar to the F19 and F31 families. Gamblin *et al.* [95] examined $1,25(OH)_2D_3$ induction of 24-hydroxylase activity in F5 and F20 fibroblasts and demonstrated complete hormone resistance. In the other cases, F1 and F3, Liberman *et al.* [88] demonstrated that the VDRs had a reduced ability to localize to the nucleus despite showing a normal affinity for DNA. Gamblin *et al.* [95] further showed that the F1 and F3 fibroblasts exhibited 24-hydroxylase activity when exposed to high concentrations of $1,25(OH)_2D_3$. Patients F1 and F3, whose fibroblasts showed a response to high concentrations of $1,25(OH)_2D_3$ *in vitro,* also exhibited a calcemic response to high doses of calciferols *in vivo*. Castells *et al.* [96] also reported on a patient (F22) who had sparse hair, rickets, and high circulating $1,25(OH)_2D_3$ levels. Studies of the VDR from the patient's fibroblasts showed that the receptor had a decreased affinity for $[^3H]1,25(OH)_2D_3$. The patient showed a marked improvement after treatment with extremely high doses of $1,25(OH)_2D_3$, apparently overcoming the low affinity binding abnormality in the VDR.

Based on the concept that VDR binding to DNA is essential for activity, Hirst *et al.* [22] showed that defective DNA binding could be the cause of resistance in cases that had normal ligand binding. In a study of a family from Haiti (F19) with two sisters with HVDRR and an unaffected sister, they showed that the fibroblasts from the affected individuals had normal $[^3H]1,25(OH)_2D_3$-binding but were resistant to $1,25(OH)_2D_3$ treatment. The fibroblasts from the two sisters with HVDRR, despite normal $[^3H]1,25(OH)_2D_3$ binding, were unresponsive to $1,25(OH)_2D_3$ treatment. The authors further demonstrated that the VDR from the patient's fibroblasts exhibited a significant decrease in affinity for general DNA. Using calf thymus DNA-cellulose chromatography, the VDR from the unaffected sister bound strongly to DNA requiring high concentrations of KCl for elution (170–173 mM KCl), whereas the patient's VDR bound weakly to DNA and eluted at lower concentrations of KCl (105–109 mM). A subsequent study by Malloy *et al.* [89] demonstrated a similar DNA binding defect in the VDR from HVDRR patients (F31) who had normal $[^3H]1,25(OH)_2D_3$ binding. DNA-cellulose chromatography clearly revealed that the patient's VDR had a low affinity for DNA. Furthermore, the authors showed that the cells from the parents had two forms of the VDR, one with a high affinity for DNA (eluting at 200 mM KC1) and the other with a low affinity for DNA (eluting at 100 mM KCl). This was the first clear evidence of heterozygosity in parents of HVDRR children. It was suspected that the defects in these cases would likely be due to point mutations in the DBD [22,89], that later proved to be correct [76].

B. Studies in Other Cell Types

In addition to studying cultured skin fibroblasts, a number of other cell types have been used to study HVDRR cases. These include peripheral mononuclear cells [97], phytohemagglutinin (PHA)-stimulated lymphocytes [98,99], myeloid progenitor cells [100], Epstein-Barr virus (EBV) immortalized B lymphoblasts [26,76,77,89], and HTLV-1 virus immortalized T lymphoblasts [101]. It is interesting to note that although EBV immortalized B lymphoblasts from normal subjects express wild-type VDR, they nevertheless fail to induce 24-hydroxylase activity or to show inhibition of cell growth in response to $1,25(OH)_2D_3$ [26]. On the other hand, PHA-stimulated lymphocytes and HTLV-1 immortalized T lymphoblasts from normal subjects do respond to $1,25(OH)_2D_3$ [101,102]. Using PHA-stimulated lymphocytes, Takeda *et al.* [99] showed that HVDRR can rapidly be diagnosed by the failure of $1,25(OH)_2D_3$ to inhibit DNA synthesis or induce 24-hydroxylase activity [98,99]. Takeda *et al.* [99] also showed that PHA-stimulated lymphocytes from parents of children with HVDRR express intermediate levels of 24-hydroxylase in response to $1,25(OH)_2D_3$, whereas other studies have failed to demonstrate defects in cells from parents or unaffected siblings.

V. MOLECULAR BASIS FOR HVDRR

A. First Description of a Genetic Defect in the Nuclear Receptor Superfamily

The biochemical and cellular data obtained from the earlier studies of HVDRR patients provided a framework to begin the search for the molecular cause of this disease. Investigations to determine the specific mutations in the VDR that cause HVDRR began shortly after the human VDR cDNA sequence was elucidated by Baker *et al.* [103]. During this same time period, the polymerase chain reaction (PCR) technique [104] was developed that provided a method to rapidly amplify genes for sequence analysis. The amino acid sequence of the VDR cDNA suggested the presence of highly conserved zinc-finger structures that were thought to be involved in DNA binding. The initial focus was HVDRR patients who had normal $[^3H]1,25(OH)_2D_3$ binding but abnormal binding to DNA, since it was suspected that this defect would arise from mutations in the VDR DBD. In 1988, Hughes *et al.* [76] used PCR to amplify exons of the VDR gene from DNA isolated from the F19 and F31 families that were defective in DNA binding [22]. The patients in the F31 family were shown to have a unique G to A single base change in exon 3 that encodes the second zinc module of the DBD. This missense mutation replaced arginine with glutamine at amino acid residue 73 (Arg73Gln) (Fig. 4). In the F19 family a G to A transition was identified in exon 2 that encodes the first zinc module of the DBD. This missense mutation changed glycine to aspartic acid at amino acid residue 33 in the first zinc finger module (Gly33Asp) (Fig. 4). Only the mutant sequences were found in the children with HVDRR. Their parents had a normal and a mutant sequence, demonstrating the genetic transmission and recessive nature of the disease. The study by Hughes *et al.* [76] was the first description of a genetic defect in any member of the steroid-thyroid-retinoid receptor gene superfamily. Mutations have now been found in many of the classical receptors including thyroid receptor (TR) [105], androgen receptor (AR) [106], estrogen receptor (ER) [107], glucocorticoid receptor (GR) [108], and mineralocorticoid receptor (MR) [109].

To determine whether the missense mutations found in these families were the cause of $1,25(OH)_2D_3$ resistance in the HVDRR patients, the Arg73Gln and the Gly33Asp mutations were generated in the wild-type VDR cDNA by site-directed mutagenesis [78]. The recreated mutant VDRs were expressed in COS-1 cells and exhibited normal $[^3H]1,25(OH)_2D_3$ binding and weak binding to DNA similar to the VDR in the patient's fibroblasts. In addition, Sone *et al.* [78] demonstrated that the mutant VDRs were transcriptionally inactive in cotransfection experiments in CV-1 cells. Using an osteocalcin-CAT reporter plasmid, they showed that CAT activity could be induced by the wild-type VDR but not by the Arg73Gln or the Gly33Asp mutant VDRs [78]. These data proved that the missense mutations caused the VDR to be transcriptionally inactive and were the cause of $1,25(OH)_2D_3$ resistance in the patients.

Since the original report by Hughes *et al.* [76], a number of VDR mutations have been identified in patients with HVDRR. Over 100 cases of HVDRR have been recorded, and a number of these have been analyzed at the biochemical and molecular level [3] (Table III). Several genetic abnormalities have been found in the VDR gene, mainly missense and nonsense mutations, but also splice site mutations and a gene deletion. A description of these mutations and the consequences of the abnormality in the VDR will be discussed below.

B. Mutations in the VDR DNA Binding Domain (DBD)

1. Description of DBD Mutations

Since the initial report by Hughes *et al.* [76], a number of mutations have been identified in the VDR DBD.

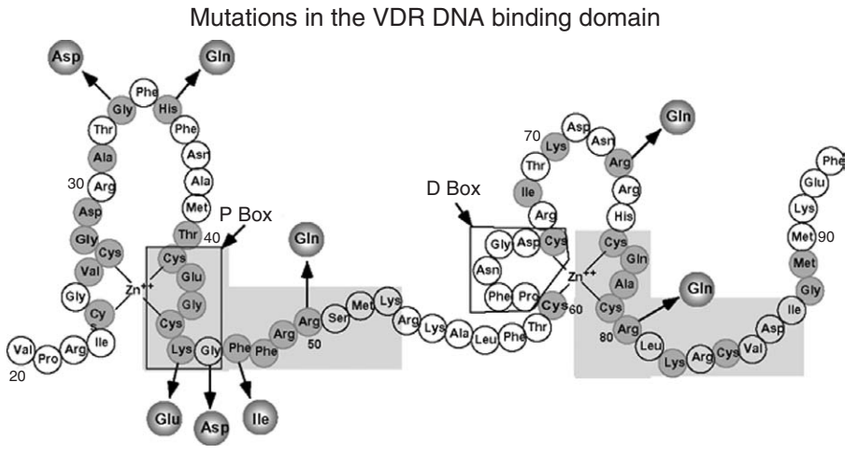

FIGURE 4 Model of the DNA-binding domain of the VDR and location of mutations causing HVDRR. The two zinc finger modules and the amino acid composition of the DBD are shown. Conserved amino acids are depicted as shaded circles. Numbers specify amino acid number.

The location of these mutations within the DBD is illustrated schematically in Fig. 4. Sone *et al.* [110] examined the VDR from two unrelated patients (F5 and F20) previously shown to exhibit a ligand-binding positive and low affinity DNA-binding phenotype by Liberman *et al.* [86,88]. In both patients, a G to A missense mutation was identified in exon 3. The mutation changed arginine to glutamine at amino acid 80 in the second zinc-finger module (Arg80Gln). The recreated Arg80Gln mutant receptor had a high affinity for [^{3}H]1,25(OH)$_2$D$_3$, bound weakly to DNA, and was unable to activate gene transcription from a reporter plasmid, demonstrating that this molecular defect is the cause of HVDRR in these cases [110]. Malloy *et al.* [25] also identified the same Arg80Gln mutation in two siblings with HVDRR (F49). The F49 family and the families (F5, F20) described by Sone *et al.* [110] both had origins in North Africa, however, no genetic relationship between these families could be established.

Saijo *et al.* [111] described a DBD mutation in three HVDRR patients from two unrelated families (F26 and F33) of Japanese origin. Earlier investigations showed that fibroblasts from the patients had normal [^{3}H]1,25-(OH)$_2$D$_3$ binding but the VDR exhibited abnormal nuclear binding [98,112,113]. A G to A mutation was found in exon 3 that converted arginine to glutamine at amino acid 50 in the at the base of the first zinc finger module (Arg50Gln). The children's parents were identified as carriers of both the normal and mutant alleles using single strand conformational polymorphism (SSCP) [111]. No data were provided to confirm the functional consequences of this defect on the VDR.

A missense mutation in the first zinc module of the VDR DBD that changed a histidine to glutamine at amino acid 35 (His35Gln) was identified by Yagi *et al.* [24]. The mutation replaced a positively charged amino acid with a neutral one. The VDR from the patient's cells (F45) exhibited normal 1,25(OH)$_2$D$_3$ binding but the receptors exhibited low affinity binding to DNA. The patient's fibroblasts were transiently transfected with a VDRE-CAT reporter plasmid to test for 1,25(OH)$_2$D$_3$ responsiveness. The transformed cells were unable to induce gene transcription. However, when the patient's fibroblasts were cotransformed with the wild-type VDR cDNA and the reporter plasmid, the cells acquired the ability to respond to hormone.

Two mutations in the VDR DBD were reported by Rut *et al.* [114]. One patient (F47) described previously by Lin and Uttley [115] had an A to G mutation in exon 2. The missense mutation resulted in lysine being replaced by glutamic acid at amino acid 45 (Lys45Glu). In the same study, Rut *et al.* [114] examined the VDR gene in a patient (F44) described previously by Simonin *et al.* [116]. They identified a unique T to C base change in exon 2 that resulted in phenylalanine being replaced by isoleucine at amino acid 47 (Phe47Ile). The recreated mutant VDRs exhibited normal ligand binding, but were transcriptionally inactive [114].

Lin *et al.* [117] examined the VDR gene for mutations in a patient (F23) with HVDRR previously described by Sakati *et al.* [118]. A unique G to A base change was found in exon 2. The mutation resulted in a glycine being changed to an aspartic acid at amino acid 46 (Gly46Asp). The recreated Gly46Asp mutant

VDR exhibited the characteristics of a DBD mutation in that the mutant receptor bound [^{3}H]1,25(OH)$_2$D$_3$ normally, but displayed a reduced affinity for DNA. The mutant receptor was also shown to be transcriptionally inactive in reporter gene assays. The authors used PCR and a restriction fragment length polymorphism (RFLP) generated by the mutation to demonstrate that the patient was homozygous for the mutation and the patient's father was a carrier of the mutant allele. In contrast to the other DBD mutations described above, the mutation at Gly46 occurs in an amino acid that is not well conserved in the steroid-thyroid-retinoid receptor superfamily. However, Gly46 is conserved among receptors that form heterodimers with RXR proteins such as TR and RAR.

2. Structural Analysis of DBD Mutations

As of this writing, the crystal structure of the VDR DBD has not been reported. However, crystallographic studies of the GR [119], RXR, and TR [120] DBD structures have been elucidated and, based on these studies, one can extrapolate the alterations created by the mutations to the VDR DBD [121,122]. Crystallographic analyses of the GR demonstrate that amino acids 457–469 (corresponding to residues 38–50 in the VDR) form an α-helix which joins the two zinc finger modules. This α-helix packs perpendicularly with a second α-helix at the base of the second zinc finger. Together, the hydrophobic residues of these two α-helices comprise the hydrophobic core of the DBD. Lys45Glu and Gly46Asp mutations are located in the P-box (aa residues 42–46), a region of the receptor likely important in contacting the DNA bases and determining the specificity of the receptor for specific VDREs (Fig. 4). Rut *et al.* [114] proposed that the Lys45Glu mutation would disturb the hydrogen bonding between Lys45 and a guanine nucleoside in the VDRE half-site. The conversion of glycine to aspartic acid, a bulky, charged amino acid, probably leads to unfavorable electrostatic interactions with the negatively-charged phosphate backbone of the DNA helix that may prevent the receptor from contacting specific nucleotide bases in the VDRE. Alternatively, the Gly46Asp mutation may eliminate the ability of the VDR to specifically recognize VDREs [117]. Similarly, the Gly33Asp mutation is expected to repel the negatively-charged phosphate backbone due to the negatively-charged aspartic acid [114]. On the other hand, the substitution of glycine for histidine in the His35Gly mutation most likely eliminates a hydrogen bond donated from the positively-charged histidine to the phosphate of a guanine nucleoside in the VDRE [114]. In the Phe47Ile mutation, the loss of the phenylalanine ring structure may disrupt the integrity of the hydrophobic core of the DBD. The mutation may obstruct the formation of the proposed α-helical structure at the base of the first zinc finger such that the VDR could not bind normally to its VDRE [114].

It is interesting to note that four of the DBD mutations, Lys45Glu, Gly46Asp, Phe47Ile, and Arg50Gln, occur in a LysXxxPhePhe[Lys/Arg]Arg sequence motif, which has been identified as a binding site for calreticulin [123–125]. Calreticulin binds to the VDR and cotransfection of calreticulin expression plasmids with a VDRE/RARE-luciferase reporter construct that causes a decrease in the reporter gene activation by VDR [124]. Since calreticulin may modulate VDR transactivation, disruption of the calreticulin binding site could lead to a decrease in VDR function. The effect of these mutations on calreticulin actions on the VDR has not been investigated.

C. Mutations Causing Premature Termination of the VDR

1. Stop Mutations

The first molecular analysis of three HVDRR cases (F18, F34, F36) that exhibited no [^{3}H]1,25(OH)$_2$D$_3$ binding was reported by Ritchie *et al.* [77]. A single unique C to A base change was found in exon 8 that changed the codon for tyrosine (TAC) to an ochre termination codon (TAA) (Tyr295stop) (Fig. 5). The location of the premature stop at amino acid 295 truncates 132 aa of the carboxy terminus of the VDR, which results in the deletion of a major portion of the LBD, thereby creating the ligand-binding negative phenotype. The recreated mutant VDR with a molecular size of 32,000 daltons was unable to bind [^{3}H]1,25(OH)$_2$D$_3$ and failed to activate gene transcription, demonstrating that this mutation was the cause of the hormone resistant state in the three patients. The Tyr295stop mutation was the first stop mutation identified in the VDR. The location of this mutation and other mutations that cause premature termination of the VDR is shown in Fig. 5 and summarized in Table III.

The three families studied by Ritchie *et al.* [77] and four additional families (F35, F37, F38, and F39) that comprise a large kindred where consanguineous marriages were common was analyzed by Malloy *et al.* [26] (Table III). A total of 8 children from this kindred exhibited HVDRR with alopecia. All of the affected children were homozygous for the Tyr295stop ochre mutation, and their parents were heterozygous as determined by analysis of a *Rsa* I restriction fragment length polymorphism (RFLP) created by the mutation [26]. Interestingly, the 32,000 molecular weight truncated protein that is predicted to be produced by this mutation

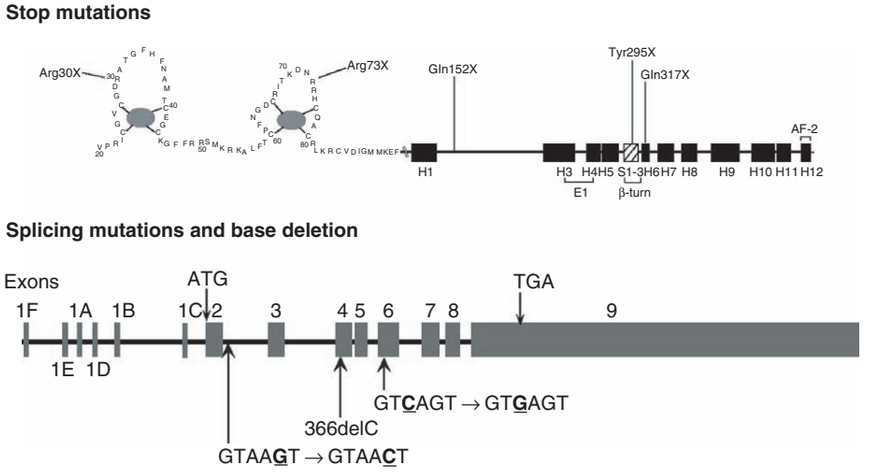

FIGURE 5 Mutations that cause premature termination of the VDR. The location of the nonsense mutations causing HVDRR are illustrated in the VDR protein. The location of the splice site mutations and the single base deletion are depicted in the VDR gene.

could not be detected in extracts of cultured dermal fibroblasts using Western blots. In addition, in all but one case, the VDR mRNA was undetectable on Northern blot using RNA isolated from the cultured fibroblasts or EBV-transformed lymphoblasts from the patients. The absence of mRNA transcripts has been reported in other genetic diseases where a premature stop mutation has been found [126–128] and, in this case, apparently accounts for the absence of the mutant VDR protein.

Since the reports by Ritchie *et al.* [77] and Malloy *et al.* [26], a number of other premature stop mutations have been identified in the VDR from patients with HVDRR [3]. The locations of these mutations are shown in Fig. 5. Family F11 described in earlier papers [20,21,29,63] had two affected children with HVDRR who exhibited the ligand-binding negative phenotype. This family of Christian Arabs live in the same town as the extended kindred (F18, F34–F39) described above who are Muslim Arabs. Although there is no known genetic relationship between family F11 and the large kindred, the Tyr295stop mutation is the cause of HVDRR in this family as well [129]. The Tyr295stop mutation was also identified by Wiese *et al.* [130] in two related patients (F29, F30) from Saudi Arabia who were previously studied by Bliziotes *et al.* [17]. These patients are apparently unrelated to the other families with the same mutation. It is not clear whether Tyr295stop mutation represents a mutational "hot spot" in the VDR gene or whether these cases all descended from a common founder mutation.

A patient with HVDRR from a Moroccan family (F46) was examined for mutations in the VDR gene by Wiese *et al.* [130]. At the cellular level, no [^{3}H]1,25(OH)$_2$D$_3$ binding was detected in the patient's cells. The authors discovered an opal mutation (CGA to TGA) in which a C to T substitution introduced a premature stop codon at amino acid 73 (Fig. 5). The Arg73stop mutation truncates the receptor in the middle of the second zinc finger module, resulting in the production of a 72 amino acid polypeptide. No evidence for the presence of a truncated VDR in the patient's cells was demonstrated since both the LBD and monoclonal antibody binding sites were deleted in the mutant protein. Interestingly, the Arg73stop mutation, occurs in the same codon that gives rise to the Arg73Gln mutation, but at a different nucleotide base [76]. In the Arg73stop mutation, CGA is mutated to TGA, while in the Arg73Gln mutation the CGA is mutated to CAA. The Arg73stop mutation has also been identified in a young boy (F56) from Greece who had HVDRR with alopecia [131].

A premature stop codon caused by an amber mutation (CAG to TAG) in exon 4 was found in a Turkish patient (F32) with HVDRR by Kristjansson *et al.* [132]. Previous studies using fibroblasts from this patient had demonstrated an absence of ligand binding and 1,25(OH)$_2$D$_3$ responses [133]. This premature stop mutation occurs at Gln152 and deletes 306 amino acids of the VDR (Fig. 5). As expected, the Gln152stop mutant VDR was unresponsive to 1,25(OH)$_2$D$_3$ in gene activation assays. The Gln152stop mutation was also identified by Wiese *et al.* [130] in a HVDRR patient (F32) previously reported by Barsony *et al.* [133].

Studies of a young boy (F53) of French-Canadian origin with HVDRR and alopecia has been reported

by Zhu *et al.* [134]. The patient's fibroblasts lacked specific [^{3}H]1,25(OH)$_2$D$_3$ binding and failed to exhibit 24-hydroxylase mRNA induction after treatment with up to 100 n*M* 1,25(OH)$_2$D$_3$. Northern blotting showed that the cells expressed a normal-sized VDR mRNA, but Western blotting failed to detect any protein. A C to T base substitution was located in exon 2, which changed the codon for arginine (CAG) at amino acid 30 to an opal stop codon (TAG) (Arg30stop) (Fig. 5). The 29 amino acid polypeptide represents the shortest truncated protein produced by a premature stop mutation in the VDR. The mutation eliminated 398 amino acids including the LBD, the monoclonal antibody epitope, the second zinc finger module, and a portion of the first zinc finger module. The same Arg30stop mutation was also identified in two children with HVDRR from a family (F54) living in Brazil [135]. One child died at 4 yr of age due to cadiorespiratory insufficiency. Interestingly, the parents, who were first cousins, were phenotypically normal, but had slightly elevated levels of serum 1,25(OH)$_2$D. The mean value for the father was 73 pg/ml and for the mother 93 pg/ml (normal range 20–80 pg/ml). The mildly elevated 1,25(OH)$_2$D values raise the possibility of mild vitamin D resistance in the heterozygotic parents, a finding that has not been documented previously in other parents of HVDRR children.

An Iranian girl (F61) whose cells were unresponsive to high doses of 1,25(OH)$_2$D$_3$ and exhibited no [^{3}H]1,25(OH)$_2$D$_3$ binding was shown to have a mutation in exon 8 that changed the codon for glutamine to a termination codon (Gln317stop) (Fig. 5) [136].

2. Splice Site Mutations

Hawa *et al.* [137] examined the VDR in a young Greek girl (F50) with HVDRR and alopecia. Using RT-PCR and DNA sequencing, they showed that the patient's RNA sequence diverged from the wild-type sequence at nucleotide 147. The sequence from exon 3 that encodes the second zinc finger module was deleted, and the sequence that followed was from exon 4. Sequence analysis of the VDR chromosomal gene found no mutations in the exons, however, a G to C base change was found in the 5′ end of intron E (Fig. 5). This single nucleotide change converts the wild-type sequence from GTGAGT to GTGACT and eliminates the 5′ donor splice site (consensus sequence: GT(A/G)(A/T)G(T/A/C)). The loss of the 5′ donor splice site caused exon 3 to be skipped in the processing of the VDR transcript and introduced a reading frameshift that resulted in a premature stop codon in exon 4. The mutant protein contains 92 amino acids of the wild-type sequence plus 6 amino acids due to the frameshift (Glu92fs) (Fig. 5). The shortened VDR had no [^{3}H]1,25(OH)$_2$D$_3$ binding and failed to induce 24-hydroxylase activity.

A splice site mutation was also identified in a German patient (F56) with HVDRR and alopecia [131]. Studies of the patient's fibroblasts showed absent [^{3}H]1,25(OH)$_2$D$_3$ binding and failure to induce 24-hydroxylase activity with 1,25(OH)$_2$D$_3$ treatment. In this case, a cryptic 5′ donor splice site was generated in exon 6. The mutation in this case, a C to G transition, changed the sequence from GTCAGT to GTGAGT. This single base change did not alter the amino acid coding sequence in exon 6, but introduced a splice site that could be recognized by the spliceosome complex during RNA processing. As a result, the mutation caused a 56 bp deletion in exon 6, which led to a frameshift 15 bases into exon 7. The mutant protein contains 233 amino acids of the wild-type sequence and an additional 4 amino acids due to the frameshift (Leu233fs) (Fig. 5). The mutation caused the truncation of 194 amino acids of the VDR leading to a loss of 1,25(OH)$_2$D$_3$ binding and hormone responsiveness.

3. Gene Deletion

There has been one report of patient (F42) with a major structural defect in the VDR gene that was the cause of HVDRR [138]. The defect, found by PCR and Southern blotting, was a deletion in the VDR gene that eliminated exons 7, 8, and 9. This is the only case thus far reported in which a partial deletion in the VDR gene has been shown to cause HVDRR.

All of the nonsense mutations and the splice site mutations that lead to frameshifts, as well as the gene deletion, result in truncated VDRs. Although some of the mutants VDRs may have intact DBDs, the loss of the LBD and therefore its ability to bind 1,25(OH)$_2$D$_3$, associate with RXR and interact with coactivators makes the receptors nonfunctional and causes complete hormone resistance.

D. Mutations in the VDR Ligand-Binding Domain (LBD)

1. Mutations That Affect 1,25(OH)$_2$D$_3$ Binding

The first missense mutation found in the VDR LBD that resulted in an amino acid substitution was described by Rut *et al.* [139] and Kristjansson *et al.* [132]. The patient from Kuwait (F21) had HVDRR, but did not have alopecia. Preliminary studies by Fraher *et al.* [18] on the patient's fibroblasts showed absent [^{3}H]1,25(OH)$_2$D$_3$ binding. However, a later study by Rut *et al.* [139] showed that the fibroblasts contained normal amounts of [^{3}H]1,25(OH)$_2$D$_3$ binding, but the affinity of the receptor for 1,25(OH)$_2$D$_3$ was significantly reduced ($K_d = 1 \times 10^{-9}$M) compared to normal controls

($K_d = 0.7 \times 10^{-10}$M). The patient's fibroblasts failed to induce 24-hydroxylase activity when treated with high doses of $1,25(OH)_2D_3$ [18, 139]. Molecular analysis of the VDR gene identified a unique G to T missense mutation in exon 7 [132, 139]. This mutation resulted in replacement of a positively-charged arginine residue by a neutral-charged leucine at amino acid 274 (Arg274Leu) (Fig. 6). The Arg274Leu mutation is located in helix H5. In transactivation assays, the recreated Arg274Leu mutant VDR was relatively resistant to vitamin D, requiring approximately 1000-fold more $1,25(OH)_2D_3$ to activate gene transcription than the wild-type receptor [132]. Although the resistance caused by the defective VDR could be overcome by treating with high concentrations of $1,25(OH)_2D_3$ *in vitro*, the patient failed to respond to massive does of the hormone and eventually died of pneumonia.

Whitfield *et al.* [140] analyzed the VDR from a girl (F4) who had the classic symptoms of HVDRR, but without alopecia. The patient's fibroblasts were originally examined by Griffin and Zerwekh [85] who showed that the cells had normal $1,25(OH)_2D_3$ binding but had defective induction of 24-hydroxylase activity. Sequencing of the VDR gene uncovered a T to G substitution in exon 8 that changed isoleucine to serine at amino acid 314 (Ile314Ser) (Fig. 6). The Ile314Ser mutation occurs in H7. The mutation causes a subtle defect in heterodimerization with RXR and decreased response to $1,25(OH)_2D_3$ in transactivation assays. This patient showed a nearly complete cure when treated with pharmacological doses of 25-hydroxyvitamin D_3.

A missense mutation in the VDR LDB was described by Malloy *et al.* [141]. The patient suffered from HVDRR and two other rare genetic disorders, congenital generalized lipoatrophic diabetes (Berardinelli-Seip syndrome) and persistent müllerian duct syndrome [142]. The patient, a Turkish boy (F51), had rickets and high $1,25(OH)_2D_3$ levels, but did not have alopecia. He was treated with extremely high doses of calcitriol (Rocaltrol 12.5 μg/day), which eventually normalized his serum calcium and ultimately improved his rickets. However, the child died of apparently unrelated problems. The patient's fibroblasts expressed normal VDR levels, but the affinity for $1,25(OH)_2D_3$ was decreased by about twofold. The patient's fibroblasts required approximately fivefold more $1,25(OH)_2D_3$ to induce 24-hydroxylase mRNA compared to control cells. Sequence analysis of the VDR gene uncovered a C to G missense mutation in exon 8. This mutation leads to replacement of histidine by glutamine at amino acid 305 (His305Gln) (Fig. 6). The His305Gln mutation occurs in the interhelical loop between H6-H7. Interestingly, $[^3H]1,25(OH)_2D_3$-binding studies of the reconstructed mutant protein demonstrated an eight-fold lower affinity for $1,25(OH)_2D_3$ compared to the wild-type VDR when the assays were performed at 24°C (vs two-fold at 0°C). In gene transactivation assays, the His305Gln mutant VDR required five-fold more $1,25(OH)_2D_3$ to achieve the same level of activity as the wild-type VDR. RFLP analysis with *AlwN* I showed that the patient and a sibling with HVDRR were homozygous for the mutation and that their parents were heterozygous. The boy's sister, who also had HVDRR and the same mutation in the VDR, did not exhibit the other genetic defects. No explanation was forthcoming for the presence of three genetic defects in a single individual. It is unclear how the His305Gln mutation in the VDR is related, if at all, to the two other genetic abnormalities that were present in this child. The congenital total lipodystrophy has been shown to be caused by a mutation in the seipin gene [143] on chromosome 11, not chromosome 12 where the VDR is located. A genetic cause for the persistent müllerian duct syndrome in this patient has not been found.

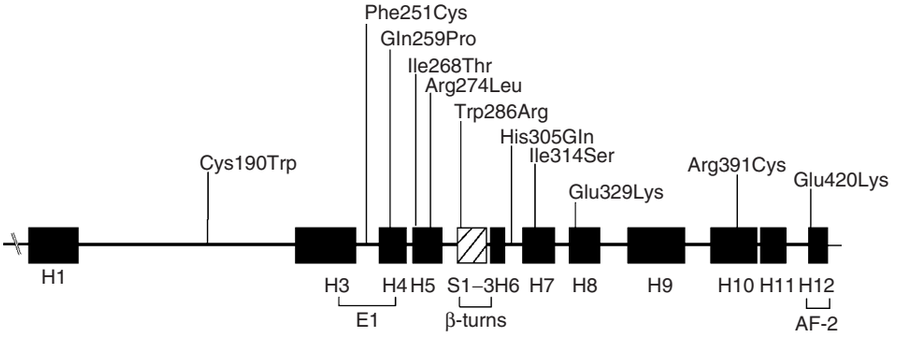

FIGURE 6 Schematic illustration of the ligand-binding domain of the VDR and location of amino acid substitutions causing HVDRR. The α-helices (H1-H12) of the VDR LBD are depicted as shaded rectangles and the β-turns are drawn as a hatched rectangle. The loops connecting the helices are drawn as solid lines. The E1 and AF-2 regions are shown below the α-helices.

An Algerian boy and his younger sister (F59) both with HVDRR without alopecia were shown to have a T to C mutation in exon 7 that changed tryptophan to arginine at amino acid 286 (Trp286Arg) (Fig. 6) [144]. Trp286 is the only tryptophan in the VDR. The patient's fibroblasts expressed a normal size VDR protein and normal length VDR mRNA but no specific [^{3}H]1,25(OH)$_2$D$_3$ binding was observed, and the cells were totally unresponsive to 1,25(OH)$_2$D$_3$ treatment. The Trp286Arg mutation is located in the β1 sheet of the three-stranded β sheet between helices H5-H6.

A young Saudi Arabian girl with HVDRR, but without alopecia, was homozygous for a unique T to C mutation in exon 7 of the VDR gene that changed the codon for isoleucine to threonine at amino acid 268 (Ile268Thr) [145]. Based on the crystallographic studies of the VDR LBD, Ile268 located in helix H5 directly interacts with 1,25(OH)$_2$D$_3$. The Ile268Thr mutant VDR exhibited a ~tenfold lower affinity for [^{3}H]1,25(OH)$_2$D$_3$ compared to the WT VDR consistent with its interaction with the ligand. However, in transactivation assays, the Ile268Thr mutant required ~100-fold higher concentrations of 1,25(OH)$_2$D$_3$ to stimulate gene transcription compared to the WT VDR. The Ile268Thr mutant also exhibited a marked decrease in RXR binding compared to the WT VDR. Ile268 is also involved in the hydrophobic stabilization of helix H12. Consistent with this activity, the Ile268Thr mutant required ~100-fold more 1,25(OH)$_2$D$_3$ to promote binding to the coactivators SRC-1 and DRIP205. These cumulative defects cause 1,25(OH)$_2$D$_3$ resistance and results in the syndrome of HVDRR in the patient [145].

A missense mutation was also described in the VDR LBD by Thompson *et al.* [138]. The HVDRR patient (F43) was shown to have a mutation in exon 5. In this case, a cysteine was changed to tryptophan at amino acid 190 (Cys190Trp) (Fig. 6). The Cys190Trp mutation occurs in the loop between H1 and H3. Further details about this case were not included in this preliminary report, however, deletion of this region of the VDR LBD has been shown to have no effect on ligand binding or transactivation. Also, we have recreated Cys190Trp mutation and have shown that it does not affect transactivation (Malloy unpublished). These data suggest that the Cys190Trp mutation was unlikely to be the sole cause of HVDRR in this case.

a. Structural Analysis of LBD Mutations That Affect 1,25(OH)$_2$D$_3$ Binding Since the crystal structure of the holo-VDR has been reported, a more definitive explanation of the effects of the LBD mutations on the VDR is possible. In one patient (F21), Arg274 was mutated to Leu (Arg274Leu) and resulted in a 1,000-fold decrease in ligand binding affinity [132]. This can now be explained by the fact that Arg274 is a contact point for the 1-hydroxyl group of 1,25(OH)$_2$D$_3$ [50]. Thus, the mutation to leucine alters the contact point and lowers the binding affinity of the VDR for 1,25(OH)$_2$D$_3$. In a different patient (F51), His305 was mutated to Gln (His305Gln) that lowered the binding affinity for 1,25(OH)$_2$D$_3$ by about five–tenfold [141]. This can now be explained by the fact that His305 is a contact point for the 25-hydroxyl group of 1,25(OH)$_2$D$_3$ [50]. Again, the mutation to glutamine alters the contact point and lowers the binding affinity of the VDR for 1,25(OH)$_2$D$_3$. In a third patient (F59), Trp286 was mutated to Arg (Trp286Arg) that totally abolished ligand binding [144]. Trp286 makes contact with the α face of the C ring in 1,25(OH)$_2$D$_3$, and is involved in forming the hydrophobic channel where the conjugated triene connecting the A and the C rings fits [50]. The Trp286Arg mutation alters the contact point with the ligand and causes resistance to 1,25(OH)$_2$D$_3$ [144]. It is evident from these data that the disruption of a ligand contact point can be the basis for HVDRR (see Chapter 15).

2. MUTATIONS THAT AFFECT VDR-RXR HETERODIMERIZATION

As mentioned above, the VDR requires heterodimerization with RXR for activity. Disruption of this protein: protein interaction can thereby cause 1,25(OH)$_2$D$_3$ resistance. The first patient found to have a mutation in the VDR that disrupted VDR-RXR heterodimerization was described by Whitfield *et al.* [140]. The patient was a young girl (F52) who had HVDRR and alopecia. Sequencing showed that the patient had a C to T base change in exon 9 that converted an arginine to cysteine at amino acid 391 (Arg391Cys) (Fig. 6). The Arg391Cys mutation is located in helix H10. Ligand binding was unaffected but transactivation of a reporter gene by the Arg391Cys mutant VDR required higher than normal concentrations of 1,25(OH)$_2$D$_3$ for activity. However, when RXR was co-transfected in the assays, the transactivation activity could be restored to normal levels. VDR-RXR interactions and VDRE binding were further examined using electrophoretic mobility shift assays (EMSA). The Arg391Cys mutant exhibited a lower capacity for forming a VDR-RXR-VDRE complex than the wild-type VDR. The Arg391Cys mutation thus reduces the interaction between the VDR and RXR. By increasing the RXR protein concentration, the affinity defect could be overcome, and the transactivation rescued. This study demonstrated the importance of both 1,25(OH)$_2$D$_3$ binding and RXR heterodimerization in VDR mediated gene transactivation. This was the first report of a mutation in the VDR that interfered with RXR binding and caused HVDRR.

Two siblings, a brother and sister from India (F57) that had HVDRR with alopecia, were studied by Cockerill *et al.* [131]. The children's parents were first cousins. Using cultured fibroblasts, [^{3}H]1,25(OH)$_2$D$_3$ binding was found to be normal but 1,25(OH)$_2$D$_3$ induction of 24-hydroxylase activity was absent. DNA sequence analysis revealed a single base alteration in exon 7 that changed glutamine to proline at amino acid 259 (Gln259Pro) (Fig. 6). The Gln259Pro mutation occurs in helix H4. Although Gln259Pro had no apparent affect on ligand binding there was evidence of impaired VDR-RXR-VDRE formation. Whereas the wild-type VDR formed two complexes (complex A and complex B) in the EMSA, the Gln259Pro mutant VDR showed a reduction in the formation of complex B and an enhancement in complex A. The authors speculated that the Gln259Pro mutation in the VDR affected protein:protein interactions, possibly by increasing the affinity of the receptor for an unidentified protein. In transactivation assays, the recreated Gln259Pro mutant VDR was functionally inactive.

Malloy *et al.* [146] examined the VDR in a young Hmong boy (F58) with HVDRR and alopecia. Analyses of the VDR demonstrated a normal size receptor on Western blots, however, the amount of VDR was decreased compared to normal. Northern blot analysis of 24-hydroxylase mRNA induction showed that the patient's fibroblasts were approximately 1,000-fold less responsive to 1,25(OH)$_2$D$_3$ than control fibroblasts confirming target organ resistance. A unique missense mutation was found in exon 6 that changed a phenylalanine to cysteine at amino acid 251 (Phe251Cys) (Fig. 6). In [^{3}H]1,25(OH)$_2$D$_3$ binding experiments, the recreated Phe251Cys mutant VDR exhibited a lower affinity for the ligand than wild-type VDR when assayed at 24°C. Using GST pull-down assays and yeast two-hybrid constructs, the Phe251Cys mutant VDR was shown to have reduced capacity to bind RXR. In transactivation assays, cotransfection of RXR partially restored the activity of the mutant receptor. The Phe251Cys mutation occurs in the E1 region (aa 244–263) of the VDR LBD. The E1 region overlaps the C-terminal portion of helix H3, loop 3–4 and the N-terminal portion of helix H4. This structural motif is highly conserved throughout the nuclear receptor superfamily. A cluster of hydrophobic amino acids within the E1 region is critical to the three-dimensional folding and formation of the ligand-binding pocket [147]. At the center of this region is an invariant aromatic phenylalanine residue which corresponds to Phe251 in the VDR. Since Phe251 is in such a critical site in the LBD, replacing the aromatic amino acid phenylalanine with a small hydrophilic cysteine residue likely disrupts the ligand-binding pocket of the VDR and interferes with the fundamental conformation required for optimal function. From the crystallographic studies, Phe251 does not form a direct contact point with the bound ligand. Indeed, at 4°C normal ligand binding was observed. However, at elevated temperatures, the binding affinity was severely decreased, suggesting that the Phe251Cys mutation disrupted the folding of the ligand-binding pocket [146].

a. Structural Analysis of VDR-RXR Heterodimerization Mutations Although the VDR-RXR dimer crystallographic studies have not been performed as of this writing, studies of RXRα, RAR-RXRα, and PPARγ-RXRα, show that the dimer interface is formed from helix H9 and helix H10 and the interhelical loops between H7–H8 and H8–H9 [50]. The Arg391Cys mutation occurs in helix H10 [140]. The mutation lowers the binding affinity for RXR causing 1,25(OH)$_2$D$_3$ resistance. However, in transactivation assays, the 1,25(OH)$_2$D$_3$ resistance could be overcome by overexpression of RXR [140].

The Phe251Cys mutation reduces the affinity of the VDR for 1,25(OH)$_2$D$_3$ and alters RXR binding [146]. The Phe251Cys mutation is located in the E1 region in the interhelical loop between H3–H4. This region of the VDR is not part of the dimer interface; however, the loop does appear to be positioned beneath the interhelical loop between H8–H9 and helix H9. The mutation apparently disrupts the formation of the dimer interface of the receptor and results in a decreased ability to heterodimerize with RXRα.

The Gln259Pro mutation is also located in the E1 region in helix H4. The Gln259Pro mutant did not affect 1,25(OH)$_2$D$_3$ binding but exhibited an abnormality in forming a complex on VDREs in EMSA [131]. A study by Whitfield *et al.* [148] has shown that a Gln259Gly mutant VDR binds ligand normally, but is defective in its ability to form a complex with RXR. They also showed that addition of increasing amounts of RXR could restore the transactivation capacity of the Gln259Gly mutant to near wild-type levels.

These single amino acid changes demonstrate the interdependency of ligand binding and heterodimer binding for transactivation.

3. Mutations That Affect Coactivator Binding

As noted above, the VDR also must recruit coactivators for transcriptional activity. It is now clear from crystallographic studies of the VDR and other members of the steroid receptor superfamily that repositioning of helix H12 is an essential event that occurs as a consequence of ligand binding and is necessary for

transactivation [50]. The repositioning of helix 12 leads to the formation of the hydrophobic cleft critical for coactivator binding. Therefore, disruption of this surface interface may cause hormone resistance and HVDRR.

A study by Malloy *et al.* [149] examined the VDR in a young boy (F60) with HVDRR. The patient did not have alopecia. The patient's fibroblasts exhibited normal ligand binding, but the cells were totally resistant to $1,25(OH)_2D_3$. A novel missense mutation was found in exon 9 that changed a glutamic acid to lysine at amino acid 420 (Glu420Lys) (Fig. 6). The Glu420Lys mutation is located in helix H12. The recreated Glu420Lys mutant VDR showed no defects in VDR-RXR heterodimerization or binding to VDREs. However, the mutation prevented the coactivator SRC-1 and DRIP205 from binding to the VDR. In transactivation assays, cotransfection of SRC-1 failed to restore transactivation by the mutant VDR. This case represents the first description of a naturally occurring mutation in the VDR that disrupts coactivator interaction and causes HVDRR [149].

The polar interactions that stabilize the repositioning of helix H12 involve a conserved salt-bridge between Lys264 in helix H4 and Glu420 in helix H12 and a hydrogen bond between Ser235 in helix H3 and Thr415 in helix H12. The Glu420Lys mutation prevents the correct repositioning of helix H12 after binding the ligand. The substitution of the negatively-charged glutamic acid (Glu420) with a positively-charged lysine residue (Lys420) would prevent the polar interaction with the positively-charged lysine (Lys264) salt bridge partner. A charge clamp formed by Lys246, Lys264, and Glu420 enables the VDR to recruit and bind coactivators through their LxxLL motifs. The Lys246 in helix 3 and Glu420 in helix H12 are thought to be indispensable for binding the LxxLL peptide on the coactivator. The Glu420Lys mutation disrupts coactivator binding and causes the hormone resistance seen in the patient.

4. OTHER MUTATIONS IN THE VDR LBD

There has been one report of a patient (F62) with HVDRR and alopecia who had two mutations in the VDR gene [150]. One mutation in exon 8 changed a glutamic acid to lysine at amino acid 329 (Glu329Lys) (Fig. 6) and the second mutation was a single base deletion of a cytosine at nucleotide 366 (366delC) (Fig. 5). The deletion of the cytosine results in a shift in the reading frame in exon 4 and leads to a premature termination signal in the VDR message. The premature termination signal truncates most of the LBD. The affect of the Glu329Lys mutation on VDR function was not reported.

E. HVDRR without Mutations in the VDR

Since the initial description of HVDRR as a genetic disorder, mutations in the VDR gene were suspected as the likely cause of $1,25(OH)_2D_3$ resistance. Although the VDR is the principle determinant in the $1,25(OH)_2D_3$ action pathway, it is possible that target organ resistance to $1,25(OH)_2D_3$ may result from mutations in other proteins that are essential to the transactivation process. The following cases highlight this possibility.

Hewison *et al.* [151] has described a case of HVDRR in which a mutation could not be found in the VDR. The patient (F48), a young girl of English descent, exhibited all of the hallmarks of HVDRR including alopecia. The patient's fibroblasts expressed a normal-sized VDR mRNA and exhibited normal $[^3H]1,25(OH)_2D_3$ binding. However, no induction of 24-hydroxylase activity was observed when the fibroblasts were treated with up to 1 μM $1,25(OH)_2D_3$. Although the fibroblasts were clearly resistant to $1,25(OH)_2D_3$, no mutations were found within the coding region of the VDR gene. The patient's VDR mRNA was reverse transcribed and amplified by PCR and then expressed in CV-1 cells. In transactivation assays, the patient's VDR exhibited a normal transactivation response to $1,25(OH)_2D_3$. These results clearly demonstrated that the patient's VDR was normal. The authors suggested that the tissue resistance was not due to a defect in the VDR and that the hormone resistance causing HVDRR was most likely the result of a mutation in an essential protein that participates in the $1,25(OH)_2D_3$ action pathway.

In a follow-up study of this interesting case, Chen *et al.* [152] proposed that the cause of $1,25(OH)_2D_3$ resistance was due to the abnormal expression of a VDRE-interacting hormone response element-binding protein. This protein is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family that binds to double-stranded DNA and modulates RNA processing. In New World primates the hormone response element-binding proteins have been shown to act as dominant negatives and are believed to cause target organ resistance to adrenal, gonadal, and vitamin D sterol/steroid hormones [153–158]. The fact that the patient had HVDRR without a mutation in the VDR highlights the importance of the complex machinery involved in VDR transactivation and that mutations in the hormone signaling pathway may result in $1,25(OH)_2D_3$ resistance.

In Cauca, Columbia, more than 200 patients have been diagnosed with a disease that somewhat resembles HVDRR without alopecia [159]. The patients exhibit lower limb deformities due to rickets but are

otherwise in good physical condition. Rickets limited to the lower extremities, as in these cases, have not been reported in other HVDRR families. The affected individuals have serum calcium levels that are within the normal range, but their serum $1,25(OH)_2D$ levels are unusually high suggesting target-organ resistance. Fibroblasts from two of the most severely affected patients showed a low induction of 24-hydroxylase activity by $1,25(OH)_2D_3$. However, no mutations were found in the VDR gene. Since the cause of vitamin D resistance in this instance was not due to mutations in the VDR and a functional response to $1,25(OH)_2D_3$ was demonstrated, it has not been clearly ascertained whether this entity is a variant of HVDRR. The high prevalence of the disease in the population and the localized distribution of the rickets raises the possibility of an environmental cause. These cases support the concept that target organ resistance to $1,25(OH)_2D_3$ may be due to mechanisms other than mutations in the VDR.

VI. THERAPY OF HVDRR

A. Vitamin D

As detailed in the preceding sections, HVDRR is almost always caused by heterogeneous mutations in the VDR that result in partial or total resistance to $1,25(OH)_2D_3$. Partially or totally inactive VDRs reduce calcium absorption from the intestine to the circulation and result in hypocalcemia. Low serum calcium levels lead to a decrease in bone mineralization and cause rickets. In order to cure rickets, calcium levels must be normalized. A number of therapies using calcium and active vitamin D metabolites aimed at increasing the serum calcium levels have been tried and the responses have varied widely. For the most part, it was thought that patients with HVDRR without alopecia were better responders to treatment with vitamin D preparations than those patients with alopecia [30]. In several of the earlier cases that were reported, patients without alopecia showed improvement both clinically and radiologically to the administration of pharmacological doses of vitamin D ranging from 5000 to 40,000 IU/day [9,10,79]; 20 to 200 μg/day of $25(OH)D_3$; and 17–20 μg/day of $1,25(OH)_2D_3$ [10]. Of the patients with HVDRR without alopecia, a few have had their VDR analyzed at the molecular level. Patient (F51) with the His305Gln mutation in the VDR LBD a contact point for the 25-hydroxyl group of $1,25(OH)_2D_3$ responded to 12.5 μg/day calcitriol [141, 142]. The treatment overcame the low affinity-binding defect and achieved adequate VDR occupancy to mediate normal $1,25(OH)_2D_3$ responses. Patient (F4) with the Ile314Ser mutation in the VDR LBD was treated with 1 mg/day of vitamin D_2 from age 2 to age 18 [79]. At age 20 following an uneventful pregnancy, the patient developed hypocalcemia and was treated successfully with 50 μg/day of $25(OH)D_3$. On the other hand, patient F21 with the Arg274Leu mutation in the VDR LBD, a contact point for the 1-hydroxyl group of $1,25(OH)_2D_3$, was unresponsive to treatment with 600,000 IU vitamin D; up to 24 μg/day of $1,25(OH)_2D_3$ (calcitriol); and 12 μg/day 1α $(OH)D_3$. The patient later died of pneumonia [18]. Fibroblasts from this patient exhibited no specific $[^3H]1,25(OH)_2D_3$ binding and were unresponsive to hormone treatment. However, the recreated Arg274Leu mutant VDR did exhibit transactivation activity at high doses of $1,25(OH)_2D_3$ [132].

In general, HVDRR patients with alopecia are more resistant to treatment with vitamin D metabolites. However, a small number of these patients have been treated successfully using vitamin D. Two patients showed signs of improvement when given vitamin D or $1\alpha(OH)D_3$ [14,160] and one patient responded to 25(OH)D as well as $1\alpha(OH)D_3$ [15]. $1\alpha(OH)D_3$ and $1,25(OH)_2D_3$ also were effective treatments in other cases [22,23,96,112,161], including patients with the Arg50Gln and Arg73Gln mutations [76, 111]. Two siblings (F32), with the Glu152stop mutation, showed no increase in serum calcium during high dose vitamin D treatment despite raising their circulating $1,25(OH)_2D$ levels to more than 100 times the mean normal range. However, notwithstanding their low serum calcium concentrations, healing of rickets and suppression of PTH was evident [162]. In one case, where vitamin D and $1,25(OH)_2D_3$ therapies failed, the patient responded to oral phosphorous treatment [11]. The molecular cause of HVDRR in this case has not been elucidated. In many cases when patients fail to respond to $1,25(OH)_2D_3$, intensive calcium therapy is used as described below.

B. Calcium

The most significant development in the treatment of HVDRR was first reported by Balsan *et al.* [163]. In their insightful study, they used long term intravenous (IV) calcium infusions to successfully treat a child with HVDRR who had failed prior treatments with large doses of vitamin D derivatives and/or oral calcium [15]. This novel therapy bypassed the calcium absorption defect in the intestine caused by the mutant VDR. The patient was infused with high IV doses of calcium during the nocturnal hours over a 9-month period. Clinical improvement including relief of bone

pain was observed within the first 2 weeks of therapy. Within 7 months, the child gained both weight and height. Eventually, the serum calcium normalized, the secondary hyperparathyroidism was reversed, and the rickets ultimately resolved as assessed by X-ray and bone biopsy. However, when the IV infusions were discontinued, the rickets returned. Several other groups have reported using IV calcium infusions as a therapy for HVDRR [17,164,165]. Weisman *et al.* [164] treated two patients with IV calcium and showed a decrease in their serum alkaline phosphatase activity and an increase in their serum calcium and phosphate over a one-year period [164]. X-ray analysis showed resolution of the rickets with the appearance of normal mineralization of bone. In some cases, after radiological healing of the rickets has been achieved with IV calcium infusions, high dose oral calcium therapy has been shown to be effective in maintaining normal serum calcium concentrations [165]. For those HVDRR children who do not respond to high dose calcitriol, this two-step protocol is initiated at an early age.

Administration of oral calcium salts to restore serum calcium also has been used as a therapy for HVDRR patients. In a study by Sakati *et al.* [118], a patient (F23) who failed to respond to calciferols received 3–4 grams of elemental calcium orally per day and showed clinical improvement during 4 months of therapy. The patient cells were later shown to be totally resistant to $1,25(OH)_2D_3$, due to a Gly46Asp mutation in the VDR DBD [117].

Although many vitamin D actions have been described on bone cells (see Chapter 32 and 37), the administration of calcium alone is sufficient to reverse the rickets. The correction of the hyperparathyroidism by IV calcium and return of phosphate to normal suggests that the hypophosphatemia was due to the secondary hyperparathyroidism. In the VDR knockout mice, a high calcium rescue diet also corrects the bone deformities (See Chapter 20). Only the alopecia is not reversed. These findings suggest that the critical defect in defective receptors is intestinal absorption of calcium.

C. Prenatal Diagnosis

Pregnant women from families with a history of HVDRR can be screened for mutations in the VDR. Cultured cells from chorionic villus samples or amniotic fluid have been used to determine whether the fetus has HVDRR using $[^3H]1,25(OH)_2D_3$ binding assays and induction of 24-hydroxylase activity. A prenatal diagnosis of HVDRR can also determined by examining DNA from chorionic villus samples for RFLPs generated by known mutations in the VDR gene [166,167].

D. Spontaneous Healing

An interesting dilemma regarding HVDRR is that several patients have had a spontaneous improvement in their disease [21,22,63]. Spontaneous healing of rickets usually happens between 7–15 years of age and is not necessarily associated with the time of puberty. Sometimes, the spontaneous recovery occurs after the patient has undergone a relatively ineffective long-term treatment with vitamin D metabolites and mineral replacement. The healing process arises spontaneously and does not appear to be related to the treatment. In some patients, spontaneous improvement occurred after treatment was discontinued [22]. The patients appear to remain eucalcemic without therapy and show no evidence of osteomalacia or rickets. Interestingly, cultured fibroblasts obtained from a skin biopsy of a patient taken after spontaneously healing of the rickets had occurred were still resistant to $1,25(OH)_2D_3$ [22]. Spontaneous improvement has been observed in patients [21,63] with the Tyr295stop mutation [25,77] and in patients [22] with the Arg73Gln mutation [76]. Despite the patient's improvement in their hypocalcemia and rickets, alopecia remained [21,22,63]. It is not uncommon for children to "outgrow" genetic diseases and, perhaps after skeletal growth has been completed, the body can more easily compensate for the defective VDR gene by other mechanisms.

Many patients can be maintained on oral calcium; however, occasionally some patients fall back to hypocalcemia due to decreased compliance or lack of tolerance to the high doses of calcium. IV calcium therapy is then reinstituted. After IV therapy normalizes the rickets and metabolic abnormalities, some patients can be switched to oral calcium. The hypocalcemia and secondary hyperparathyroidism can often be controlled with high dose oral calcium. A number of observations about the patients have been obtained in personal communications with Dr. Zeev Hochberg, the physician caring for several patients with the Tyr295stop mutation. As the patients get older they appear normal on physical exam and X-ray. Bone mineral density is reduced but slowly improving over time. When calcium treatment is commenced at an early age, their eventual height is normal. Although the females have normal menstruation, their reproductive function is unknown at this time. Immunologically, they appear to experience no more infections than normal. No cases of cancer have been observed.

E. Future Therapy Using Vitamin D Analogs

Vitamin D analogs have been proposed as a potential therapy for patients with HVDRR, especially those with mutations in the VDR LBD [168–171]. The use of vitamin D analogs is based on the rationale that they bind to the VDR at different amino acids than $1,25(OH)_2D_3$. Using cultured fibroblasts from patients and *in vitro* transactivation assays, the vitamin D analogs 20-epi-$1,25(OH)_2D_3$ and 1β-hydroxymethyl-3-epi-16-ene-26α,27α-bishomo-$25(OH)D_3$ were shown to partially or completely restore the responsiveness to the Arg274Leu and His305Gln mutant VDRs, but were less effective in activating the Phe251Cys mutant [168]. These results suggest that amino acids that are involved in ligand binding rather than amino acids that are involved in heterodimerization or coactivator binding are more likely to respond to analogs. Future therapy using vitamin D analogs could be based on a rationale drug design for individual patients with specific types of VDR mutations.

VII. ALOPECIA

The role of vitamin D in the regulation of hair growth is still unclear. However, analysis of patients with HVDRR and VDR knockout mice has revealed a number of interesting facts concerning alopecia (see Chapters 20 and 35). First, alopecia is not found in patients with 1α-hydroxylase deficiency (VDDR I) and other forms of vitamin D deficiency. In the 1α-hydroxylase knockout mouse model, abnormalities develop in skeletal, reproductive and immune function [172]. However, the 1α-hydroxylase knockout mice do not develop alopecia. These findings suggest that $1,25(OH)_2D_3$ itself is not required for hair development. On the other hand, VDR knockout mice develop alopecia, indicating that the VDR is essential for hair growth [44,45]. Furthermore, targeting of the WT VDR to keratinocytes of the VDR knockout mouse prevents alopecia [173]. These findings raise the question of how vitamin D and the VDR are involved in regulating hair growth. Patients with DBD mutations and premature stop mutations all have alopecia and are totally hormone resistant. Alopecia remains unchanged even after patients undergo successful therapy or show spontaneous improvement in rickets. The patients that did not develop alopecia all had missense mutations in the VDR LBD. Some of these mutations affect ligand binding (Arg274Leu, His305Gln, Ile314Ser, and Trp286Arg). Three of these amino acids Arg274, His305, Trp286 are contact points for $1,25(OH)_2D_3$. Since the patients with the His305Gln and Ile314Ser mutations were somewhat responsive to vitamin D therapy, it is reasonable to speculate that the limited VDR function may have prevented the development of alopecia after birth. On the other hand, the patients with the Arg274Leu and Trp286Arg mutations that severely diminished or abolished $[^3H]1,25(OH)_2D_3$ binding were totally resistant to $1,25(OH)_2D_3$, yet did not have alopecia. In contrast, in patients with LBD mutations that reduced heterodimerization with RXRα (Phe251Cys, Gln259Pro, and Arg391Cys), but had little or no effect on ligand binding, alopecia was present. Although these mutations caused $1,25(OH)_2D_3$ resistance in the patient, the function of the mutant VDRs could be restored *in vitro* by supraphysiological doses of the hormone and addition of excess RXR. Perhaps most interestingly was the patient with the Glu420Lys mutation that prevents coactivator binding but not ligand binding or RXR heterodimerization. The patient did not have alopecia, yet was clearly resistant to high doses of hormone. Based on these findings, one can conclude that mutations that affect DNA binding, VDR-RXR heterodimerization, or that truncate the LBD are linked to alopecia while mutations that affect ligand binding or coactivator binding are not. This suggests that VDR-RXR heterodimerization and DNA binding are critical for VDR function in hair development. These findings also suggest that ligand binding and coactivator binding are not essential functions of the VDR for hair growth and/or to prevent alopecia. A role for RXR is clearly demonstrated since targeted inactivation of RXRα in keratinocytes also causes alopecia [174].

The alopecia associated with HVDRR is clinically and pathologically indistinguishable from the generalized atrichia with papules found in patients with mutations in the *hairless* (*hr*) gene [150,175]. Like the VDR, HR is a zinc finger protein suggesting that it interacts with DNA. The *hr* gene is expressed in many tissues, especially in the skin and brain [176]. HR has recently been shown to interact with the VDR, and coexpression of HR and VDR blocks VDR mediated transactivation [177].

The data on the VDR mutations, combined with the findings in the 1α-hydroxylase knockout mouse model, suggests that the role of the VDR in the hair cycle is to repress the expression of some gene(s) in a ligand-independent manner. This activity requires RXR heterodimerization and DNA binding, but not interaction with coactivators. HR may also be required as a corepressor for this negative regulatory activity of the VDR. The VDR is a negative regulator of a number of genes, and the loss of a negative regulatory activity by the unliganded VDR could potentially lead to the derepression of those genes that could ultimately lead to alopecia. Since the VDR is a negative regulator of the PTHrP gene [178] and overexpression of PTHrP

has been shown to cause alopecia and is involved in hair cycle regulation [179,180], dysregulation of the PTHrP gene may be a likely candidate in the development of alopecia in HVDRR.

VIII. CONCLUDING REMARKS

HVDRR is a rare recessive genetic disorder caused by heterogeneous mutations in the VDR that result in end-organ resistance to $1,25(OH)_2D_3$ action. The major manifestation of the defective VDR on the vitamin D endocrine system is to decrease intestinal calcium and phosphate absorption that results in decreased bone mineralization and rickets. Secondary hyperparathyroidism results as a consequence of the low serum calcium. The classical role of $1,25(OH)_2D$ is to regulate mineral homeostasis, achieved through its coordinated actions on intestine, kidney, bone, and parathyroid gland [181,182]. The VDR is also expressed in a wide variety of tissues, including kidney, skin, liver, pancreas, muscle, breast, prostate, adrenal, thyroid, and cells of mesenchymal or hematopoietic origin [31,32,35,36,80,183,184]. From the ubiquitous nature of the VDR, it appears that the role of the VDR in cellular function is not homeostatic but rather pleiotropic. The expanded scope of vitamin D actions include stimulation of differentiation, inhibition of cell proliferation, and suppression of the immune response [35,36,39,184]. In addition, the regulation of cellular proliferation and differentiation by $1,25(OH)_2D$ is a common feature in many tissues examined, and it is likely that this regulatory feature is a fundamental component of all biological responses to $1,25(OH)_2D$. Notwithstanding the complexity and diversity of biological responses elicited by $1,25(OH)_2D$, the profound skeletal abnormalities demonstrated in patients with HVDRR emphasizes the fundamental and essential role of $1,25(OH)_2D$ in calcium homeostasis. It is interesting to note that despite the many pleiotropic processes regulated by $1,25(OH)_2D_3$, children with HVDRR exhibit only symptoms that relate to their calcium deficiency and/or alopecia. After treatment, they appear normal in all respects except for the alopecia. Analysis of HVDRR patients provides many interesting insights into vitamin D physiology and the role of the VDR in mediating $1,25(OH)_2D_3$ action.

Since 1978, more than 60 families with HVDRR have been reported. A number of cases have been examined for mutations in the VDR. However, since some of these earlier cases of HVDRR presented late in life, it is possible that they may have been due to other conditions unrelated to genetic defects in the VDR or to some form of acquired resistance to $1,25(OH)_2D_3$. Presently, eight missense mutations have been identified in the VDR DBD. All of the DBD mutations prevent the VDR from binding to DNA, causing total $1,25(OH)_2D_3$ resistance even though $1,25(OH)_2D_3$ binding is normal. In the LBD, 10 missense mutations have been identified. These mutations disrupt ligand binding, VDR-RXR heterodimerization, or modify coactivator-binding sites. They result in partial or total hormone resistance. In addition, premature termination codons and splice site mutations truncate the VDR and cause total $1,25(OH)_2D_3$ resistance.

The successful use of IV calcium infusion or oral calcium therapy for HVDRR raises interesting questions about the role of vitamin D in bone homeostasis. Correction of hypocalcemia and secondary hyperparathyroidism leads to healing of the rickets as assessed by X-ray and bone biopsy. However, careful analysis of bone histomorphometry has not been accomplished in HVDRR cases. Recent investigations comparing the 1α-hydroxylase knockout mice ($1\alpha(OH)ase^{-/-}$) with the VDR knockout mice ($VDR^{-/-}$) has revealed interesting differences in phenotypes [185]. Defects of a subtle nature were found in bone histomorphometry suggesting incomplete normalization of osteoblastic function. Growth plates were more widened and disorganized in the $1\alpha(OH)ase^{-/-}$ mice, while the $VDR^{-/-}$ mice were slightly thickened and more organized. However, when the animals were fed a rescue diet of 2% calcium and 20% lactose, the cartilaginous growth plate was normalized in the $VDR^{-/-}$ mice, but remained larger and more disorganized in the $1\alpha(OH)ase^{-/-}$ mice. The parathyroid glands were enlarged in both mutants, but fed the rescue diet, the parathyroid gland size normalized in the $VDR^{-/-}$ mice, but remained increased in the $1\alpha(OH)ase^{-/-}$ mice [185].

Thus, although there are many well-defined actions of vitamin D on osteoblasts, the response to normalization of serum calcium in HVDRR patients and $VDR^{-/-}$ mice suggests that $1,25(OH)_2D_3$ action on osteoblasts is not essential to form bone, although the bone is not completely normal. The implication is that $1,25(OH)_2D_3$ action on bone is mainly due to its effects on intestinal mineral absorption to provide calcium and phosphate for bone formation. The same conclusion was reached by Underwood and DeLuca [186], who showed that rickets could be prevented in vitamin D–deficient rats by calcium and phosphate infusions. Also, normalizing serum calcium by IV infusion is sufficient to suppress PTH overproduction in HVDRR children. This suggests that the hypophosphatemia in these patients is mainly the result of secondary hyperparathyroidism and not inadequate intestinal phosphate absorption.

The findings also suggest that $1,25(OH)_2D_3$ action is not essential to suppress PTH in secondary hyperparathyroidism.

The mechanism for the spontaneous improvement exhibited by some HVDRR children as they get older is an interesting dilemma. One hypothesis, that explains the normalization of the $1,25(OH)_2D_3$ endocrine system in the face of inactive VDRs, is that some other transcription factor can substitute for the defective vitamin D system. Possibly RAR, RXR, or TR can substitute for a nonfunctional VDR and activate the appropriate target genes to reverse the hypocalcemia and restore the bones to normal. In this context, Whitfield *et al.* [140] have shown *in vitro* that addition of RXR can rescue mutant VDR with defects in the heterodimerization domain and restore hormone responsiveness. Another possibility is that a diet sufficient in calcium may bypass the need for vitamin D action when the demand for calcium for bone growth is diminished at an older age.

In conclusion, the biochemical and genetic analysis of the VDR in HVDRR patients has yielded important insights into the structure and function of the receptor in mediating $1,25(OH)_2D_3$ action. Similarly, studies of the affected children with HVDRR continues to provide further insight into the biological role of $1,25(OH)_2D_3$ *in vivo*. A concerted investigative approach of HVDRR at the clinical, cellular, and molecular level has proven exceedingly valuable in understanding the mechanism of action of $1,25(OH)_2D_3$ and improving the diagnostic and clinical management of this rare genetic disease.

References

1. Feldman D, Malloy PJ 1990 Hereditary 1,25-dihydroxyvitamin D–resistant rickets: molecular basis and implications for the role of $1,25(OH)_2D_3$ in normal physiology. Mol Cell Endocrinol **72**:C57–62.
2. Hughes MR, Malloy PJ, O'Malley BW, Pike JW, Feldman D 1991 Genetic defects of the 1,25-dihydroxyvitamin D_3 receptor. J Recept Res **11**:699–716.
3. Malloy PJ, Pike JW, Feldman D 1999 The vitamin D receptor and the syndrome of hereditary 1,25-dihydroxyvitamin D–resistant rickets. Endocr Rev **20**:156–188.
4. Albright F, Butler AM, Bloomberg E 1937 Rickets resistant to vitamin D therapy. Am J Dis Child **54**:531–547.
5. Prader VA, Illig R, Heierli E 1961 Eine besondere form der primaren vitamin D–resistenten rachitis mit hypocalcamie und autosomal-dominantem erbgang: die hereditare pseudomangelrachitis. Helvetica Paediatrica Acta **16**:452–468.
6. Takeyama K, Kitanaka S, Sato T, Kobori M, Yanagisawa J, Kato S 1997 25-Hydroxyvitamin D_3 1alpha-hydroxylase and vitamin D synthesis. Science **277**:1827–1830.
7. Fu GK, Lin D, Zhang MY, Bikle DD, Shackleton CH, Miller WL, Portale AA 1997 Cloning of human 25-hydroxyvitamin D-1 alpha-hydroxylase and mutations causing vitamin D–dependent rickets type 1. Mol Endocrinol **11**: 1961–1970.
8. Kitanaka S, Takeyama K, Murayama A, Sato T, Okumura K, Nogami M, Hasegawa Y, Niimi H, Yanagisawa J, Tanaka T, Kato S 1998 Inactivating mutations in the 25-hydroxyvitamin D_3 1(alpha)-hydroxylase gene in patients with pseudovitamin D–deficiency rickets. N Engl J Med **338**: 653–661.
9. Brooks MH, Bell NH, Love L, Stern PH, Orfei E, Queener SF, Hamstra AJ, DeLuca HF 1978 Vitamin D–dependent rickets type II. Resistance of target organs to 1,25-dihydroxyvitamin D. N Engl J Med **298**:996–999.
10. Marx SJ, Spiegel AM, Brown EM, Gardner DG, Downs RW, Jr., Attie M, Hamstra AJ, DeLuca HF 1978 A familial syndrome of decrease in sensitivity to 1,25-dihydroxyvitamin D. J Clin Endocrinol Metab **47**:1303–1310.
11. Rosen JF, Fleischman AR, Finberg L, Hamstra A, DeLuca HF 1979 Rickets with alopecia: an inborn error of vitamin D metabolism. J Pediatr **94**:729–735.
12. Liberman UA, Samuel R, Halabe A, Kauli R, Edelstein S, Weisman Y, Papapoulos SE, Clemens TL, Fraher LJ, O'Riordan JL 1980 End-organ resistance to 1,25-dihydroxycholecalciferol. Lancet **1**:504–506.
13. Sockalosky JJ, Ulstrom RA, DeLuca HF, Brown DM 1980 Vitamin D–resistant rickets: end-organ unresponsiveness to $1,25(OH)_2D_3$. J Pediatr **96**:701–703.
14. Kudoh T, Kumagai T, Uetsuji N, Tsugawa S, Oyanagi K, Chiba Y, Minami R, Nakao T 1981 Vitamin D–dependent rickets: decreased sensitivity to 1,25-dihydroxyvitamin D. Eur J Pediatr **137**:307–311.
15. Balsan S, Garabedian M, Liberman UA, Eil C, Bourdeau A, Guillozo H, Grimberg R, Le Deunff MJ, Lieberherr M, Guimbaud P, Broyer M, Marx SJ 1983 Rickets and alopecia with resistance to 1,25-dihydroxyvitamin D: two different clinical courses with two different cellular defects. J Clin Endocrinol Metab **57**:803–811.
16. Laufer D, Benderly A, Hochberg Z 1987 Dental pathology in calcitirol resistant rickets. J Oral Med **42**:272–275.
17. Bliziotes M, Yergey AL, Nanes MS, Muenzer J, Begley MG, Viera NE, Kher KK, Brandi ML, Marx SJ 1988 Absent intestinal response to calciferols in hereditary resistance to 1,25-dihydroxyvitamin D: documentation and effective therapy with high dose intravenous calcium infusions. J Clin Endocrinol Metab **66**:294–300.
18. Fraher LJ, Karmali R, Hinde FR, Hendy GN, Jani H, Nicholson L, Grant D, O'Riordan JL 1986 Vitamin D–dependent rickets type II: extreme end organ resistance to 1,25-dihydroxy vitamin D_3 in a patient without alopecia. Eur J Pediatr **145**:389–395.
19. Beer S, Tieder M, Kohelet D, Liberman OA, Vure E, Bar-Joseph G, Gabizon D, Borochowitz ZU, Varon W, Modai D 1981 Vitamin D–resistant rickets with alopecia: A form of end organ resistance to 1,25-dihydroxyvitamin D. Clin Endocrinol **14**:395–402.
20. Feldman D, Chen T, Cone C, Hirst M, Shani S, Benderli A, Hochberg Z 1982 Vitamin D–resistant rickets with alopecia: cultured skin fibroblasts exhibit defective cytoplasmic receptors and unresponsiveness to $1,25(OH)_2D_3$. J Clin Endocrinol Metab **55**:1020–1022.
21. Hochberg Z, Benderli A, Levy J, Vardi P, Weisman Y, Chen T, Feldman D 1984 1,25-Dihydroxyvitamin D resistance, rickets, and alopecia. Am J Med **77**:805–811.
22. Hirst MA, Hochman HI, Feldman D 1985 Vitamin D resistance and alopecia: a kindred with normal 1,25-dihydroxyvitamin D binding, but decreased receptor affinity for deoxyribonucleic acid. J Clin Endocrinol Metab **60**: 490–495.
23. Takeda E, Kuroda Y, Saijo T, Naito E, Kobashi H, Yokota I, Miyao M 1987 1 alpha-hydroxyvitamin D_3 treatment of three

patients with 1,25-dihydroxyvitamin D–receptor-defect rickets and alopecia. Pediatrics **80**:97–101.
24. Yagi H, Ozono K, Miyake H, Nagashima K, Kuroume T, Pike JW 1993 A new point mutation in the deoxyribonucleic acid-binding domain of the vitamin D–receptor in a kindred with hereditary 1,25-dihydroxyvitamin D-resistant rickets. J Clin Endocrinol Metab **76**:509–512.
25. Malloy PJ, Weisman Y, Feldman D 1994 Hereditary 1 alpha,25-dihydroxyvitamin D–resistant rickets resulting from a mutation in the vitamin D receptor deoxyribonucleic acid-binding domain. J Clin Endocrinol Metab **78**:313–316.
26. Malloy PJ, Hochberg Z, Tiosano D, Pike JW, Hughes MR, Feldman D 1990 The molecular basis of hereditary 1,25-dihydroxyvitamin D_3–resistant rickets in seven related families. J Clin Invest **86**:2071–2079.
27. Heaney RP 1997 Vitamin D: role in the calcium economy. In: Feldman D, Glorieux F, Pike JW (eds) Vitamin D. Academic Press: San Diego, pp 485–497.
28. Heaney RP, Barger-Lux MJ, Dowell MS, Chen TC, Holick MF 1997 Calcium absorptive effects of vitamin D and its major metabolites. J Clin Endocrinol Metab **82**:4111–4116.
29. Hochberg Z, Gilhar A, Haim S, Friedman-Birnbaum R, Levy J, Benderly A 1985 Calcitriol-resistant rickets with alopecia. Arch Dermatol **121**:646–647.
30. Marx SJ, Bliziotes MM, Nanes M 1986 Analysis of the relation between alopecia and resistance to 1,25-dihydroxyvitamin D. Clin Endocrinol **25**:373–381.
31. Stumpf WE, Sar M, Reid FA, Tanaka Y, DeLuca HF 1979 Target cells for 1,25-dihydroxyvitamin D_3 in intestinal tract, stomach, kidney, skin, pituitary, and parathyroid. Science **206**:1188–1190.
32. Berger U, Wilson P, McClelland RA, Colston K, Haussler MR, Pike JW, Coombes RC 1988 Immunocytochemical detection of 1,25-dihydroxyvitamin–D receptors in normal human tissues. J Clin Endocrinol Metab **67**:607–613.
33. Reichel H, Koeffler HP, Norman AW 1989 The role of the vitamin D endocrine system in health and disease. N Engl J Med **320**:980–991.
34. Pike JW 1991 Vitamin D_3 receptors: structure and function in transcription. Ann Rev Nutr **11**:189–216.
35. Walters MR 1992 Newly identified actions of the vitamin D endocrine system. Endocrine Rev **13**:719–764.
36. Bikle DD 1992 Clinical counterpoint: Vitamin D: new actions, new analogs, new therapeutic potential. Endocrine Rev **13**:765–784.
37. Darwish H, DeLuca HF 1993 Vitamin D–regulated gene expression. Crit Rev Eukaryot Gene Expr **3**:89–116.
38. MacDonald PN, Dowd DR, Haussler MR 1994 New insight into the structure and functions of the vitamin D receptor. Semin Nephrol **14**:101–118.
39. Feldman D, Malloy PJ, Gross C 2001 Vitamin D: biology, action, and clinical implications. In: Marcus R, Feldman D, Kelsey J (eds) Osteoporosis. Academic Press: San Diego, pp 257–303.
40. Hochberg Z, Borochowitz Z, Benderli A, Vardi P, Oren S, Spirer Z, Heyman I, Weisman Y 1985 Does 1,25-dihydroxyvitamin D participate in the regulation of hormone release from endocrine glands? J Clin Endocrinol Metab **60**: 57–61.
41. Even L, Weisman Y, Goldray D, Hochberg Z 1996 Selective modulation by vitamin D of renal response to parathyroid hormone: a study in calcitriol-resistant rickets. J Clin Endocrinol Metab **81**:2836–2840.
42. Manolagas SC, Yu XP, Girasole G, Bellido T 1994 Vitamin D and the hematolymphopoietic tissue: a 1994 update. Semin Nephrol **14**:129–143.
43. Etzioni A, Hochberg Z, Pollak S, Meshulam T, Zakut V, Tzehoval E, Keisari Y, Aviram I, Spirer Z, Benderly A, Weisman Y 1989 Defective leukocyte fungicidal activity in end-organ resistance to 1,25-dihydroxyvitamin D. Pediatr Res **25**:276–279.
44. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S 1997 Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia, and growth retardation after weaning. Nat Genet **16**:391–396.
45. Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R, Demay MB 1997 Targeted ablation of the vitamin D receptor: an animal model of vitamin D–dependent rickets type II with alopecia. Proc Natl Acad Sci USA **94**:9831–9835.
46. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, *et al.* 1995 The nuclear receptor superfamily: the second decade. Cell **83**:835–839.
47. Berg JM 1988 Proposed structure for the zinc-binding domains from transcription factor IIIA and related proteins. Proc Natl Acad Sci USA **85**:99–102.
48. Mader S, Kumar V, de Verneuil H, Chambon P 1989 Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. Nature **338**:271–274.
49. Umesono K, Evans RM 1989 Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell **57**:1139–1146.
50. Rochel N, Wurtz JM, Mitschler A, Klaholz B, Moras D 2000 The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. Mol Cell **5**:173–179.
51. Blanco JC, Wang IM, Tsai SY, Tsai MJ, O'Malley BW, Jurutka PW, Haussler MR, Ozato K 1995 Transcription factor TFIIB and the vitamin D receptor cooperatively activate ligand-dependent transcription. Proc Natl Acad Sci USA **92**:1535–1539.
52. MacDonald PN, Sherman DR, Dowd DR, Jefcoat SC, Jr., DeLisle RK 1995 The vitamin D receptor interacts with general transcription factor IIB. J Biol Chem **270**:4748–4752.
53. Onate SA, Tsai SY, Tsai MJ, O'Malley BW 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science **270**:1354–1357.
54. Masuyama H, Brownfield CM, St-Arnaud R, MacDonald PN 1997 Evidence for ligand-dependent intramolecular folding of the AF-2 domain in vitamin D receptor-activated transcription and coactivator interaction. Mol Endocrinol **11**:1507–1517.
55. Gill RK, Atkins LM, Hollis BW, Bell NH 1998 Mapping the domains of the interaction of the vitamin D receptor and steroid receptor coactivator-1. Mol Endocrinol **12**:57–65.
56. DeLuca HF, Zierold C 1998 Mechanisms and functions of vitamin D. Nutr Rev **56**:S4–10; discussion S54–75.
57. Haussler MR, Whitfield GK, Haussler CA, Hsieh J, Thompson PD, Selznick SH, Dominguez CE, Jurutka PW 1998 The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Min Res **13**:325–349.
58. Rachez C, Freedman LP 2000 Mechanisms of gene regulation by vitamin D_3 receptor: a network of coactivator interactions. Gene **246**:9–21.
59. Minghetti PP, Norman AW 1988 1,25$(OH)_2$-vitamin D_3 receptors: gene regulation and genetic circuitry. FASEB J **2**: 3043–3053.
60. Racz A, Barsony J 1999 Hormone-dependent translocation of vitamin D receptors is linked to transactivation. J Biol Chem **274**:19352–19360.

61. Jurutka PW, Hsieh JC, MacDonald PN, Terpening CM, Haussler CA, Haussler MR, Whitfield GK 1993 Phosphorylation of serine 208 in the human vitamin D receptor. The predominant amino acid phosphorylated by casein kinase II, *in vitro*, and identification as a significant phosphorylation site in intact cells. J Biol Chem **268**:6791–6799.
62. Freedman LP 1999 Increasing the complexity of coactivation in nuclear receptor signaling. Cell **97**:5–8.
63. Chen TL, Hirst MA, Cone CM, Hochberg Z, Tietze HU, Feldman D 1984 1,25-dihydroxyvitamin D resistance, rickets, and alopecia: analysis of receptors and bioresponse in cultured fibroblasts from patients and parents. J Clin Endocrinol Metab **59**:383–388.
64. Kerner SA, Scott RA, Pike JW 1989 Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D_3. Proc Natl Acad Sci USA **86**:4455–4459.
65. Morrison NA, Shine J, Fragonas JC, Verkest V, McMenemy ML, Eisman JA 1989 1,25-dihydroxyvitamin D–responsive element and glucocorticoid repression in the osteocalcin gene. Science **246**:1158–1161.
66. Ozono K, Liao J, Kerner SA, Scott RA, Pike JW 1990 The vitamin D–responsive element in the human osteocalcin gene. Association with a nuclear proto-oncogene enhancer. J Biol Chem **265**:21881–21888.
67. Lian J, Stewart C, Puchacz E, Mackowiak S, Shalhoub V, Collart D, Zambetti G, Stein G 1989 Structure of the rat osteocalcin gene and regulation of vitamin D–dependent expression. Proc Natl Acad Sci USA **86**:1143–1147.
68. Demay MB, Gerardi JM, DeLuca HF, Kronenberg HM 1990 DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D_3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA **87**:369–373.
69. Terpening CM, Haussler CA, Jurutka PW, Galligan MA, Komm BS, Haussler MR 1991 The vitamin D–responsive element in the rat bone Gla protein gene is an imperfect direct repeat that cooperates with other cis-elements in 1,25-dihydroxyvitamin D_3-mediated transcriptional activation. Mol Endocrinol **5**:373–385.
70. Noda M, Vogel RL, Craig AM, Prahl J, DeLuca HF, Denhardt DT 1990 Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D_3 receptor and 1,25-dihydroxyvitamin D_3 enhancement of mouse secreted phosphoprotein 1 (SPP-1 or osteopontin) gene expression. Proc Natl Acad Sci USA **87**:9995–9999.
71. Darwish HM, DeLuca HF 1992 Identification of a 1,25-dihydroxyvitamin D_3–response element in the 5′-flanking region of the rat calbindin D-9k gene. Proc Natl Acad Sci USA **89**:603–607.
72. Gill RK, Christakos S 1993 Identification of sequence elements in mouse calbindin-D28k gene that confer 1,25-dihydroxyvitamin D_3- and butyrate-inducible responses. Proc Natl Acad Sci USA **90**:2984–2988.
73. Ohyama Y, Ozono K, Uchida M, Shinki T, Kato S, Suda T, Yamamoto O, Noshiro M, Kato Y 1994 Identification of a vitamin D–responsive element in the 5′-flanking region of the rat 25-hydroxyvitamin D_3 24-hydroxylase gene. J Biol Chem **269**:10545–10550.
74. Chen KS, DeLuca HF 1995 Cloning of the human 1 alpha,25-dihydroxyvitamin D_3 24-hydroxylase gene promoter and identification of two vitamin D–responsive elements. Biochim Biophys Acta **1263**:1–9.
75. Zierold C, Darwish HM, DeLuca HF 1995 Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter. J Biol Chem **270**:1675–1678.
76. Hughes MR, Malloy PJ, Kieback DG, Kesterson RA, Pike JW, Feldman D, O'Malley BW 1988 Point mutations in the human vitamin D receptor gene associated with hypocalcemic rickets. Science **242**:1702–1705.
77. Ritchie HH, Hughes MR, Thompson ET, Malloy PJ, Hochberg Z, Feldman D, Pike JW, O'Malley BW 1989 An ochre mutation in the vitamin D receptor gene causes hereditary 1,25-dihydroxyvitamin D_3–resistant rickets in three families. Proc Natl Acad Sci USA **86**:9783–9787.
78. Sone T, Scott RA, Hughes MR, Malloy PJ, Feldman D, O'Malley BW, Pike JW 1989 Mutant vitamin D receptors which confer hereditary resistance to 1,25-dihydroxyvitamin D_3 in humans are transcriptionally inactive *in vitro*. J Biol Chem **264**:20230–20234.
79. Zerwekh JE, Glass K, Jowsey J, Pak CY 1979 An unique form of osteomalacia associated with end organ refractoriness to 1,25-dihydroxyvitamin D and apparent defective synthesis of 25-hydroxyvitamin D. J Clin Endocrinol Metab **49**:171–175.
80. Colston K, Hirst M, Feldman D 1980 Organ distribution of the cytoplasmic 1,25-dihydroxycholecalciferol receptor in various mouse tissues. Endocrinology **107**:1916–1922.
81. Feldman D, Chen T, Hirst M, Colston K, Karasek M, Cone C 1980 Demonstration of 1,25-dihydroxyvitamin D_3 receptors in human skin biopsies. J Clin Endocrinol Metab **51**:1463–1465.
82. Simpson RU, DeLuca HF 1980 Characterization of a receptor-like protein for 1,25-dihydroxyvitamin D_3 in rat skin. Proc Natl Acad Sci USA **77**:5822–5826.
83. Eil C, Liberman UA, Rosen JF, Marx SJ 1981 A cellular defect in hereditary vitamin-D–dependent rickets type II: defective nuclear uptake of 1,25-dihydroxyvitamin D in cultured skin fibroblasts. N Engl J Med **304**:1588–1591.
84. Clemens TL, Adams JS, Horiuchi N, Gilchrest BA, Cho H, Tsuchiya Y, Matsuo N, Suda T, Holick MF 1983 Interaction of 1,25-dihydroxyvitamin-D_3 with keratinocytes and fibroblasts from skin of normal subjects and a subject with vitamin D–dependent rickets, type II: A model for study of the mode of action of 1,25-dihydroxyvitamin D_3. J Clin Endocrinol Metab **56**:824–830.
85. Griffin JE, Zerwekh JE 1983 Impaired stimulation of 25-hydroxyvitamin D-24-hydroxylase in fibroblasts from a patient with vitamin D–dependent rickets, type II. A form of receptor-positive resistance to 1,25-dihydroxyvitamin D_3. J Clin Invest **72**:1190–1199.
86. Liberman UA, Eil C, Marx SJ 1983 Resistance to $1,25(OH)_2D_3$. Association with heterogeneous defects in cultured skin fibroblasts. J Clin Invest **71**:192–200.
87. Liberman UA, Eil C, Holst P, Rosen JF, Marx SJ 1983 Hereditary resistance to 1,25-dihydroxyvitamin D: defective function of receptors for 1,25-dihydroxyvitamin D in cells cultured from bone. J Clin Endocrinol Metab **57**: 958–962.
88. Liberman UA, Eil C, Marx SJ 1986 Receptor-positive hereditary resistance to 1,25-dihydroxyvitamin D: chromatography of receptor complexes on deoxyribonucleic acid-cellulose shows two classes of mutation. J Clin Endocrinol Metab **62**:122–126.
89. Malloy PJ, Hochberg Z, Pike JW, Feldman D 1989 Abnormal binding of vitamin D receptors to deoxyribonucleic acid in a kindred with vitamin D–dependent rickets, type II. J Clin Endocrinol Metab **68**:263–269.
90. Pike JW, Dokoh S, Haussler MR, Liberman UA, Marx SJ, Eil C 1984 Vitamin D_3–resistant fibroblasts have immunoassayable 1,25-dihydroxyvitamin D_3 receptors. Science **224**: 879–881.

91. Dokoh S, Haussler MR, Pike JW 1984 Development of a radioligand immunoassay for 1,25-dihydroxycholecalciferol receptors utilizing monoclonal antibody. Biochem J **221**:129–136.
92. Pike JW, Donaldson CA, Marion SL, Haussler MR 1982 Development of hybridomas secreting monoclonal antibodies to the chicken intestinal 1 alpha,25-dihydroxyvitamin D_3 receptor. Proc Natl Acad Sci USA **79**:7719–7723.
93. Pike JW, Marion SL, Donaldson CA, Haussler MR 1983 Serum and monoclonal antibodies against the chick intestinal receptor for 1,25-dihydroxyvitamin D_3. Generation by a preparation enriched in a 64,000-dalton protein. J Biol Chem **258**:1289–1296.
94. Pike JW 1984 Monoclonal antibodies to chick intestinal receptors for 1,25-dihydroxyvitamin D_3. Interaction and effects of binding on receptor function. J Biol Chem **259**:1167–1173.
95. Gamblin GT, Liberman UA, Eil C, Downs RWJ, Degrange DA, Marx SJ 1985 Vitamin D–dependent rickets type II: Defective induction of 25-hydroxyvitamin D_3-24-hydroxylase by 1,25-dihydroxyvitamin D_3 in cultured skin fibroblasts. J Clin Invest **75**:954–960.
96. Castells S, Greig F, Fusi MA, Finberg L, Yasumura S, Liberman UA, Eil C, Marx SJ 1986 Severely deficient binding of 1,25-dihydroxyvitamin D to its receptors in a patient responsive to high doses of this hormone. J Clin Endocrinol Metab **63**:252–256.
97. Koren R, Ravid A, Liberman UA, Hochberg Z, Weisman Y, Novogrodsky A 1985 Defective binding and function of 1,25-dihydroxyvitamin D_3 receptors in peripheral mononuclear cells of patients with end-organ resistance to 1,25-dihydroxyvitamin D. J Clin Invest **76**:2012–2015.
98. Takeda E, Kuroda Y, Saijo T, Toshima K, Naito E, Kobashi H, Iwakuni Y, Miyao M 1986 Rapid diagnosis of vitamin D–dependent rickets type II by use of phytohemagglutinin-stimulated lymphocytes. Clin Chim Acta **155**:245–250.
99. Takeda E, Yokota I, Ito M, Kobashi H, Saijo T, Kuroda Y 1990 25-Hydroxyvitamin D-24-hydroxylase in phytohemagglutinin-stimulated lymphocytes: intermediate bioresponse to 1,25-dihydroxyvitamin D_3 of cells from parents of patients with vitamin D–dependent rickets type II. J Clin Endocrinol Metab **70**:1068–1074.
100. Nagler A, Merchav S, Fabian I, Tatarsky I, Hochberg Z 1987 Myeloid progenitors from the bone marrow of patients with vitamin D–resistant rickets (type II) fail to respond to $1,25(OH)_2D_3$. Brit J Haematol **67**:267–271.
101. Koeffler HP, Bishop JE, Reichel H, Singer F, Nagler A, Tobler A, Walka M, Norman AW 1990 Lymphocyte cell lines from vitamin D–dependent rickets type II show functional defects in the 1 alpha,25-dihydroxyvitamin D_3 receptor. Mol Cell Endocrinol **70**:1–11.
102. Takeda E, Yokota I, Saijo T, Kawakami I, Ito M, Kuroda Y 1990 Effect of long-term treatment with massive doses of 1 alpha-hydroxyvitamin D_3 on calcium-phosphate balance in patients with vitamin D–dependent rickets type II. Acta Paediatr Jpn **32**:39–43.
103. Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW 1988 Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci USA **85**:3294–3298.
104. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA 1988 Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
105. Refetoff S, Weiss RE, Usala SJ 1993 The syndromes of resistance to thyroid hormone. Endocrine Rev **14**: 348–399.
106. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS 1995 Androgen receptor defects: historical, clinical, and molecular perspectives. Endocrine Rev **16**:271–321.
107. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med **331**:1056–1061.
108. Werner S, Bronnegard M 1996 Molecular basis of glucocorticoid-resistant syndromes. Steroids **61**:216–221.
109. Geller DS, Rodriguez-Soriano J, Vallo Boado A, Schifter S, Bayer M, Chang SS, Lifton RP 1998 Mutations in the mineralocorticoid receptor gene cause autosomal dominant pseudohypoaldosteronism type I. Nat Genet **19**:279–281.
110. Sone T, Marx SJ, Liberman UA, Pike JW 1990 A unique point mutation in the human vitamin D receptor chromosomal gene confers hereditary resistance to 1,25-dihydroxyvitamin D_3. Mol Endocrinol **4**:623–631.
111. Saijo T, Ito M, Takeda E, Huq AH, Naito E, Yokota I, Sone T, Pike JW, Kuroda Y 1991 A unique mutation in the vitamin D receptor gene in three Japanese patients with vitamin D–dependent rickets type II: utility of single-strand conformation polymorphism analysis for heterozygous carrier detection. Am J Hum Genet **49**:668–673.
112. Takeda E, Yokota I, Kawakami I, Hashimoto T, Kuroda Y, Arase S 1989 Two siblings with vitamin D–dependent rickets type II: no recurrence of rickets for 14 years after cessation of therapy. Eur J Pediatr **149**:54–57.
113. Yokota I, Takeda E, Ito M, Kobashi H, Saijo T, Kuroda Y 1991 Clinical and biochemical findings in parents of children with vitamin D–dependent rickets Type II. J Inherit Metab Dis **14**:231–240.
114. Rut AR, Hewison M, Kristjansson K, Luisi B, Hughes MR, O'Riordan JL 1994 Two mutations causing vitamin D–resistant rickets: modelling on the basis of steroid hormone receptor DNA-binding domain crystal structures. Clin Endocrinol **41**:581–590.
115. Lin JP, Uttley WS 1993 Intra-atrial calcium infusions, growth, and development in end organ resistance to vitamin D. Arch Dis Child **69**:689–692.
116. Simonin G, Chabrol B, Moulene E, Bollini G, Strouc S, Mattei JF, Giraud F 1992 Vitamin D–resistant rickets type II: apropos of 2 cases. Pediatrie **47**:817–820.
117. Lin NU-T, Malloy PJ, Sakati N, Al-Ashwal A, Feldman D 1996 A novel mutation in the deoxyribonucleic acid-binding domain of the vitamin D receptor gene causes hereditary 1,25-dihydroxyvitamin D–resistant rickets. J Clin Endocrinol Metab **81**:2564–2569.
118. Sakati N, Woodhouse NJY, Niles N, Harfi H, de Grange DA, Marx S 1986 Hereditary resistance to 1,25-dihydroxyvitamin D: clinical and radiological improvement during high-dose oral calcium therapy. Hormone Res **24**:280–287.
119. Luisi BF, Xu WX, Otinowski Z, Freedman LP, Yamamoto KR, Sigler PB 1991 Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature **352**: 497–505.
120. Rastinejad F, Perlmann T, Evans RM, Sigler PB 1995 Structural determinants of nuclear receptor assembly on DNA direct repeats. Nature **375**:203–211.
121. Freedman LP, Luisi BF 1993 On the mechanism of DNA binding by nuclear hormone receptors: a structural and functional perspective. J Cell Biochem **51**:140–150.

122. Luisi BF, Schwabe JW, Freedman LP 1994 The steroid/nuclear receptors: from three-dimensional structure to complex function. Vitam Horm **49**:1–47.
123. Burns K, Duggan B, Atkinson EA, Famulski KS, Nemer M, Bleackley RC, Michalak M 1994 Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. Nature **367**:476–480.
124. Cao X, Teitelbaum SL, Dedhar S, Zhang L, Ross FP 1994 Retinoic acid and calreticulin, a novel ER-derived transcription factor inhibits $1,25(OH)_2D_3$-induced gene transcription. J Bone Miner Res **9**:S145.
125. Dedhar S, Rennie PS, Shago M, Leung Hagesteijn C-Y, Yang H, Filmus J, Hawley RG, Bruchovsky N, Cheng H, Matusik RJ, Giguere V 1994 Inhibition of nuclear hormone receptor activity by calreticulin. Nature **367**:480–483.
126. Orkin SH 1984 The mutation and polymorphism of the human ß-globin gene and its surrounding DNA. Ann Rev Genet **18**:131–171.
127. Neufeld EF 1989 Natural history and inherited disorders of a lysosomal enzyme, β-hexosaminidase. J Biol Chem **264**:10927–10930.
128. Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ 1989 The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science **244**: 1578–1580.
129. Malloy PJ, Hughes MR, Pike JW, Feldman D 1991 Vitamin D receptor mutations and hereditary 1,25-dihydroxyvitamin D–resistant rickets. In: Norman AW, Bouillon R, Thomasset M (eds) Vitamin D: gene regulation, structure-function analysis, and clinical application. Eighth workshop on vitamin D. Walter de Gruyter: New York, pp 116–124.
130. Wiese RJ, Goto H, Prahl JM, Marx SJ, Thomas M, al-Aqeel A, DeLuca HF 1993 Vitamin D-dependency rickets type II: truncated vitamin D–receptor in three kindreds. Mol Cell Endocrinol **90**:197–201.
131. Cockerill FJ, Hawa NS, Yousaf N, Hewison M, O'Riordan JL, Farrow SM 1997 Mutations in the vitamin D receptor gene in three kindreds associated with hereditary vitamin D–resistant rickets. J Clin Endocrinol Metab **82**:3156–3160.
132. Kristjansson K, Rut AR, Hewison M, O'Riordan JL, Hughes MR 1993 Two mutations in the hormone binding domain of the vitamin D receptor cause tissue resistance to 1,25 dihydroxyvitamin D_3. J Clin Invest **92**:12–16.
133. Barsony J, McKoy W, DeGrange DA, Liberman UA, Marx SJ 1989 Selective expression of a normal action of the 1,25-dihydroxyvitamin D_3 receptor in human skin fibroblasts with hereditary severe defects in multiple actions of that receptor. J Clin Invest **83**:2093–2101.
134. Zhu WJ, Malloy PJ, Delvin E, Chabot G, Feldman D 1998 Hereditary 1,25-dihydroxyvitamin D–resistant rickets due to an opal mutation causing premature termination of the vitamin D receptor. J Bone Miner Res **13**:259–264.
135. Mechica JB, Leite MO, Mendonca BB, Frazzatto ES, Borelli A, Latronico AC 1997 A novel nonsense mutation in the first zinc finger of the vitamin D receptor causing hereditary 1,25-dihydroxyvitamin D_3–resistant rickets. J Clin Endocrinol Metab **82**:3892–3894.
136. Malloy PJ, Zhu W, Bouillon R, Feldman D 2002 A novel nonsense mutation in the ligand binding domain of the vitamin D receptor causes hereditary 1,25-dihydroxyvitamin D–resistant rickets. Mol Genet Metab **77**:314–318.
137. Hawa NS, Cockerill FJ, Vadher S, Hewison M, Rut AR, Pike JW, O'Riordan JL, Farrow SM 1996 Identification of a novel mutation in hereditary vitamin D–resistant rickets causing exon skipping. Clin Endocrinol **45**:85–92.
138. Thompson E, Kristjansson K, Hughes M Molecular scanning methods for mutation detection: application to the 1,25-dihydroxyvitamin D receptor. Eighth workshop on Vitamin D, Paris, France, 1991, p 6.
139. Rut AR, Hewison M, Rowe P, Hughes M, Grant D, O'Riordan JLH 1991 A novel mutation in the steroid binding region of the vitamin D receptor (VDR) gene in hereditary vitamin D–resistant rickets (HVDRR). In: Norman AW, Bouillon R, Thomasset M (eds) Vitamin D: gene regulation, structure-function analysis, and clinical application. Eighth workshop on vitamin D. Walter de Gruyter: New York, pp 94–95.
140. Whitfield GK, Selznick SH, Haussler CA, Hsieh JC, Galligan MA, Jurutka PW, Thompson PD, Lee SM, Zerwekh JE, Haussler MR 1996 Vitamin D receptors from patients with resistance to 1,25-dihydroxyvitamin D_3: point mutations confer reduced transactivation in response to ligand and impaired interaction with the retinoid X receptor heterodimeric partner. Mol Endocrinol **10**:1617–1631.
141. Malloy PJ, Eccleshall TR, Gross C, Van Maldergem L, Bouillon R, Feldman D 1997 Hereditary vitamin D–resistant rickets caused by a novel mutation in the vitamin D receptor that results in decreased affinity for hormone and cellular hyporesponsiveness. J Clin Invest **99**:297–304.
142. Van Maldergem L, Bachy A, Feldman D, Bouillon R, Maassen J, Dreyer M, Rey R, Holm C, Gillerot Y 1996 Syndrome of lipoatrophic diabetes, vitamin D–resistant rickets, and persistent Müllerian ducts in a Turkish boy born to consanguineous parents. Am J Med Genet **64**:506–513.
143. Magre J, Delepine M, Khallouf E, Gedde-Dahl T, Jr., Van Maldergem L, Sobel E, Papp J, Meier M, Megarbane A, Bachy A, Verloes A, d'Abronzo FH, Seemanova E, Assan R, Baudic N, Bourut C, Czernichow P, Huet F, Grigorescu F, de Kerdanet M, Lacombe D, Labrune P, Lanza M, Loret H, Matsuda F, Navarro J, Nivelon-Chevalier A, Polak M, Robert JJ, Tric P, Tubiana-Rufi N, Vigouroux C, Weissenbach J, Savasta S, Maassen JA, Trygstad O, Bogalho P, Freitas P, Medina JL, Bonnicci F, Joffe BI, Loyson G, Panz VR, Raal FJ, O'Rahilly S, Stephenson T, Kahn CR, Lathrop M, Capeau J 2001 Identification of the gene altered in Berardinelli-Seip congenital lipodystrophy on chromosome 11q13. Nat Genet **28**:365–370.
144. Nguyen TM, Adiceam P, Kottler ML, Guillozo H, Rizk-Rabin M, Brouillard F, Lagier P, Palix C, Garnier JM, Garabedian M 2002 Tryptophan missense mutation in the ligand-binding domain of the vitamin D receptor causes severe resistance to 1,25-dihydroxyvitamin D. J Bone Miner Res **17**:1728–1737.
145. Malloy PJ, Xu R, Peng L, Peleg S, Ashwal A, Feldman D 2004 Heredity 1,25-dihydroxyvitamin D resistant rickets due to a mutation causing multiple defects in vitamin D receptor function. Endocrinology, in press.
146. Malloy PJ, Zhu W, Zhao XY, Pehling GB, Feldman D 2001 A novel inborn error in the ligand-binding domain of the vitamin D receptor causes hereditary vitamin D–resistant rickets. Mol Genet Metab **73**:138–148.
147. Wurtz JM, Bourguet W, Renaud JP, Vivat V, Chambon P, Moras D, Gronemeyer H 1996 A canonical structure for the ligand-binding domain of nuclear receptors. Nat Struct Biol **3**:87–94.
148. Whitfield GK, Hsieh JC, Nakajima S, MacDonald PN, Thompson PD, Jurutka PW, Haussler CA, Haussler MR 1995 A highly conserved region in the hormone-binding domain of the human vitamin D receptor contains residues vital for heterodimerization with retinoid X receptor and for transcriptional activation. Mol Endocrinol **9**:1166–1179.

149. Malloy PJ, Xu R, Peng L, Clark PA, Feldman D 2002 A novel mutation in helix 12 of the vitamin D receptor impairs coactivator interaction and causes hereditary 1,25-dihydroxyvitamin D–resistant rickets without alopecia. Mol Endocrinol **16**:2538–2546.
150. Miller J, Djabali K, Chen T, Liu Y, Ioffreda M, Lyle S, Christiano AM, Holick M, Cotsarelis G 2001 Atrichia caused by mutations in the vitamin D receptor gene is a phenocopy of generalized atrichia caused by mutations in the hairless gene. J Invest Dermatol **117**:612–617.
151. Hewison M, Rut AR, Kristjansson K, Walker RE, Dillon MJ, Hughes MR, O'Riordan JL 1993 Tissue resistance to 1,25-dihydroxyvitamin D without a mutation of the vitamin D receptor gene. Clin Endocrinol **39**:663–670.
152. Chen H, Hewison M, Hu B, Adams JS 2003 Heterogeneous nuclear ribonucleoprotein (hnRNP) binding to hormone response elements: A cause of vitamin D resistance. Proc Natl Acad Sci USA **100**:6109–6114.
153. Chrousos GP, Renquist D, Brandon D, Eil C, Pugeat M, Vigersky R, Cutler GB, Jr., Loriaux DL, Lipsett MB 1982 Glucocorticoid hormone resistance during primate evolution: receptor-mediated mechanisms. Proc Natl Acad Sci USA **79**:2036–2040.
154. Chrousos GP, Loriaux DL, Brandon D, Shull J, Renquist D, Hogan W, Tomita M, Lipsett MB 1984 Adaptation of the mineralocorticoid target tissues to the high circulating cortisol and progesterone plasma levels in the squirrel monkey. Endocrinology **115**:25–32.
155. Chrousos GP, Brandon D, Renquist DM, Tomita M, Johnson E, Loriaux DL, Lipsett MB 1984 Uterine estrogen and progesterone receptors in an estrogen- and progesterone-"resistant" primate. J Clin Endocrinol Metab **58**:516–520.
156. Adams JS, Gacad MA, Baker AJ, Kheun G, Rude RK 1985 Diminished internalization and action of 1,25-dihydroxyvitamin D_3 in dermal fibroblasts cultured from New World primates. Endocrinology **116**:2523–2527.
157. Takahashi N, Suda S, Shinki T, Horiuchi N, Shiina Y, Tanioka Y, Koizumi H, Suda T 1985 The mechanism of end-organ resistance to 1 alpha,25-dihydroxycholecalciferol in the common marmoset. Biochem J **227**:555–563.
158. Reynolds PD, Ruan Y, Smith DF, Scammell JG 1999 Glucocorticoid resistance in the squirrel monkey is associated with overexpression of the immunophilin FKBP51. J Clin Endocrinol Metab **84**:663–669.
159. Giraldo A, Pino W, Garcia-Ramirez LF, Pineda M, Iglesias A 1995 Vitamin D–dependent rickets type II and normal vitamin D receptor cDNA sequence. A cluster in a rural area of Cauca, Colombia, with more than 200 affected children. Clin Genet **48**:57–65.
160. Tsuchiya Y, Matsuo N, Cho H, Kumagai M, Yasaka A, Suda T, Orimo H, Shiraki M 1980 An unusual form of vitamin D–dependent rickets in a child: alopecia and marked end-organ hyposensitivity to biologically active vitamin D. J Clin Endocrinol Metab **51**:685–690.
161. Fujita T, Nomura M, Okajima S, Furuya H 1980 Adult-onset vitamin D–resistant osteomalacia with the unresponsiveness to parathyroid hormone. J Clin Endocrinol Metab **50**: 927–931.
162. Kruse K, Feldmann E 1995 Healing of rickets during vitamin D therapy despite defective vitamin D receptors in two siblings with vitamin D–dependent rickets type II. J Pediatr **126**:145–148.
163. Balsan S, Garabedian M, Larchet M, Gorski AM, Cournot G, Tau C, Bourdeau A, Silve C, Ricour C 1986 Long-term nocturnal calcium infusions can cure rickets and promote normal mineralization in hereditary resistance to 1,25-dihydroxyvitamin D. J Clin Invest **77**:1661–1667.
164. Weisman Y, Bab I, Gazit D, Spirer Z, Jaffe M, Hochberg Z 1987 Long-term intracaval calcium infusion therapy in end-organ resistance to 1,25-dihydroxyvitamin D. Am J Med **83**:984–990.
165. Hochberg Z, Tiosano D, Even L 1992 Calcium therapy for calcitriol-resistant rickets. J Pediatr **121**:803–808.
166. Weisman Y, Jaccard N, Legum C, Spirer Z, Yedwab G, Even L, Edelstein S, Kaye AM, Hochberg Z 1990 Prenatal diagnosis of vitamin D–dependent rickets, type II: response to 1,25-dihydroxyvitamin D in amniotic fluid cells and fetal tissues. J Clin Endocrinol Metab **71**:937–943.
167. Weisman Y, Malloy PJ, Krishnan AV, Jaccard N, Feldman D, Hochberg Z Prenatal diagnosis of calcitriol resistant rickets (CRR) by $1,25(OH)_2D_3$ binding, 24-hydroxylase induction and RFLP analysis. Ninth workshop on Vitamin D, Orlando, 1994, p 106.
168. Gardezi SA, Nguyen C, Malloy PJ, Posner GH, Feldman D, Peleg S 2001 A rationale for treatment of hereditary vitamin D–resistant rickets with analogs of 1 alpha,25-dihydroxyvitamin D_3. J Biol Chem **276**:29148–29156.
169. Swann SL, Bergh J, Farach-Carson MC, Ocasio CA, Koh JT 2002 Structure-based design of selective agonists for a rickets-associated mutant of the vitamin D receptor. J Am Chem Soc **124**:13795–13805.
170. Swann SL, Bergh JJ, Farach-Carson MC, Koh JT 2002 Rational design of vitamin D_3 analogues which selectively restore activity to a vitamin D receptor mutant associated with rickets. Org Lett **4**:3863–3866.
171. Kittaka A, Kurihara M, Peleg S, Suhara Y, Takayama H 2003 2alpha-(3-Hydroxypropyl)- and 2alpha-(3-Hydroxypropoxy)-1alpha,25-dihydroxyvitamin D(3) Accessible to vitamin D receptor mutant related to hereditary vitamin D–resistant rickets. Chem Pharm Bull (Tokyo) **51**:357–358.
172. Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, Goltzman D 2001 Targeted ablation of the 25-hydroxyvitamin D 1alpha-hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. Proc Natl Acad Sci USA **98**:7498–7503.
173. Chen CH, Sakai Y, Demay MB 2001 Targeting expression of the human vitamin D receptor to the keratinocytes of vitamin D receptor null mice prevents alopecia. Endocrinology **142**:5386–5389.
174. Li M, Chiba H, Warot X, Messaddeq N, Gerard C, Chambon P, Metzger D 2001 RXR-alpha ablation in skin keratinocytes results in alopecia and epidermal alterations. Development **128**:675–688.
175. Ahmad W, Faiyaz ul Haque M, Brancolini V, Tsou HC, ul Haque S, Lam H, Aita VM, Owen J, deBlaquiere M, Frank J, Cserhalmi-Friedman PB, Leask A, McGrath JA, Peacocke M, Ahmad M, Ott J, Christiano AM 1998 Alopecia universalis associated with a mutation in the human hairless gene. Science **279**:720–724.
176. Cichon S, Anker M, Vogt IR, Rohleder H, Putzstuck M, Hillmer A, Farooq SA, Al-Dhafri KS, Ahmad M, Haque S, Rietschel M, Propping P, Kruse R, Nothen MM 1998 Cloning, genomic organization, alternative transcripts, and mutational analysis of the gene responsible for autosomal recessive universal congenital alopecia. Hum Mol Genet **7**:1671–1679.
177. Hsieh JC, Sisk JM, Jurutka PW, Haussler CA, Slater SA, Haussler MR, Thompson CC 2003 Physical and functional

interaction between the vitamin D receptor and hairless corepressor, two proteins required for hair cycling. J Biol Chem **7**:7.
178. Ikeda K, Lu C, Weir EC, Mangin M, Broadus AE 1989 Transcriptional regulation of the parathyroid hormone-related peptide gene by glucocorticoids and vitamin D in a human C-cell line. J Biol Chem **264**:15743–15746.
179. Wysolmerski JJ, Broadus AE, Zhou J, Fuchs E, Milstone LM, Philbrick WM 1994 Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development. Proc Natl Acad Sci USA **91**:1133–1137.
180. Cho YM, Woodard GL, Dunbar M, Gocken T, Jimenez JA, Foley J 2003 Hair-cycle–dependent expression of parathyroid hormone-related protein and its type I receptor: evidence for regulation at the anagen to catagen transition. J Invest Dermatol **120**:715–727.
181. Haussler MR, McCain TA 1977 Basic and clinical concepts related to vitamin D metabolism and action. N Engl J Med **297**:1041–1050.
182. DeLuca HF 1979 The vitamin D system in the regulation of calcium and phosphorus metabolism. Nutr Rev **37**:161–193.
183. Clemens TL, Garrett KP, Zhou XY, Pike JW, Haussler MR, Dempster DW 1988 Immunocytochemical localization of the 1,25-dihydroxyvitamin D_3 receptor in target cells. Endocrinology **122**:1224–1230.
184. Skowronski R, Peehl D, Feldman D 1993 Vitamin D and prostate cancer: 1,25-dihydroxyvitamin D_3 receptors and actions in prostate cancer cell lines. Endocrinology **132**: 1952–1960.
185. Panda DK, Miao D, Bolivar I, Li J, Huo R, Hendy GN, Goltzman D 2004 Inactivation of the 25-hydroxyvitamin D 1 alpha-hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis. J Biol Chem **279**:16754–16766.
186. Underwood JL, DeLuca HF 1984 Vitamin D is not directly necessary for bone growth and mineralization. Am J Physiol **246**:E493–498.
187. Miyamoto K, Kesterson RA, Yamamoto H, Taketani Y, Nishiwaki E, Tatsumi S, Inoue Y, Morita K, Takeda E, Pike JW 1997 Structural organization of the human vitamin D receptor chromosomal gene and its promoter. Mol Endocrinol **11**:1165–1179.

CHAPTER 73

Glucocorticoids and Vitamin D

PHILIP SAMBROOK Institute of Bone & Joint Research, University of Sydney, Sydney, Australia

I. INTRODUCTION

This chapter gives a general overview of the current understanding of glucocorticoid induced osteoporosis (GIO) with special reference to vitamin D metabolism. This chapter also includes a review of steroid hormone receptor expression in bone cells with specific reference to glucocorticoid and vitamin D hormones and their interaction. Clinical aspects of GIO and the role of vitamin D metabolites as therapeutic agents in GIO are also discussed.

Bone loss resulting from long-term glucocorticoid therapy is a common clinical problem. A number of different glucocorticoid mediated effects are responsible for GIO, including 1) direct inhibitory effects of glucocorticoids upon osteoblast, osteocyte, and osteoclast function leading to reduced bone remodeling and diminished repair of microdamage in bone as well as enhanced osteoblast and osteocyte apoptosis; 2) antagonism by glucocorticoids of gonadal function and inhibition of the osteoanabolic action of sex steroids; 3) increased renal excretion and reduced intestinal absorption of calcium leading to negative calcium balance that can promote secondary hyperparathyroidism; and 4) potential effects on vitamin D metabolism [1–3]. From a mechanistic point of view, all of these effects have long been considered to be mediated at the molecular level exclusively by genomic actions. However, there is now increasing interest in the existence of rapid glucocorticoid effects that are incompatible with this classical mode of action [1]. These rapid effects, termed nongenomic effects, appear to be mediated by glucocorticoid interactions with biological membranes, either through binding to membrane receptors or by physicochemical interactions [1].

II. STEROID RECEPTORS AND ACTIONS IN BONE

A. Receptors

The skeleton is a complex tissue and hormonal control of bone remodeling is elaborate (see Chapter 28). The important role that steroid hormones play in bone cell development and in the maintenance of normal bone architecture is well established, but it is only relatively recently that it has become possible to describe the precise mechanism of action of steroid hormones including glucocorticoids and related hormones such as vitamin D, all of which act via structurally homologous nuclear receptors that form part of the steroid/thyroid receptor superfamily (described in Section II of this book). The action of all these hormones are mediated by hormone binding to these nuclear receptors, which act as ligand-dependent transcription factors to either activate or repress target gene expression [4].

Thus, for glucocorticoids, it is currently believed that most of their biological activities are mediated via binding to the glucocorticoid receptor (GR). By this classic genomic mechanism, lipophilic glucocorticoid passes across the cell membrane, attaches to the cytosolic GR and after dimerization, the GR binds to conserved sequence motifs (glucocorticoid response elements or GREs) to positively or negatively regulate specific gene transcription [5]. However, it is also now recognized that some biological activities of glucocorticoids may be mediated via other transcription factors, such as AP-1 and NFkB, independent of GR binding to DNA but dependent upon interaction with these factors [5]. Similarly, the diverse biological activities of vitamin D which include effects on bone, cancer, and immune cells are mediated by binding of the ligand, calcitriol,

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or 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$) to the ubiquitously expressed vitamin D receptor (VDR). The VDR functions as a heterodimer with retinoid X receptors (RXR) to bind vitamin D response elements (VDREs) within gene promoters and interact with a number of cofactors that are critical for transcriptional activation. It appears that both GR and VDR share a large number of coactivators and corepressors, raising the possibility that shared components for certain functions could be a limiting effect.

In humans, alternative splicing of GR mRNA produces two similar isoforms, GRα and GRβ. However GRβ does not bind ligand and, although it may act as a dominant negative regulator of GRα activity, most studies consequently have focused on the GRα isoform [4]. In terms of glucocorticoid effects on bone cells, cytosolic binding studies have shown specific glucocorticoid binding sites predominantly in cells of osteoblastic lineage. This glucocorticoid binding occurs in animal and human osteoblasts and human osteosarcoma cells [6–14]. The effects of glucocorticoid on bone cells appear to be species specific. For example, in rats glucocorticoids increase differentiation of osteoblasts and osteoblasts progenitors [15–17]. The increase in osteoblast differentiation is associated with induction of osteoblast marker genes such as alkaline phosphatase, osteocalcin, osteopontin, and bone sialoprotein [18–20]. In mice, however, the principal effect of glucocorticoids is to stimulate bone resorption and osteoclast formation [21,22]. In osteoblast cultures in human cells, glucocorticoids appear necessary for osteoblast differentiation and may induce some osteoblast marker genes such as alkaline phosphatase [22–25], while inhibiting others such as osteocalcin [22–25], although these effects may differ depending upon the age and stage of differentiation of the cultured cells [25,26].

B. Calcitriol

Vitamin D also has predominant effects on osteoblasts and modulates the expression of a number of osteoblastic genes including alkaline phosphatase, osteopontin, and osteocalcin [27–32], as well as inhibiting collagen synthesis [33–35]. However, the osteoblastic response to $1,25(OH)_2D$ appears to vary in relation to the stage of osteoblast development [26] (see Chapters 32 and 37).

Since osteocalcin, a protein produced by osteoblasts, is induced by $1,25(OH)_2D$ (see Chapter 41) but suppressed by glucocorticoids, study of this gene has provided many insights into the interactions of vitamin D and glucocorticoids at the genomic level. For example, transcription of the rat osteocalcin gene is controlled by basal and hormone response elements located in proximal and distal sites, respectively [36]. $1,25(OH)_2D$ acts on the osteocalcin gene via the distal VDRE within the promoter at nucleotides −465 to −437. This VDRE functions as an enhancer and cannot induce transcription but requires basal expression. Morrison *et al*. [37] have shown that glucocorticoids repress both $1,25(OH)_2D$ induction and basal activity of the osteocalcin promoter through a region distinct from the VDRE. Indeed, the rat and human osteocalcin promoters contain multiple GREs [38–41]. In the rat osteocalcin gene, GREs in the distal (nucleotides −697 to −683) and proximal promoter regions (−16 to −1) bind GR and suppress $1,25(OH)_2D$ induced transcription of osteocalcin [41]. Shalhoub *et al.* [20] have shown the influence of glucocorticoids on osteocalcin transcription is dependent on the stage of osteoblast maturation. In proliferating early stage osteoblasts, dexamethasone increased osteocalcin transcriptional rates, although not to the extent of vitamin D, and only in cells continuously treated with dexamethasone for long periods. In mature cells, acute treatment with dexamethasone resulted in decreased transcription and mRNA levels. The results indicate that transcriptional control of basal and hormone-regulated osteocalcin expression predominates in immature osteoblasts prior to matrix mineralization. However, in mature osteoblasts, osteocalcin expression was controlled primarily by post-transcriptional mechanisms reflected by elevated mRNA levels with a decline in transcription. Vitamin D, alone or in combination with dexamethasone, was a significant factor contributing to mRNA stabilization in mature osteoblasts with a mineralized extracellular matrix. Transcriptional modifications in response to dexamethasone were reflected by quantitative differences between proliferating and mature osteoblasts in the formation of GR binding complexes at the proximal osteocalcin GRE. Both VDR and GR basal mRNA levels were significantly higher in mature osteoblasts than in early stage bone cells. Dexamethasone significantly increased VDR transcription on day 7 (proliferation stage) and day 20 (differentiated osteoblasts) in fetal rat calvariae, but this resulted in a small increase in VDR mRNA accumulation on day 7 and a decrease on day 20. These results suggest there are developmental, stage-specific effects of steroid hormone on transcriptional regulation of bone-expressed genes, and inverse relationships between levels of transcription and cellular representation of mRNA with osteocalcin message stabilized in mature osteoblasts. Further studies by the same group [42] were consistent with selective influences of $1,25(OH)_2D$ and glucocorticoids as a function of osteoblast maturation and species-specific responsiveness of mouse bone-expressed genes, to steroid

hormones during osteoblast differentiation. These findings were in contrast to findings from other osteoblast culture systems.

Binding of the VDR by $1,25(OH)_2D$ induces conformational changes in the receptor that enable it to interact with several types of cofactors that are necessary for transcriptional activation (see Chapter 16). It has been shown that osteocalcin gene activation by $1,25(OH)_2D$ at the VDRE region is accompanied by changes in chromatin structure and that such chromatin remodeling is a prerequisite for transcription [43]. Indeed, unlike the GR, the VDR-RXR appears unable to bind its target sequence until nucleosomal remodeling occurs, to allow occupancy of binding sites in the distal region of the osteocalcin gene promoter by the regulatory factors responsible for $1,25(OH)_2D$ dependent enhancement of transcription.

The extent to which such transcriptional coactivators are involved in modulating tissue-specific functions of the VDR is unclear. Recent studies by Issa *et al*. [44] have investigated the role of p160 coactivators in regulating VDR function and interaction with the heterodimeric partner of VDR, RXR. Two p160 coactivators, glucocorticoid receptor interacting protein-1 (GRIP1) and receptor associated coactivator-3 (RAC3) [45,46], appear to interact directly with the VDR, but only in the presence of the ligand. Deletional analyses of VDR suggest that GRIP1 and RAC3 required an intact VDR activation function domain (AF-2) for efficient interaction. Co-expression studies indicated that both GRIP1 and RAC3 co-assemble with the VDR to form an active transcriptional complex. They also form ternary complexes with VDR homodimers and VDR:RXRα heterodimers. Consistent with a role in modulating VDR function in bone, GRIP1 potentiated transactivation of the osteocalcin promoter, whereas RAC3 enhanced VDR activation indirectly through RXR. These data suggest different p160 coactivators regulate VDR function via different mechanisms and that the VDR recruits different coactivators depending on specific gene and cellular contexts.

III. EFFECT OF GLUCOCORTICOIDS ON VITAMIN D METABOLISM

The metabolism of serum 25-hydroxy vitamin D (25OHD) occurs mainly in the kidney where it is converted to $1,25(OH)_2D$ by the enzyme 1-α-hydroxylase and to 24,25-dihydroxyvitamin D by the enzyme vitamin D-24-hydroxylase (see Chapters 5 and 6). It has been suggested that chronic glucocorticoid therapy can increase renal expression of vitamin D-24-hydroxylase and decrease expression or renal 1-α-hydroxylase [47]. Kurahashi *et al.* [48] investigated the mechanisms of this increase in UMR-106 osteoblast-like cells and found dexamethasone dose dependently increased 24-hydroxylase mRNA expression and enzymatic activity in the presence of $1,25(OH)_2D$. The mechanism of this effect appeared to involve activation of the AP-1 site by increased c-fos protein. However, the significance of this observation is uncertain since changes in circulating vitamin D metabolites with glucocorticoid therapy are not clearly established.

As noted above, although multiple different glucocorticoid mediated effects are responsible for GIO, consistent findings from many studies of increased renal calcium excretion and reduced intestinal absorption of calcium, which could lead to secondary hyperparathyroidism, have lead to numerous studies to look for alterations in circulating vitamin D metabolites in patients receiving exogenous glucocorticoids or with Cushing's syndrome. However, just as the published data looking for increased serum PTH concentrations with glucocorticoids are conflicting, there is even less evidence that changes in vitamin D metabolism are involved in the pathophysiology of GIO. At the same time it must be acknowledged that interpretation of the published data is difficult because many of the studies have been cross-sectional in nature with small samples, although some prospective studies have been performed (Table I).

Long-term excess glucocorticoids have been reported to produce varied effects on vitamin D metabolites such as 25OHD or $1,25(OH)_2D$, including reductions [49–51], no change [52–55], or small increases [56–59]. Chesney *et al.* [49] reported reductions in serum $1,25(OH)_2D$ in 22 children receiving long-term glucocorticoid treatment for various glomerular diseases, including nephrotic syndrome (mean $\pm$ SD serum $1,25(OH)_2D$: 20 ± 4 pg/ml vs. 53 ± 5 pg/ml in controls, $p < 0.005$). Moreover, the reduction in concentration of serum $1,25(OH)_2D$ correlated with the dose of corticosteroid administered and with reduction in forearm bone mineral content. In contrast, in 10 children with chronic glomerulonephritis not treated with glucocorticoids, who had similar serum creatinine to those children treated with glucocorticoids, serum $1,25(OH)_2D$ concentrations were similar to those in 18 healthy controls, indicating that glomerular renal disease per se did not account for the observed reduction.

In a subsequent study, Chesney *et al*. [50] measured vitamin D metabolites in 8 children with chronic glomerulonephritis not treated with prednisone (group I), 9 nonedematous children with nephrotic syndrome treated with prednisone for more than 18 months (group 2), and in 5 children with nephrotic edema also treated with prednisone (group 3). Reductions in

TABLE I Studies of Serum Vitamin D Metabolites in Glucocorticoid-Induced Osteoporosis

Reference	Type	Disease population	Sample size	Findings	
				25(OH)D	$1,25(OH)_2D$
Chesney [49]	Cross-sectional	Renal, pediatric	22	N	L
Chesney [50]	Cross-sectional	Renal, pediatric	21	L if edema	L
Klein [61]	Cross-sectional	Rheumatic	27	L	–
Seeman [52]	Cross-sectional	Mixed	14	L	N
Slovik [53]	Cross-sectional	Asthma	48	L-N	–
Cannigia [62]	Cross-sectional	Not stated	15	L-N	–
Hahn [56]	Prospective	Normal	12	N	small H
Hahn [60]	Prospective	Rheumatic	17	N	–
Findling [55]	Prospective	Cushing's	7	N	N
Morris [51]	Cross-sectional	Adult, miscellaneous	60	-	L-N
Prummel [52]	Prospective	Graves disease	10	N	N
Cosman [59]	Prospective	Multiple sclerosis	56	N	H

L = low; N = normal; H = high or elevated.

serum calcium, albumin, and 25(OH)D were found in group 3 only, whereas both group 2 and group 3 patients showed reduced values of $1,25(OH)_2D$ ($p < 0.001$ vs. group I or controls). It was concluded that chronic glucocorticoid administration in children with glomerulonephritis and minimally impaired renal function (group 2) was associated with a reduction in the circulating level of $1,25(OH)_2D$, since children with comparable type and degree of renal disease but nonglucocorticoid treatment (group I) had normal $1,25(OH)_2D$ values. Children with nephrotic edema (group 3) had greater reduction of $1,25(OH)_2D$ values, as well as lower 25(OH)D values and serum calcium values, considered possibly related to a urinary loss of vitamin D–binding protein. No changes in PTH were evident in either glucocorticoid-treated or edematous patients, suggesting that the acute elevation in PTH seen after prednisone treatment is an acute phenomenon.

Morris *et al.* [51] examined serum $1,25(OH)_2D$ levels in 60 postmenopausal women on glucocorticoid therapy (29 with and 31 without vertebral compression fractures), and they compared these results with those from 31 normal age-matched postmenopausal women. Serum $1,25(OH)_2D$ levels were slightly reduced in both glucocorticoid treated groups (mean $\pm$ SE: 80 ± 8.4 and 92 ± 7.9 pmol/l respectively) compared to normal subjects (107 ± 7.3 pmol/l), but these differences were not significant.

Seeman *et al.* [52] studied circulating levels of vitamin D metabolites in 6 patients with endogenous Cushing's syndrome and 8 patients treated with prednisone (mean dose 50 mg/day, range 30–60 mg/day) for one month for various connective tissue diseases. Comparing "euglucocorticoid" levels (i.e., after surgical correction in the Cushing's patients or before treatment in the prednisone therapy group) with the hyperglucocorticoid state (i.e., before surgery or after prednisone therapy, respectively), they observed a nonsignificant reduction in serum $1,25(OH)_2D$ (mean $\pm$ SD: 32 ± 8 vs. 23 ± 6 pg/ml), but significantly lower serum 25(OH)D (22 ± 2 vs. 18 ± 2 ng/ml) in the hyperglucocorticoid state. Kinetic studies using tritiated $1,25(OH)_2D$ in 10 hyperglucocorticoid patients and 14 normal controls revealed no evidence for altered production or degradation.

Slovik *et al.* [53] observed that mean serum 25(OH)D and PTH levels in 48 adult asthmatic patients on chronic glucocorticoid therapy were not significantly different from a disease control group of 12 asthmatic patients not on glucocorticoids, but nine such patients had abnormally low 25(OH)D levels.

Hahn *et al.* [56] studied 12 normal adults treated with prednisone 20 mg daily for 2 weeks. Serum 25(OH)D did not change significantly but serum $1,25(OH)_2D$ rose significantly as did serum PTH, but the latter rise was not significant. In another prospective study of the effect of administration of 25(OH)D in GIO (discussed below), Hahn *et al.* [59] observed no difference in baseline serum 25(OH)D in 17 patients treated with chronic glucocorticoids (dose greater than 7.5 mg prednisone equivalent for at least 18 months) compared to 15 normal subjects.

Thus, cross-sectional studies have generally reported no change in vitamin D metabolites in response to glucocorticoid therapy. Findling *et al.* [55] studied vitamin D metabolites in 7 patients with spontaneous ACTH-dependent Cushing's syndrome. Remission of hypercortisolism resulted in a significant increase in tubular reabsorption of phosphate and serum phosphate. Serum PTH levels were normal during Cushing's syndrome, but fell significantly after remission. Plasma 25(OH)D and $1,25(OH)_2D$ did not differ from measurements in 97 normal subjects. After treatment, serum 25(OH)D did not change, but serum $1,25(OH)_2D$ fell (mean 44 to 22 pg/ml, $p < 0.02$) and was inversely correlated with serum phosphate ($r = 0.59$; $p < 0.01$), but did not correlate with serum PTH. It was concluded that impairment of intestinal calcium absorption in Cushing's syndrome could not be attributed to any decrease in the circulating levels of $1,25(OH)_2D$.

In a small study, Prummel *et al.* [54] prospectively measured biochemical markers of bone turnover in 10 euthyroid patients with Graves ophthalmopathy treated with tapering prednisone (initial dose 60 mg/day) over 19 weeks. There were no significant changes in serum 25(OH)D or $1,25(OH)_2D$, although serum intact PTH fell slightly. In another larger prospective study, Cosman *et al.* [59] studied various biochemical markers of bone turnover in a larger population of 56 patients with multiple sclerosis, also treated with tapering corticosteroids (initially intravenous methylprednisolone 1 g daily for 10 days, then 500 mg/day for 2 days, 250 mg/day for 2 days followed by oral prednisone 80 mg/day reducing in dose over 28 days). During and after treatment, there were no changes in serum 25(OH)D, but serum $1,25(OH)_2D$ increased and serum phosphate decreased within 3 days of commencing corticosteroid. Serum PTH increased later to a peak at 2 weeks and serum $1,25(OH)_2D$ subsequently declined (Fig. 1). The early changes in serum $1,25(OH)_2D$ were considered to represent a direct effect of glucocorticoids on the kidney, rather than being secondary to the decrease in serum phosphate, with the later decline secondary to the change in PTH.

IV. VITAMIN D AS A TREATMENT FOR GIO

Even though the literature is controversial as to whether circulating metabolites of vitamin D are affected by glucocorticoid therapy, the original rationale for use of vitamin D in GIO was based upon the demonstration of impaired calcium absorption in glucocorticoid treated subjects and the assumption that this could be

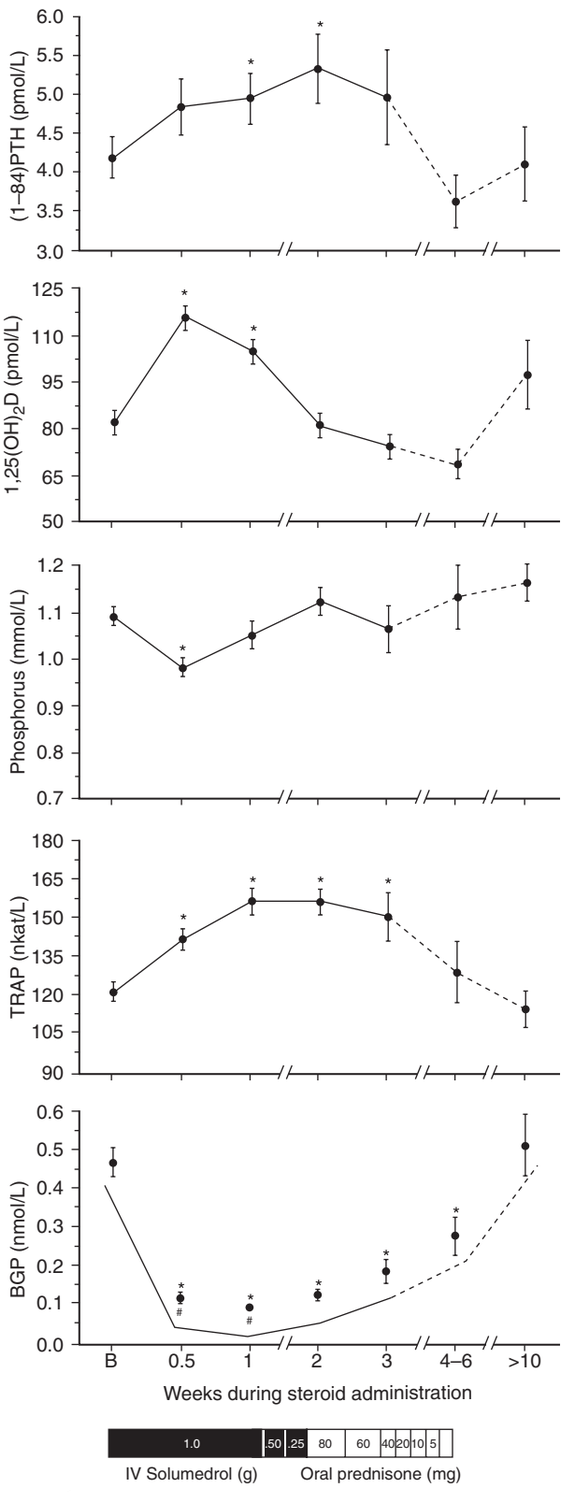

FIGURE 1 Effects of intravenous followed by oral glucocorticoid over 10 weeks on serum indices of mineral and skeletal metabolism. Reproduced from J Bone Miner Res 1994, **9**: 1097–1105 with permission of the American Society for Bone and Mineral Research.

reversed by vitamin D [60–63]. Therapeutic studies of vitamin D have examined both changes in calcium absorption and bone mass or density. Some of the earlier studies have been performed with simple vitamin D or calciferols; however, the active metabolites, calcitriol and alfacalcidol (1α-hydroxy vitamin D), have also been studied. Recent interest has also focused on the nonskeletal benefits of vitamin D (such as reducing the risk of falls; see Chapter 102) as a further mechanism of reduced fracture risk, although this has not been specifically studied in GIO.

A. Calcium Absorption

Klein *et al.* [61] compared fractional calcium absorption in 27 patients receiving prednisone compared to 27 age- and sex-matched controls. In patients receiving high doses of prednisone (15–100 mg/day) calcium absorption and serum 25OHD were decreased. However, in patients receiving low doses (8–10 mg/day), or high doses (30–100 mg) on an alternate day schedule, both of these parameters were normal. Calcium absorption correlated inversely with daily prednisone dosage. Administration of 0.4 μg of calcitriol daily for 7 days in 5 patients led to an increase in calcium absorption. A study by Hahn *et al.* [60] examined the effect of treatment with calcidiol (25 hydroxyvitamin D) 40 μg/day plus calcium in 17 patients with GIO compared to 15 controls. The glucocorticoid group had reduced calcium absorption and increased serum PTH, but similar serum 25(OH)D levels and reduced forearm bone mass compared to controls. Treatment increased calcium absorption by 46%. Caniggia *et al.* [62] reported that low to normal serum 25(OH)D in patients treated with chronic glucocorticoids and impaired calcium absorption could be reversed by treatment with either calcidiol or calcitriol.

Braun *et al.* [64] performed a double-blind placebo controlled study of alfacalcidol 2 μg daily for 6 months in 14 patients receiving long-term glucocorticoid therapy. Treatment with alfacalcidol increased calcium absorption at 3 and 6 months and, on repeat iliac crest biopsy, showed a decrease in active resorption and a trend for increased trabecular bone volume, suggesting no suppression of bone formation was occurring.

Morris *et al.* [51] examined the relation between calcium absorption and serum $1,25(OH)_2D$ levels in a set of 60 postmenopausal women on glucocorticoid therapy (29 with and 31 without vertebral compression fractures) and compared these results with those from 31 normal postmenopausal women age-matched women. Calcium absorption was reduced in glucocorticoid treated patients and shown to be linearly related to serum $1,25(OH)_2D$ in both glucocorticoid treated groups and in the glucocorticoid set as a whole. However only about one-third of the impairment in calcium absorption was accounted for by serum $1,25(OH)_2D$ levels. This apparent resistance to the intestinal action of $1,25(OH)_2D$ was quantified by a Z score, which expressed the difference between the measured calcium absorption and that predicted from the $1,25(OH)_2D$ level. The calcium absorption was significantly reduced in the fracture group by −0.52 standard deviation.

Despite the consistency in findings about impaired calcium absorption, its mechanism remains unclear. Studies of changes in intestinal $1,25(OH)_2D$ receptor expression have yielded conflicting results. Dexamethasone administration in male rats caused a reduction in receptor number in jejunal villous cells; however, administration of prednisone in dogs increased duodenal concentrations of a $1,25(OH)_2D$ specific binding protein [65,66]. Moreover, no changes in plasma $1,25(OH)_2D$ or in renal hydroxylase mRNA abundance or enzyme activity occur in response to dexamethasone [47].

Although the demonstration of impaired calcium absorption with glucocorticoids provided a rationale for use of vitamin D in GIO, other data from animal and human studies also suggested a role for vitamin D as a therapeutic agent. In a rat model, when calcitriol was coadministered with glucocorticoids, suppression of serum osteocalcin was delayed, and effects of glucocorticoids on osteoid volume, tetracycline labeled surface, and trabecular bone volume, assessed histomorphometrically, were prevented [67]. In clinical studies, coadministration of calcitriol with prednisone produced less suppression of serum osteocalcin than administration of prednisone alone. Nielsen *et al.* [68] treated 7 healthy volunteers with prednisone—10 mg daily for 2 days with or without 2 μg calcitriol in a cross-over design. Prednisone inhibited and reversed the nocturnal rise in serum osteocalcin, whereas after calcitriol plus prednisone, the time course of serum osteocalcin almost paralleled the placebo (see Fig. 2).

Lems *et al.* [69] examined the effect of low dose prednisone (10 mg/day) and the possible preventative effects of calcitriol on bone metabolism in 8 healthy, young male volunteers. The study consisted of four observation periods: in the first period, prednisone was administered for one week; in the second, third, and fourth periods, calcium (500 mg/day), calcitriol (0.5 μg bd), and calcium in combination with calcitriol respectively, were added to prednisone. Treatment with prednisone alone led to a decrease in osteocalcin and a (nonsignificant) increase in PTH, but PTH decreased during cotreatment with calcitriol (−16%) and calcium plus calcitriol (−44%; $p < 0.01$). It was concluded that the increase in PTH during prednisone

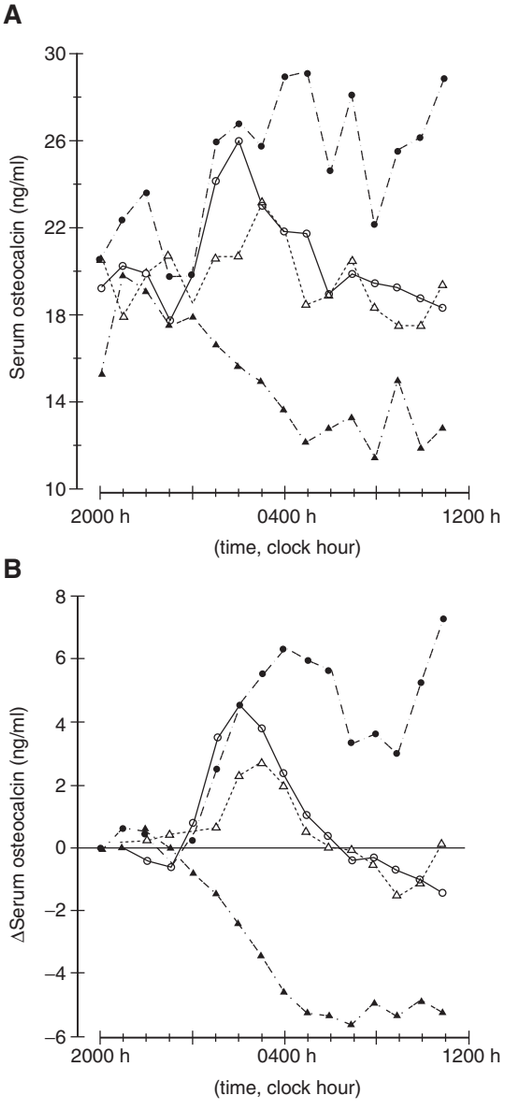

FIGURE 2 Mean serum osteocalcin levels against time in five normal subjects (**A**) mean curves using raw data; (**B**) mean smoothed (moving average technique) data transformed by subtracting the 2000 h values of each day from all subsequent times: (○) placebo; (•) 2 μg $1,25(OH)_2D_3$ + placebo; (Δ) 2 μg $1,25(OH)_2D_3$ + 10 mg prednisone; and (▲) 10 mg prednisone. Reproduced from J Bone Miner Res 1991, **5**:435–441 with permission of the American Society for Bone and Mineral Research.

could be prevented by taking calcitriol combined with calcium supplementation.

B. Bone Mass

These observations led inevitably to studies of the effects of vitamin D on bone mass. Vitamin D in various formulations, usually in combination with calcium, has been studied as a treatment for GIO in studies dating from the 1970s. These studies can be broadly divided into primary prevention (in patients commencing glucocorticoid therapy) or treatment (in patients on chronic glucocorticoids) (Table II). Hahn *et al.* [70] examined the effect of treatment with calcium 500 mg/day and vitamin D 50,000 units per week on bone mass in patients on chronic glucocorticoid therapy. A significant increase in forearm bone mass was observed with vitamin D treatment, but the study was not randomized. Since bone mass was only assessed in the radius (as the measurement of spinal bone density was not available at that time), the clinical relevance of these findings to other skeletal sites is unclear. Another study by Hahn *et al.* [60] examined the effect of treatment with calcidiol (40 μg/day) plus calcium in 17 patients receiving chronic glucocorticoids compared to 15 controls. Treatment with vitamin D improved bone mass over 12 months by 13.2 % at the metaphyseal site and 2.1% at the diaphyseal site.

Bijlsma *et al.* [71] studied the effect of calcium 500 mg daily versus calcium plus a vitamin D preparation (dihydrotachysterol 4000 IU on alternate days) in 21 patients on long-term glucocorticoid therapy (mean daily prednisone dose 14 mg/day) for 2 years. A small increase in lumbar spine BMD was noted in both groups, but there was no significant difference between groups.

Adachi *et al.* [72] compared combination calcium plus vitamin D (1000 mg daily plus 50,000 units weekly, respectively) against placebo over 3 years in 62 patients initiating corticosteroids (i.e., primary prevention). Bone loss at the lumbar spine was reduced by treatment with calcium/vitamin D, but the difference was not significantly different from the placebo (Fig. 3). However, a secondary prevention study by Buckley *et al.* [73], in 65 patients receiving chronic low-dose corticosteroids for rheumatoid arthritis observed an annual spinal loss of 2.0% in placebo-treated patients compared to 0.7% gain in calcium/vitamin D_3-treated patients (1000 mg plus 500 IU/day, respectively). As the patients were receiving a chronic low dose of corticosteroids, the BMD rise may have been explicable as a "remodeling transient", and the results may not necessarily be applicable to patients commencing corticosteroids (i.e., primary prevention) or those treated with higher doses.

Sambrook *et al.* [74] examined the effect of 12 months of calcium, calcitriol, or calcitonin in 103 patients starting corticosteroids. Patients treated with calcium lost bone rapidly at the lumbar spine (–4.3% in the first year), whereas patients treated with either calcitriol alone (mean dose 0.6 μg/day) or calcitriol

TABLE II Trials of Vitamin D Metabolites on Bone Mineral Density

Reference	Type	Prevention/ treatment	Agent	Sample size	BMD effect size (% change vs. control)*	
					Forearm	Spine
Hahn [70]	Nonrandomized, open	Treatment	Vit D 50,000 IU/wk	26	4.4	–
Hahn [60]	Nonrandomized, open	Treatment	Calcidiol 40 µg/d	32	5.8	–
Dykman [78]	Randomized,DB	Treatment	Calcitriol 0.4 µg/d	23	NS**	–
Biljsma [71]	Nonrandomized, open	Treatment	Vit D 2000 IU/d	21	–	5.4
Sambrook [74]	Randomized,DB	Prevention	Calcitriol 0.75 µg/d	103	–	3.0
Adachi [72]	Randomized	Prevention	Vit D 50,000 U/wk	62	–	–0.69
Buckley [73]	Randomized	Treatment	Vit D 500 IU/d	65	–	2.65
Reginster [75]	Randomized,DB	Prevention	Alfacalcidol 1 µg/d	145	–	6.06
Lambrindouki [79]	Randomized,DB	Treatment	Calcitriol 0.5 µg/d	81	–	1.6
Ringe [80]	Nonrandomized, open	Treatment	Alfacalcidol 1 µg/d*	85	–	2.0
Sambrook [82]	Randomized, open	Mixed	Calcitriol 0.5 µg/d*	198	–	–0.2
Ringe [81]	Randomized, open	Treatment	Alfacalcidol 1 µg/d*	204	–	3.2

* vs simple vitamin D.
Transplant studies not included as immunosuppressives other than glucocorticoids included.
** Small increase both groups, % difference not stated.

plus calcitonin lost at a much reduced rate (–1.3% and –0.2% per year, respectively (Fig. 4). Both groups were significantly different from the calcium group. Approximately 25% of patients developed mild hypercalcemia, probably related to coadministration of calcium, which settled with a reduction in the calcitriol dosage. A randomized double-blind controlled trial in 145 patients starting corticosteroids compared 1 µg/day of alfacalcidol with calcium [75]. After 12 months, the change in spinal bone density with alfacalcidol was +0.4% compared to –5.7% with calcium. Hypercalcemia occurred in only 6.7% of alfacalcidol-treated patients. A recent randomized, double-blind prospective trial found that prophylactic treatment with calcitriol in 66 patients undergoing cardiac or single lung transplantation was able to prevent or markedly reduce bone

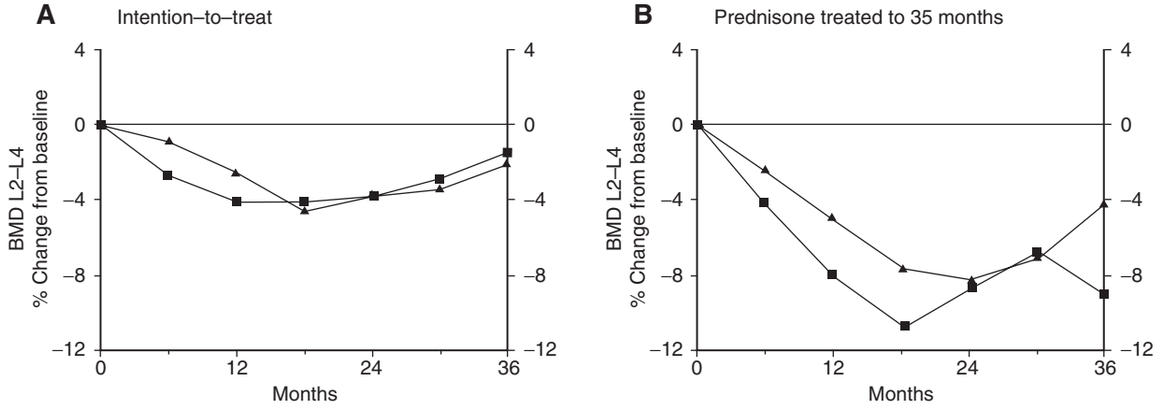

FIGURE 3 Lumbar spine BMD, percentage change from baseline (**A**) intention to treat analysis (**B**) prednisone treated to 36 months. (**A**) Intention to treat analysis (**B**) prednisone treated to 36 months; ■ Placebo treatment; ▲ Vitamin D and calcium treatment group. Reproduced from J Rheumatol 1996, **23**:995–1000 with permission.

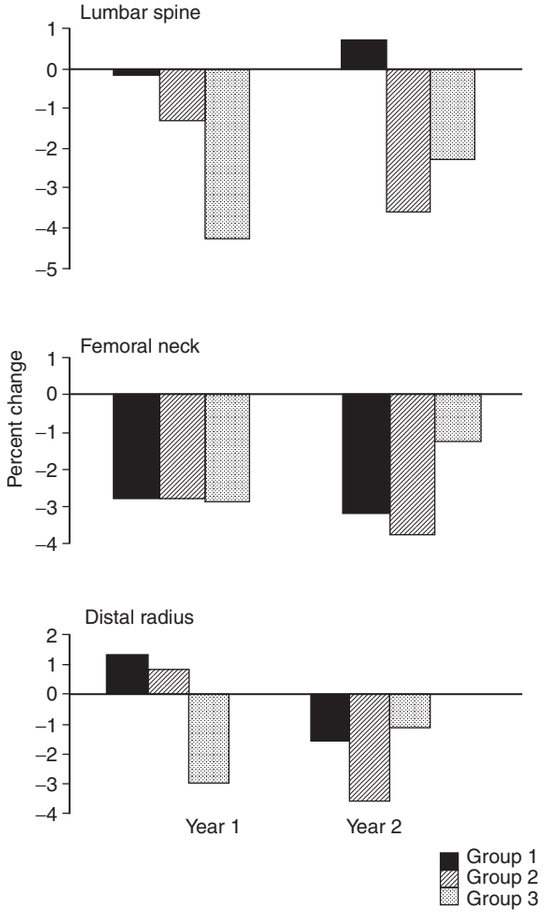

FIGURE 4 Mean bone mineral density of the lumbar spine, femoral neck, and distal radius as the percent change per year in corticosteroid treated patients. Reproduced from New Engl J Med 1993, **328**:1747–1752 with permission.
Group 1 received calcitriol, calcitonin, and calcium; group 2, calcitriol and calcium; and group 3, calcium alone. After one year, with regard to bone loss in the lumbar spine, P = 0.0035 for the overall difference between groups; P = 0.0001 for the difference between groups 1 and 3; P = 0.026 for the difference between groups 2 and 3; P = 0.94 for the difference between groups 1 and 2. After two years, P = 0.044 for the overall difference between groups; P = 0.17 for the difference between groups 1 and 3, P = 0.94 for the difference between groups 2 and 3, and P = 0.014 for the difference between groups 1 and 2. The one-year results represented 92 patients, and the two-year results 64 patients.

loss over 24 months [76]. Similarly, Neuhaus *et al.* [77] have also reported that calcitriol was effective in increasing spine and femoral BMD in patients after liver transplantation. However, the mechanisms of post transplant bone loss are likely to reflect not just the effects of glucocorticoids but also other immunosuppressive agents, such as cyclosporine (see Chapter 74) and so the findings of these two studies should be interpreted in this context.

Not all studies with active metabolites have shown positive effects on bone density. Dykman *et al.* [78] studied 23 rheumatic disease patients with GIO in an 18-month double-blind, randomized study to assess the effect of oral calcium and calcitriol (mean dose 0.4 μg/day) or calcium versus placebo. Intestinal calcium absorption was increased and serum PTH levels were suppressed by calcitriol; however, no significant gain in forearm bone mass occurred, and fractures were as frequent in both groups. In the calcitriol group, histomorphometric analysis of iliac crest biopsy specimens demonstrated a decrease in osteoclasts/mm^2 of trabecular bone ($p < 0.05$) and parameters of osteoblastic activity ($p < 0.05$), indicating that calcitriol reduced both bone resorption and formation.

Lambrinoudaki *et al.* [79] studied the effect of calcitriol in GIO in a double-blind, placebo-controlled study of 81 premenopausal women with systemic lupus erythematosus on chronic steroid therapy (mean cumulative prednisone dose of 28 g). They were randomly allocated to three groups: Group 1: 0.5 μg calcitriol and 1200 mg calcium daily; Group 2: 1,200 mg calcium and placebo calcitriol; and Group 3: both placebo calcitriol and placebo calcium. At the end of two years, patients in the calcitriol group exhibited a significant increase of 2.1% in BMD at the lumbar spine compared to baseline value ($p < 0.05$). This change was not significantly different from the respective change in either calcium or placebo group (0.4% and 0.3%, respectively). No significant changes were observed in any treatment group in BMD at the hip or radius. It was concluded that premenopausal women with lupus taking prolonged glucocorticoid therapy had lower bone density, but showed no significant bone loss over the two-year study period. The beneficial effect of calcitriol treatment in these premenopausal women was small when it was instituted late in the course of glucocorticoid therapy.

C. Efficacy of Different Vitamin D Preparations

Whether plain vitamin D is less efficacious than active metabolites in GIO is unclear, but three recent studies have addressed this question. Ringe *et al.* [80] evaluated the efficacy of alfacalcidol compared with simple vitamin D in patients on chronic corticosteroids. Eighty-five patients on long-term corticosteroid therapy were allocated to either 1 μg alfacalcidol plus calcium 500 mg daily or 1000 IU vitamin D_3 plus 500 mg calcium in a nonrandomized parallel group study. The two groups were similar in age, sex, underlying diseases, initial BMD (lumbar spine: mean T-score −3.28 and −3.25, respectively), and rates of vertebral and

nonvertebral fractures. During the three-year study, a small but significant increase was seen in lumbar spine BMD in the alfacalcidol group (+2.0%, $p < 0.0001$) with no significant changes at the femoral neck. In the vitamin D group, there were no significant changes at either site. More recently, Ringe *et al.* [81] have repeated this study with the same treatment regimen in a larger study sample and as a randomized open label trial. In 204 patients on chronic glucocorticoids over three years, significant increases in BMD were seen at lumbar spine (+2.4%) and the femoral neck (+1.2%) in the alfacalcidol group with no significant changes in the plain vitamin D group at either site (–0.7% and +0.7%, respectively). By the end of the study, 16 new vertebral fractures had occurred in 10 patients of the alfacalcidol group and 25 in 25 patients of the vitamin D group. These studies suggest alfacalcidol is superior to simple vitamin D in the treatment of established glucocorticoids osteoporosis.

In contrast, Sambrook [82] compared treatment with calcitriol with ergocalciferol or alendronate in 198 patients commencing or already being treated with chronic glucocorticoids. Patients were randomized to one of three groups: calcitriol 0.5 to 0.75 μg/day; simple vitamin D (ergocalciferol 30,000 IU weekly) plus calcium carbonate (600 mg daily); or alendronate 10 mg/day plus calcium carbonate (600 mg daily). Over two years, mean lumbar BMD change was +5.9% with alendronate, –0.5% with ergocalciferol, and –0.7% with calcitriol ($p < 0.001$). At the femoral neck, there was no significant difference in BMD change between the treatments over two years, alendronate (+0.9%), ergocalciferol (–2.2%), and calcitriol (–3.2%). The calcitriol group was treated with a higher cumulative glucocorticoid dose, but after adjustment, no significant difference was seen between calcitriol or ergocalciferol in the prevention of bone loss, but both were inferior to alendronate.

V. SUMMARY

To summarize, evidence from all these randomized trials taken together suggests that patients receiving glucocorticoids, who are at risk of rapid bone loss and consequent fracture, should be actively considered for prophylactic measures that include a vitamin D metabolite [83]. However, based upon the available evidence (not reviewed here), first-line therapy would be a bisphosphonate, with vitamin D as adjunctive therapy or second-line therapy. Moreover, although vitamin D metabolites appear to confer an additional therapeutic effect in GIO, it remains unclear whether the active metabolites are superior to simple vitamin D in this context and further studies are required.

References

1. Patschan D, Loddenkemper K, Buttgereit F 2001 Molecular mechanisms of glucocorticoid-induced osteoporosis. Bone **29**:498–505.
2. Sambrook P, Lane NE 2001 Corticosteroid osteoporosis, Balliere's best practice and research. Clin Rheumatol **15**:3, 401–413.
3. Weinstein RS, Jilka RL, Parfitt AF, Manolagas SC 1998 Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. J Clin Invest **102**:272–282.
4. Bland R 2000 Steroid hormone receptor expression and action in bone. Clin Science **98**:217–240.
5. Karin M 1998 New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? Cell **15**:93, 487–490.
6. Chen TL, Aronow L, Feldman D 1997 Glucocorticoid receptors and inhibition of bone cell growth in primary culture. Endocrinology **100**:619–628.
7. Manolagas SC, Anderson DC 1978 Detection of high-affinity glucocorticoid binding in rat bone. J Endocrinol **76**: 379–380.
8. Chen TL, Feldman D 1979 Glucocorticoid receptors and actions in subpopulations of cultured rat bone cells. J Clin Invest **63**:750–757.
9. Haussler MR, Manolagas SC, Deftos LJ 1980 Glucocorticoid receptor in clonal osteosarcoma cell lines: a novel system for investigating bone active hormones. Biochem Biophys Res Commun **94**:373–380.
10. Masuyama A, Ouchi Y, Sato F, Hosoi T, Nakamura T, Orimo H 1992 Characteristics of steroid hormone receptors in cultured MC3T3-E1 osteoblastic cells and effect of steroid hormones on cell proliferation. Calcif Tissue Int **51**:376–381.
11. Suzuki S, Koga Takaoka K, Ono K, Sato B 1993 Effects of retinoic acid on steroid and vitamin D_3 receptors in cultured mouse osteosarcoma cells. Bone **14**:7–12.
12. Liesegang P, Romalo G, Sudmann M, Wolf L, Schweikert HU 1994 Human osteoblast-like cells contain specific, saturable, high-affinity glucocorticoid, androgen, oestrogen, and 1alpha, 25-hydroxycholecalciferol receptors. J Androl **15**:194–199.
13. Song LN 1994 Effects of retinoic acid and dexamethasone on proliferation, differentiation, and glucocorticoid receptor expression in cultured human osteosarcoma cells. Oncol Res **6**:111–118.
14. Bland R, Worker CA, Noble BS, *et al.* 1999 Characterisation of 1 alpha-hydroxysteroid dehydrogenase activity and corticosteroid receptor expression in human osteosarcoma cell lines. J Endocrinol **161**:455–464.
15. Bellows CG, Ciaccia A, Heersche JNM 1998 Osteoprogenitor cells in cell populations derived from mouse and rat calvaria differ in the response to corticosterone, cortisol and cortisone. Bone **23**:119–125.
16. Boden SD, Hair G, Titus L, Racine M, McCuaig K, Wozney JM, Nanes MS 1997 Glucocorticoid-induced differentiation of fetal rat calvarial osteoblasts is mediated by bone morphogenetic protein 6. Endocrinol **138**:2820–2828.
17. Ishida Y, Heersche JM 1997 Progesterone stimulates proliferation and differentiation of osteoprogenitor cells in bone cell populations derived from adult female but not from adult male rats. Bone **20**:17–25.
18. Green E, Todd B, Heath D 1990 Mechanism of glucocorticoid regulation of alkaline phosphatase gene expression in osteoblast-like cells. Eur J Biochem **188**:147–153.

19. Ogata T, Yamauchi M, Kim RH, Li JJ, Freedman LP, Sodek J 1995 Glucocorticoid regulation of bone sialoprotein (BSP) gene-expression—identification of a glucocorticoid response element in the bone sialoprotein gene promoter. Eur J Biochem **230**:183–192.
20. Shalhoub V, Aslam F, Breen E, van Wijnen A, Bortell R, Stein GS, Stein JL, Lian JB 1998 Multiple levels of steroid hormone-dependent control of osteocalcin during osteoblast differentiation: glucocorticoid regulation of basal and vitamin D–stimulated gene expression. J Cell Biochem **69**:154–68.
21. Conaway HH, Grigorie D, Lerner UH 1996 Stimulation of neonatal mouse calvarial bone resorption by the glucocorticoids hydrocortisone and dexamethasone. J Bone Miner Res **11**:1419–1429.
22. Kaji H, Sugimoto T, Kanatani M, Nishiyama K, Chihara K 1997 Dexamethasone stimulates osteoclast-like cell formation by directly acting on hemopoietic blast cells and enhances osteoclast-like cell formation stimulated by parathyroid hormone and prostaglandin E_2. J Bone Miner Res **12**: 734–741.
23. Wong MM, Rao LG, Ly H, Hamilton L, Tong J, Sturtridge W, McBroom R, Aubin JE, Murray TM 1990 Long-term effects of physiologic concentrations of dexamethasone in human bone-derived cells. J Bone Miner Res **5**:803–813.
24. Subramaniam M, Colvard D, Keeting PE, Rasmussen K, Riggs BL, Spelsberg TC 1992 Glucocorticoid regulation of alkaline phosphatase, osteocalcin, and protooncogenes and normal human osteoblast-like cells. J Cell Biochem **50**: 411–424.
25. Sutherl MS, Rao LG, Muzaffar SA, Wylie JN, Wong MM, McBroom RJ, Murray TM 1995 Age-dependent expression of osteoblastic phenotypic markers in normal human osteoblasts cultured long-term in the presence of dexamethasone. Osteop Inter **5**:335–343.
26. Pockwinse SM, Stein JL, Lian JB, Stein GS 1995 Developmental stage-specific cellular responses to vitamin D glucocorticoids during differentiation of the osteoblast phenotype: interelationship of morphology and gene expression by *in situ* hybridization. Exp Cell Res **216**:244–260.
27. Skjodt H, Gallagher JA, Beresford JN, Couch M, Poser JW, Russell RGG 1985 Vitamin D metabolites regulate osteocalcin synthesis and proliferation of human bone cells *in vitro*. J Endocrinol **105**:391–396.
28. Demay MM, Gerardi JM, DeLuca HF, Kronenberg HM 1990 DNA sequences in the rat osteocalcin gene that bond the 1,25-dihydroxyvitamin D_3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci (USA) **87**: 369–373.
29. Williams GR, Bland R, Sheppard MC 1995 Retinoids modify regulation of endogenous gene expression by vitamin D_3 and thyroid hormone in three osteosarcoma cell lines. Endocrinology **136**:4304–4314.
30. Noda M, Vogel RL, Craig AM, Prahl J, DeLuca HF, Denhardt DT 1990 Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D_3 receptor and 1,25-dihydroxyvitamin d_3 enhancement of mouse secreted phosphoprotein 1 (spp-1 or osteopontin) gene expression. Proc Natl Acad Sci (USA) **87**:9995–9999.
31. Pols HAP, Schilte HP, Herrmann-Erlee NMP, Visser TJ, Birkenhager JC 1986 The effect of 1,25-dihydroxyvitamin D_3 on growth, alkaline phosphatase, and adenylate cyclase of rate osteoblast-like cells. Bone Miner **1**:397–405.
32. Owen TA, Arnow MS, Barone LM, Bettencourt B, Stein GS, Lian JB 1991 Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix. Endocrinol **128**:1496–1504.
33. Rowe DW, Kream BE 1982 Regulation of collagen synthesis in rat calvaria by 1,25-dihydroxyvitamin D_3. J Biol Chem **257**:8009–8015.
34. Kream BE, Rowe D, Smith MD, Maher V, Majeska R 1986 Hormonal regulation of collagen synthesis in a clonal rat osteosarcoma cell line. Endocrinol **119**:1922–1928.
35. Harrison JR, Peterson DN, Lichtler AC, Mador AT, Rowe DW, Kream BE 1989 1,25-dihydroxyvitamin D_3-inhibits transcription of type 1 collagen genes in the rat osteosarcoma cell line ROS 17/2.8. Endocrinol **125**:327–333.
36. Montecino M, Pockwinse S, Lian J, Stein G, Stein J 1994 DNase I hypersensitive sites in promoter elements associated with basal and vitamin D–dependent transcription of the bone-specific osteocalcin gene. Biochem **33**:348–353.
37. Morrison NA, Shine J, Fragonas JC, Verkest V, McMenemy ML, Eisman JA 1989 1,25-dihydroxyvitamin D–responsive element and glucocorticoid repression in the osteocalcin gene. Science **246**:1158–61.
38. Stromstedt PE, Poellinger L, Gustafsson JA, Carlstedt-Duke J 1991 The glucocorticoid receptor binds to a sequence overlapping the TATA box of the human osteocalcin promoter: a potential mechanism for negative regulation. Mol Cell Biol **11**(6): 3379–3383.
39. Morrison N, Eisman J 1993 Role of the negative glucocorticoid regulatory element in glucocorticoid repression of the human osteocalcin promoter. J Bone Min Res **8**(8):969–975.
40. Aslam F, Shalhoub V, van Wijnen AJ, Banerjee C, Bortell R, Shakoori AR, Litwack G, Stein JL, Stein GS, Lian JB 1995 Contributions of distal and proximal promoter elements to glucocorticoid regulation of osteocalcin gene transcription. Molecular Endocrinology **9**(6):679–90.
41. Heinrichs AA, Bortell R, Rahman S, Stein JL, Alnemri ES, Litwack G, Lian JB, Stein GS 1993 Identification of multiple glucocorticoid receptor-binding sites in the rat osteocalcin gene promoter. Biochemistry **32**(42):11436–11444.
42. Lian JB, Shalhoub V, Aslam F, Frenkel B, Green J, Hamrah M, Stein GS, Stein JL 1997 Species-specific glucocorticoid and 1,25-dihydroxyvitamin D responsiveness in mouse MC3T3-E1 osteoblasts: dexamethasone inhibits osteoblast differentiation and vitamin D down-regulates osteocalcin gene expression. Endocrinol **138**: 2117–2127.
43. Paredes R, Gutierrez J, Gutierrez S, Allison L, Puchi M, Imschenetzky M, van Wijnen A, Lian J, Stein G, Stein J, Montecino M 2002 Interaction of the 1alpha,25-dihydroxyvitamin D_3 receptor at the distal promoter region of the bone-specific osteocalcin gene requires nucleosomal remodeling. Biochemical J **363**:667–676.
44. Issa LL, Leong GM, Barry JB, Sutherland RL, Eisman JA 2001 Glucocorticoid receptor-interacting protein-1 and receptor-associated coactivator-3 differentially interact with the vitamin D receptor (VDR) and regulate VDR-retinoid X receptor transcriptional cross-talk. Endocrinol **142**:1606–1615.
45. Hong H, Kohli K, Garabedian MJ, Stallcup MR 1997 GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. Molec Cell Biol **17**:2735–2744.
46. Li H, Gomes PJ, Chen JD 1997 RAC3, a steroid/nuclear receptor-associated coactivator, that is related to SRC-1 and TIF2. Proc Natl Acad Sci USA **94**:8479–8484.

47. Akeno N, Matsunuma A, Maeda T, Kawane T, Horiuchi N 2000 Regulation of vitamin D-1alpha-hydroxylase and -24-hydroxylase expression by dexamethasone in mouse kidney. J Endocrinol **164**:339–348.
48. Kurahashil I, Matsunuma A, Kawane T, Abe M, Horiuchi N 2002 Dexamethasone enhances vitamin D-24-hydroxylase expression in osteoblastic (UMR-106) and renal (LLC-PK1) cells treated with 1alpha,25-dihydroxyvitamin D_3. Endocrine **17**:109–118.
49. Chesney RW, Hamstra A, Rose P, DeLuca HF 1984 Vitamin D and parathyroid hormone status in children with the nephrotic syndrome and chronic mild glomerulonephritis. Inter J Pediat Neph **5**:1–4.
50. Chesney RW, Mazess RB, Hamstra AJ, DeLuca HF, O'Reagan S 1978 Reduction of serum-1, 25-dihydroxyvitamin-D_3 in children receiving glucocorticoids. Lancet **2**:1123–1125.
51. Morris HA, Need AG, O'Loughlin PD, Horowitz M, Bridges A, Nordin BE 1990 Malabsorption of calcium in corticosteroid-induced osteoporosis. Calcif Tiss Int **46**:305–308.
52. Seeman E, Kumar R, Hunder GG, Scott M, Heath H, 3rd, Riggs BL 1980 Production, degradation, and circulating levels of 1,25-dihydroxyvitamin D in health and in chronic glucocorticoid excess. J Clin Invest **66**:664–669.
53. Slovik DM, Neer RM, Ohman JL, Lowell FC, Clark MB, Segre GV, Potts JT Jr 1980 Parathyroid hormone and 25-hydroxyvitamin D levels in glucocorticoid-treated patients. Clin Endocrinol **12**:243–248.
54. Prummel MF, Wiersinga WM, Lips P, Sanders GTB, Sauerwein HP 1991 The course of biochemical parameters of bone turnover during treatment with corticosteroids. J Clin Endocrinol Metab **72**:382–367.
55. Findling JW, Adams ND, Lemann J Jr, Gray RW, Thomas CJ, Tyrrell JB 1982 Vitamin D metabolites and parathyroid hormone in Cushing's syndrome: relationship to calcium and phosphorus homeostasis. J Clin Endoc Metab **54**: 1039–1044.
56. Hahn TJ, Halstead LR, Baran DT 1981 Effects of short-term glucocorticoid administration on intestinal calcium absorption and circulating vitamin D metabolite concentrations in man. J Clin Endoc Metab **1**:111–115.
57. Braun JJ, Juttmann JR, Visser TJ, Birkenhager JC 1982 Short-term effect of prednisone on serum 1,25-dihydroxyvitamin D in normal individuals and in hyper- and hyperparathyroidism. Clin Endocrinol **17**:21–28.
58. Bikle DD, Halloran B, Fong L, Steinbach L, Shellito J 1993 Elevated 1,25-dihydroxyvitamin D levels in patients with chronic obstructive pulmonary disease treated with prednisone. J Clin Endocr Metab **76**:456–461.
59. Cosman F, Nieves J, Herbert J, Shen V, Lindsay R 1994 High-dose glucocorticoids in multiple sclerosis patients exert direct effects on the kidney and skeleton. J Bone Miner Res **9**:1097–1105.
60. Hahn TJ, Halstead LR, Teitelbaum SL, Hahn BH 1979 Altered mineral metabolism in glucocorticoid-induced osteopenia. Effect of 25-hydroxyvitamin D administration. J Clin Invest **64**:655–665.
61. Klein RG, Arnaud SB, Gallagher JC, De Luca HF, Riggs BL 1977 Intestinal calcium absorption in exogenous hypercortisolism, Role of 25-hydroxyvitamin D and corticosteroid dose. J Clin Invest **60**:253–259.
62. Caniggia A, Nuti R, Lore F, Vattimo A 1981 Pathophysiology of the adverse effects of glucoactive corticosteroids on calcium metabolism in man. J Ster Biochem **15**:153–161.
63. Colette C, Monnier L, Pares Herbute N, Blotman F, Mirouze J 1987 Calcium absorption in corticoid-treated subjects—effects of a single oral dose of calcitriol. Hormone Metab Res **19**:335–338.
64. Braun JJ, Birkenhager-Frenkel DH, Rietveld AH, Juttmann JR, Visser TJ, Birkenhager JC 1983 Influence of 1 alpha-(OH)D_3 administration on bone and bone mineral metabolism in patients on chronic glucocorticoid treatment; a double-blind controlled study. Clin Endocrinol **19**:265–273.
65. Chan SD, Chiu DK, Atkins D 1984 Mechanism of the regulation of the 1alpha,25-dihydroxyvitamin D_3 receptor in the rat jejunum by glucocorticoids. J Endocrinol **103**:295–300.
66. Korkor AB, Kuchibotla J, Arrieh M, Gray RW, Gleason WA Jr. 1985 The effects of chronic prednisone administration on intestinal receptors for 1,25-dihydroxyvitamin D_3 in the dog. Endocrinol **117**:2267–2273.
67. Jowell PS, Epstein S, Fallon MD, Reinhardt TA, Ismail F 1987 1,25-Dihydroxyvitamin D_3 modulates glucocorticoid-induced alteration in serum bone Gla protein and bone histomorphometry. Endocrinol **120**:531–536.
68. Nielsen HK, Brixen K, Mustapha K, Mosekilde L 1991 Acute effect of 1,25-dihydroxyvitamin D_3, prednisone, and 1,25 dihydroxyvitamin D_3 plus prednisone on serum osteocalcin in normal individuals. J Bone Miner Res **6**:435–441.
69. Lems WF, Van Veen GJ, Gerrits MI, Jacobs JW, Houben HH, Van Rijn HJ, Bijlsma JW 1998 Effect of low-dose prednisone (with calcium and calcitriol supplementation) on calcium and bone metabolism in healthy volunteers. Brit J Rheumatol **37**:27–33.
70. Hahn TJ, Hahn BH 1976 Osteopenia in subjects with rheumatic diseases, principles of diagnosis and therapy. Semin Arthritis Rheum **6**:165–188.
71. Bijlsma JW, Raymakers JA, Mosch C, Hoekstra A, Derksen RH, Baart de la Faille H, Duursma SA 1988 Effect of oral calcium and vitamin D on glucocorticoid-induced osteopenia. Clin Exp Rheumatol **6**:113–119.
72. Adachi JD, Bensen WG, Bianchi F, Cividino A, Pillersdorf S, Sebaldt RJ, Tugwell P, Gordon M, Steele M, Webber C, Goldsmith CH 1996 Vitamin D and calcium in the prevention of corticosteroid-induced osteoporosis: a three-year follow-up study. J Rheumatol **23**:995–1000.
73. Buckley LM, Leib ES, Cartularo KS, Vacek PM, Cooper SM 1996 Calcium and vitamin D_3 supplementation prevents bone loss in the spine secondary to low-dose corticosteroids in patients with rheumatoid arthritis. Ann Intern Med **125**:961–968.
74. Sambrook PN, Birmingham J, Kelly PJ, Kempler S, Pocock NA, Eisman JA 1993 Prevention of corticosteroid osteoporosis; a comparison of calcium, calcitriol, and calcitonin. New Engl J Med **328**:1747–1752.
75. Reginster JY, Kuntz D, Verdickt W, Wouters M, Guillevin L, Menkes CJ, Nielsen K 1999 Prophylactic use of alfacalcidol in corticosteroid-induced osteoporosis. Osteop Inter **9**:75–81.
76. Sambrook PN, Henderson NK, Keogh A, MacDonald P, Glanville A, Spratt P, Bergin P, Ebeling P, Eisman JA 2000 Effect of calcitriol on bone loss after cardiac or lung transplantation. J Bone Miner Res **15**:1818–1824.
77. Neuhaus R, Kubo A, Lohmann R, Rayes N, Hierholzer J, Neuhaus P 1999 Calcitriol in prevention and therapy of osteoporosis after liver transplantation. Transplant Proc **31**:472–473.
78. Dykman TR, Haralson KM, Gluck OS, Murphy WA, Teitelbaum SL, Hahn TJ, Hahn BH 1984 Effect of oral 1,25-dihydroxyvitamin D and calcium on glucocorticoid-induced osteopenia in patients with rheumatic diseases. Arthritis Rheum **27**:1336–1343.
79. Lambrinoudaki I, Chan DT, Lau CS, Wong RW, Yeung SS, Kung AW 2000 Effect of calcitriol on bone mineral density in premenopausal Chinese women taking chronic steroid therapy.

A randomized, double-blind, placebo-controlled study. J Rheumatol **27**:1759–1765.
80. Ringe JD, Coster A, Meng T, Schacht E, Umbach R 1999 Treatment of glucocorticoid-induced osteoporosis with alfacalcidol/calcium versus vitamin D/calcium. Calcif Tiss Int **65**:337–340.
81. Ringe JD, Dorst A, Faber H, Schacht E 2003 Treatment of established glucocorticoid-induced osteoporosis with alfacalcidol or plain vitamin D. Calcif Tiss Int **72**:4, Abs 037.
82. Sambrook PN, Kotowicz M, Nash P, Styles CB, Naganathan V, Henderson-Briffa KN, Eisman JA, Nicholson GC 2003 Prevention and treatment of glucocorticoid-induced osteoporosis: a comparison of calcitriol, vitamin D plus calcium and alendronate plus calcium. J Bone Miner Res **18**:919–924.
83. Amin S, Lavalley MP, Simms RW, Felson DT 2002 The comparative efficacy of drug therapies used for the management of corticosteroid induced osteoporosis: a meta regression. J Bone Miner Res **17**:1512–1526.

CHAPTER 74

Drug and Hormone Effects on Vitamin D Metabolism

SOL EPSTEIN AND ADINA E. SCHNEIDER
Division of Endocrinology, Diabetes, and Bone Diseases, Department of Medicine, Mount Sinai School of Medicine, New York, NY

I. INTRODUCTION

This chapter will discuss the effects that drugs and hormones have on vitamin D metabolism. The level of active vitamin D metabolite and the activity of the renal hydroxylase enzymes that are responsible for regulating its production are governed by a variety of hormones and cations. Parathyroid hormone (PTH), phosphate, and calcium are the principal controlling factors of the renal hydroxylases (discussed in Chapter 5), yet several other hormones may also play a role in the metabolism of vitamin D. Among those discussed here are parathyroid hormone-related protein (PTHrp), calcitonin, prolactin, growth hormone and insulin-like growth factor (IGF), sex steroids (estrogen, testosterone, and progesterone), insulin, thyroid hormone, prostaglandin, interferon-γ, and tumor necrosis factor-α. The findings are summarized in Table I. Along with the endogenous regulators of the vitamin D endocrine system, some exogenous ones are also capable of exerting an effect on the Vitamin D endocrine system. Drugs are being increasingly recognized in this regard. Three that have received the most attention are the anticonvulsants, ethanol, and corticosteroids, but many others have also been implicated. The mechanisms by which these drugs affect vitamin D metabolism are discussed in detail and the findings summarized in Table II (see Section III below). It should be realized that the introduction of new drugs and molecular technology may expand this area considerably.

II. HORMONE EFFECTS ON VITAMIN D METABOLISM

A. Parathyroid Hormone and Parathyroid Hormone-Related Protein

This section summarizes the relationship between exogenous PTH or stimulated PTH secretion and vitamin D metabolism. (For a more extensive review, refer to Chapter 78.) A large body of evidence has established PTH as one of the main regulating hormones of vitamin D metabolism. Early rodent and chicken work demonstrated that PTH was necessary for the stimulation of 1α-hydroxylase and synthesis of $1,25(OH)_2D_3$ [1–7]. Similarly, most *in vitro* studies have shown increased production $1,25(OH)_2D_3$ [8–10], stimulation of 1α-hydroxylase, and inhibition of 24-hydroxylase in the presence of PTH [11–15].

The mechanism by which PTH enhances renal 1α-hydroxylase and inhibits 24-hydroxylase is most probably via second messengers. PTH increases cyclic AMP (cAMP) production in proximal tubules [16–18] and in renal slices from adult rats treated with PTH [12,13,15]. Further evidence for a role of cAMP emerged following studies by Horiuchi and co-workers [19]. They found that an infusion of cAMP and dibutyryl cAMP into vitamin D–deficient rats increases the conversion of tritiated 25-hydroxyvitamin D_3 ($25OHD_3$) to $1,25(OH)_2D_3$ [7,19]. Furthermore, cAMP [8,9,14] and forskolin [20,21], a direct activator of adenylate cyclase which increases intracellular cAMP levels [22], have been shown, *in vitro*, to enhance the production of $1,25(OH)_2D_3$ and inhibit 24-hydroxylase activity [7]. The application of molecular technology has led to important mechanistic insights of PTH action. The promotor region of the gene for 1α-hydroxylase has been shown to contain cAMP-response elements which respond to PTH [23,24]. PTH has also been shown to decrease 24-hydroxylase activity through suppression of cytochrome P450 24-hydroxylase gene expression [25], thus strengthening the association of PTH, cAMP, and $1,25(OH)_2D_3$. Another mechanism of down-regulation of 24-hydroxylase appears to be by altering the stability of its mRNA [26].

Along with cAMP, investigators have demonstrated that the phospholipase C/protein kinase C (PKC) second messenger system may also mediate PTH stimulation

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TABLE I Hormone Effects on Vitamin D Metabolism

Hormone	Study	$25OHD_3$	$1,25OH_2D_3$	$24,25OH_2D_3$
PTH	*In vitro*		⇑	⇑
	Animal		⇑	⇑
	Human		⇑	⇑
PTHrP	*In vitro*		⇑	
	Animal		⇑	
	Human		⇑	
Calcitonin	*In vitro*		⇓⇑	
	Animal	⇔	⇑	⇓
	Human	⇔	⇑⇔	⇑⇔
Growth Hormone	*In vitro*		⇔	
	Animal		⇑	⇓
	Human	⇔	⇑⇓	
IGF-1	*In vitro*		⇑	
	Animal		⇑	
Prolactin	*In vitro*		⇑	
	Bird		⇑	
	Mammal		⇑⇔	
	Human	⇔	⇔⇑	
Insulin	*In vitro*		⇑	⇓
	Animal	⇔	⇑	⇓
	Human	⇔	⇔⇑	⇔⇓
Estrogen	*In vitro*		⇑⇓⇔	⇓⇑⇔
	Animal	⇔	⇑⇔	
	Human	⇔	⇑⇔	
Testosterone	*In vitro*		⇑	⇓
	Animal	⇔	⇔⇑	
	Human	⇔	⇑	
Progesterone	*In vitro*		⇑	⇑
	Animal	⇑	⇔	
	Human	⇔	⇔⇑	
Thyroid Hormone	*In vitro*	⇑	⇓	⇑
	Animal	⇔	⇓	⇑
	Human	⇓	⇓	⇑
Prostaglandins	*In vitro*		⇑	⇓
	Animal		⇑⇔	
	Human		⇑	
Tumor Necrosis Factor α	*In vitro*		⇑	
Interferon γ	*In vitro*		⇑	
	Animal		⇔	

Symbols denote effects on serum levels of vitamin D metabolites: ⇑, increased; ⇓, decreased; ⇔ unchanged. See text for details and references.

of $1,25(OH)_2D_3$ secretion by mammalian renal proximal tubules *in vitro* [27,28]. This occurs at concentrations of PTH that are insufficient to raise cAMP content [27]. It is conceivable that PTH activation of $1,25(OH)_2D_3$ may involve both signaling pathways, with the PKC pathway being responsive to lower concentrations of PTH [27,29].

Clinically, patients with primary hyperparathyroidism have mean plasma concentrations of $1,25(OH)_2D_3$ that are significantly increased in approximately one-third of cases versus controls [30–33]. In patients with Paget's bone disease, $1,25(OH)_2D_3$ elevation in response to mithramycin-induced hypocalcemia also corresponds to an increase in PTH secretion [34].

In addition, treatment with $1,25(OH)_2D_3$ will correct the hypocalcemia found in hypoparathyroid patients [35–37]. Moreover, patients with pseudohypoparathyroidism, a disease characterized by metabolic unresponsiveness to PTH, have low circulating levels of $1,25(OH)_2D_3$ [38]. $1,25(OH)_2D_3$ is also widely used to

TABLE II Drug Effect on Vitamin D Metabolism

Drug	Study	$25OHD_3$	$1,25(OH)_2D_3$	$24,25(OH)_2D_3$
Anticonvulsants	Animal	⇔	⇔	⇓
	Human	⇓ ⇔	⇑ ⇔ ⇓	
Corticosteroids	Animal	⇔	⇓ ⇔	⇔
	Human	⇓ ⇔	⇓ ⇔ ⇑	⇔
Ethanol	Animal	⇑	⇓	
	Human	⇓ ⇔	⇓ ⇔	
Ketoconazole	*In vitro*	⇔	⇓	⇓
	Human		⇓	
Statins	Human	⇔	⇔	
Cholestyramine	Animal	⇓	⇔	
	Human	⇓ ⇔		
Fibric Acid	Human	⇓	⇑	
Ezetimibe	Animal	⇔		
Bisphosponates	*In vitro*		⇓	
	Animal		⇑	
	Human	⇔ ⇓	⇑ ⇓	⇓
Thiazide diuretics	Human	⇑	⇓	⇑
Calcium channel blockers	Animal		⇓	
	Human	⇔	⇔	
Heparin	Animal		⇓	
	Human	⇔	⇓	⇔
Cimetidine	Animal	⇓		
	Human	⇔ ⇓	⇔	⇔
Aluminum	Animal	⇔	⇔ ⇓	
Oral Parenteral	Human	⇔	⇓	
	Human		⇑	
Antituberculous agents	Human	⇔ ⇓	⇔ ⇓	
Caffeine	*In vitro*		⇓	
	Animal		⇔ ⇑	
Theophylline	*In vitro*		⇓	⇑
	Animal	⇓	⇔	
Immunosuppressants	Animal		⇔ ⇑ ⇓	⇑
	Human	⇔	⇔	
Flouride	Animal	⇔	⇔	
	Human	⇔	⇔	
Olestra	Animal	⇔		
	Human	⇔		
Orlistat	Human	⇔ ⇓		
Lithium	Human	⇔	⇔ ⇓	

Symbols denote effects on serum levels of vitamin D metabolites: ⇑, increased; ⇓, decreased; ⇔ unchanged. See text for details and references.

decrease PTH levels in uremic patients with secondary hyperparathyroidism [39].

Another major influence on vitamin D metabolism is PTHrP (see also Chapter 43). PTHrP is a protein that is produced by a number of solid tumors, especially squamous and renal cell carcinomas, and is thought to be responsible for many cases of hypercalcemia of malignancy. However, it is also seen in the normal physiological situation [40]. PTHrP binds to the classic type I PTH/PTHrP receptor, which is expressed in many tissues, such as bone and lung, as well as to alternative type II receptors in several nonclassic PTH target tissues, such as keratinocytes and squamous carcinoma cell lines [41]. PTHrP has a similar activity to PTH [42,43]; however, unlike in primary hyperparathyroidism where patients have normal or elevated

$1,25(OH)_2D_3$ levels (see above), $1,25(OH)_2D_3$ is not elevated and may be decreased in patients with hypercalcemia of malignancy and increased urinary cAMP excretion [44,45]. Thus, the hypercalcemia associated with humoral hypercalcemia of malignancy (HHM) is thought to be caused by increased renal calcium reabsorption [46,47] and/or increased bone resorption [33,48], rather than increased intestinal calcium absorption. Unlike the human scenario of HHM, rodent studies have shown an elevation in serum calcium and $1,25(OH)_2D_3$ levels following tumor transplantation [49] and infusion of synthesized N-terminal fragments of PTHrP [50,51]. Walker *et al.* [51] also demonstrated direct stimulation of 1α-hydroxylase in rodent kidney slices *in vitro* by PTHrP (l–36). Conversely, Michigami *et al*. recently described an animal model of HHM in which serum $1,25(OH)_2D_3$ levels were markedly reduced in the setting of severe hypercalcemia [52]. In this model, PTHrP-producing infantile fibrosarcomas were innoculated into nude rats. Administration of a bisphosphonate to these rats normalized their serum calcium and increased their $1,25(OH)_2D_3$ levels. Moreover, 1α-hydroxylase activity was decreased in the hypercalcemic rats and rose after treatment with a bisphosphonate. In contrast, administration of a neutralizing antibody to PTHrP led to a reduction in serum calcium without an increase in $1,25(OH)_2D_3$ levels [52]. These results suggest that PTHrP stimulates 1α-hydroxylase in this animal model.

Because of the differences observed between HHM and infusion of synthesized PTHrP, additional factors, other than PTHrP, may be involved in the inhibition of renal $1,25(OH)_2D_3$ production associated with HHM [53]. It has been suggested that hypercalcemia itself may be the cause of low $1,25(OH)_2D_3$ levels [54]. However, Nakayama *et al.* [33] reported that low $1,25(OH)_2D_3$ levels were demonstrated at any serum calcium level in patients with hypercalcemia of malignancy compared to patients with primary hyperparathyroidism. This suggests that the reduction in serum $1,25(OH)_2D_3$ observed in these patients cannot be explained solely by an elevation in serum calcium [33]. As in animals, a study by Everhart-Caye *et al.* [55] revealed a dose-related increase in renal production of $1,25(OH)_2D_3$ following a 6-hr infusion of human PTHrP (1–36) into healthy subjects. This confirms a previous report by Fraher and co-workers [56], who observed a similar increase in $1,25(OH)_2D_3$ production following human PTHrP (1–34) infusion in healthy subjects. Although the *in vivo* human studies of Fraher and Everhart-Caye do indeed demonstrate PTHrP stimulation of $1,25(OH)_2D_3$ production, they were conducted in normal healthy subjects and do not mirror the clinical situation as seen in hypercalcemia of malignancy, in which a host of factors may be at play (as discussed below). They were also short-term infusion studies, and it is quite possible that equivalent findings may not be produced with longer-term studies. More recently, Horowitz *et al*. [57] directly compared the effects of 48-hour infusions of human PTHrP (1–36) versus human PTH (1–34) in healthy subjects. Although the calcemic, renal handling of calcium and phosphaturic effects were similar, PTH was significantly more effective at stimulating renal $1,25(OH)_2D_3$ than PTHrP [57]. These findings, however, do not explain the low $1,25(OH)_2D_3$ levels seen in HHM.

Possible explanations for the disparate effect of PTHrP in hypercalcemia of malignancy are that other regions, or alternatively the full length of the PTHrP (1–141) molecule, may act differently from the foreshortened synthetic molecules administered in previous studies [56]. This may modify or impair the capacity of the N-terminal moiety of PTHrP to stimulate 1α-hydroxylase, as might other factors also produced by tumors, such as the cytokines, interleukin-1 (IL-1), and tumor necrosis factor, and/or growth factors [58].

The other clinical scenario in which PTHrP may play an important role is during lactation. PTHrP may regulate mammary blood flow, and is a potential mediator of the changes in calcium metabolism seen during lactation [59]. Mather *et al*. [60] reported a case of a woman with hypoparathyroidism whose need for calcium and calcitriol supplementation abated during lactation Her $1,25(OH)_2D_3$ levels remained in the normal range, despite a cessation of supplemental calcitriol and a lack of endogenous PTH. This was associated with a rise in PTHrP and suggests that PTHrP was able to stimulate production of $1,25(OH)_2D_3$ in this setting. This lends further support to the hypothesis that the decrease in $1,25(OH)_2D_3$ seen in HHM is related to other tumor-related factors.

In summary, available evidence, both *in vitro* and *in vivo* in animal models and humans, clearly defines the role of PTH as an activator of $1,25(OH)_2D_3$ synthesis and an inhibitor of $24,25(OH)_2D_3$ production. Amino-terminal fragments of PTHrP also stimulate $1,25(OH)_2D_3$ production experimentally, both *in vitro* and *in vivo* in animals and humans. However, it is as yet unclear as to why patients with HHM and elevated PTHrP have reduced $1,25(OH)_2D_3$.

B. Calcitonin

The role of calcitonin in vitamin D metabolism has been thoroughly investigated in both *in vivo* and *in vitro* studies (see Chapter 39). Initial *in vivo* studies demonstrated a pronounced increase in $1,25OH_2D_3$

levels and decrease in $24,25(OH)_2D_3$ levels after the administration of synthetic salmon calcitonin to vitamin D–deficient rats [61]. Subsequent studies attempted to clarify if this effect was independent of PTH. Lorenc and co-workers [62] found that following thyroparathyroidectomy (TPTX), the calcitonin effect was eliminated. In contrast, subsequent studies involving vitamin D–deficient [63] and vitamin D–replete [64] TPTX rats demonstrated increased production of $1,25(OH)_2D_3$ following calcitonin administration. Furthermore, the actions of PTH and calcitonin were additive, suggesting independent effects [63]. This effect was seen in rats fed regular diets as well as calcium-free diets. There were no significant changes in $25OHD_3$ levels [64]. However, Shinki *et al*. [65] demonstrated that calcitonin administered to sham and TPTX normocalcemic rats caused an increase in the expression of renal 25-hydroxylase and increased the conversion of $25OHD_3$ to $1,25(OH)_2D_3$. This effect was not seen in hypocalcemic rats whose 25-hydroxylase increased in response to PTH injection [65].

Calcitonin also appears to act as a negative regulator of intestinal 24-hydroxylase. The inhibition of intestinal 24-hydroxylase activity and expression may spare $1,25(OH)_2D_3$ from deactivation and thereby result in enhanced $1,25(OH)_2D_3$ mediated activity [66]. The majority of *in vitro* data have supported the positive effect of calcitonin on $1,25OH_2D_3$ levels [9,67,68]. Similar to *in vivo* data, 1α-hydroxylase was stimulated by calcitonin in vitamin D–deficient rat kidneys, post-TPTX [68]. This effect was independent of the adenylate cyclase system which, as mentioned earlier, is believed to be the mechanism of PTH stimulation of 1α-hydroxylase [19,63]. These findings suggest that calcitonin acts independently of PTH.

In human studies, patients with medullary carcinoma of the thyroid and excess calcitonin had serum $1,25(OH)_2D_3$ levels that were elevated [69]. However, therapeutic use of calcitonin has produced mixed results. Injectable [70] and nasal calcitonin [62,63] treatment daily for postmenopausal osteoporosis produce either no changes [70–72] or an increase in $1,25(OH)_2D_3$ levels at 6 months, which returned to normal at 1 year, with PTH and $25OHD_3$ levels being unchanged during the study period [73].

No changes in $1,25(OH)_2D_3$ levels were observed in patients with Paget's disease of bone treated for 3 months with calcitonin, although in these patients $24,25(OH)_2D_3$ was elevated, most probably due to decreased consumption by osteoclasts and osteoblasts [74]. Other studies involving the treatment of Paget's disease of bone with nasal calcitonin did show a rise in $1,25(OH)_2D_3$ after 18 months duration, yet longer term therapy had no effect. Levels of other vitamin D metabolites such as $24,25(OH)_2D_3$ and $25OHD_3$ were unchanged [75].

In summary, *in vivo* and most *in vitro* work suggest that calcitonin plays an important role in stimulating $1,25(OH)_2D_3$ production. The less than convincing evidence seen in clinical studies may be related to the doses used or the route of calcitonin administration.

C. Growth Hormone and Insulin-like Growth Factor

The recent advent of recombinant growth hormone (GH) has spurred an increased interest into the role of this hormone and its intermediary, insulin like growth factor (IGF-1), in bone metabolism. One of the many functions of GH is to stimulate intestinal calcium absorption, which has been shown in both rats [76] and humans [77,78]. Although the exact mechanism of this effect is unknown, it may be a $1,25(OH)_2D_3$ dependent phenomenon. Hypophysectomy and subsequent GH deficiency in rats was shown to reduce the level of $1,25(OH)_2D_3$ and to increase the conversion of $25OHD_3$ to $24,25(OH)_2D_3$ [79–82]. GH replacement led to the restoration of normal levels of vitamin D metabolites [79–82]. No increase in the metabolic clearance or increase in tissue metabolism of $1,25(OH)_2D_3$ occurred; thus, the most likely explanation is that hypophysectomy decreases the renal 1α-hydroxylase activity [81]. Furthermore, hypophysectomized rats fed a low phosphate diet and replaced with rat GH had increased levels of $1,25(OH)_2D_3$, as a result of stimulation of 1α-hydroxylase activity [83]. In other animal work, exogenous porcine GH also increased $1,25(OH)_2D_3$ in intact pigs [84]. Of note, a more recent study in rats found that hypophysectomy also modulated the regulation of 24-hydroxylase by phosphate [84].

Clinical studies have yielded conflicting results. Acromegalic subjects, with endogenous GH excess, have increased levels of $1,25(OH)_2D_3$ [32,84–86], which are reduced by treatment with bromocriptine, which decreases GH [84–86]. Short-term recombinant GH therapy in healthy young [87] or elderly people [88] caused significant increases in $1,25(OH)_2D_3$, and increased vitamin D levels in response to phosphate depletion were found to be dependent on the presence of GH [89]. However, other studies, have found that chronic GH therapy does not seem to cause a rise in $1,25(OH)_2D_3$ levels [90,91]. GH replacement given to children with GH deficiency did not alter $25OHD_3$, $1,25(OH)_2D_3$, or PTH levels [92]. Interestingly, a recent study in children with end-stage renal disease found that higher doses of calcitriol were needed in those children treated with GH [93].

If GH does, in fact, increase $1,25(OH)_2D_3$ levels in humans, the possible mechanism remains controversial. Although Marcus *et al.* [88] found that the increase is mediated by an increase in PTH, other studies have not arrived at the same conclusion [91,94]. More recent studies continue to find the effect to be independent of PTH. Wei *et al.* [95] reported that children treated with GH experienced an increase in $1,25(OH)_2D_3$ after one month of therapy, which subsequently decreased at 3 months. 24,25-Dihydroxyvitamin D decreased at both one and three months and returned to baseline at 6 months. No change in $25OHD_3$ was seen. IFG-1 levels increased in this study, while PTH levels declined, suggesting the effect of GH on vitamin D metabolism may be mediated by IGF-1 [95]. A small, randomized crossover study of IGF-1 versus GH found that IGF-1 increased the free calcitriol index, while calcium, phosphate, and PTH levels were unchanged [96]. Similarly, Wright *et al.* [97] found that GH's positive effect on $1,25(OH)_2D_3$ levels was independent of PTH and likely mediated by IGF-1.

In vitro evidence supports the above clinical findings and suggests that the effects of GH *in vivo* on $1,25(OH)_2D_3$ production are indirect, as GH fails to stimulate $1,25(OH)_2D_3$ production in chick renal preparations [98]. Insulin-like growth factor type I (IGF-I) receptors are present on the basolateral membrane of renal proximal tubules [99], and low concentrations of exogenous IGF-I enhance $1,25(OH)_2D_3$ synthesis when phosphate concentrations are low [100]. *In vivo* animal studies also show that the GH-dependent increases in serum $1,25(OH)_2D_3$ levels induced by dietary phosphate restriction may be mediated by IGF-I, as administration of IGF-I to hypophysectomized rats increases serum $1,25(OH)_2D_3$ to approximately the same degree as GH [101,102]. In addition, Gray [103] showed that serum concentrations of $1,25(OH)_2D_3$ are directly related to the serum levels of IGF-I in rats fed a low phosphate diet. IGF-1 was also shown to stimulate renal 1α-hydroxylase activity in a time- and dose-dependent manner in weanling mice that were phosphate depleted [104], but this was also demonstrated to be independent of changes in serum calcium or phosphate [104]. Currently, no data suggest that IGF-II affects vitamin D metabolism.

The majority of *in vivo* data seem to suggest that GH, probably via IGF-I and especially during hypophosphatemia, is an important regulator of serum $1,25(OH)_2D_3$ levels. Furthermore, it is possible that hormone- and phosphate-dependent enzyme stimulation occur by different mechanisms. Data from human clinical studies are more conflicting but increasingly support the theory that GH, via IGF-I, regulates serum $1,25(OH)_2D_3$. This may partially explain the positive effects of GH on bone mass in patients with GH deficiency.

D. Prolactin

Initial work involving prolactin (PRL) as a possible stimulator of $1,25(OH)_2D_3$ synthesis came from *in vitro* studies of chick cell cultures. Spanos *et al.* [105–107] and Bickle *et al.* [98] found that ovine PRL could stimulate 1α-hydroxylase. Unlike the case in birds, however, in mammals, fish, and amphibians PRL does not seem to play a significant role in vitamin D metabolism [79,83,108,109].

In certain physiological situations, such as lactation, however, PRL may play a role in vitamin D metabolism. In rats, PRL stimulates intestinal transport of calcium [110], especially during lactation, when increased serum levels of $1,25(OH)_2D_3$ are found [111,112]. Suppression of PRL by bromocriptine decreases $1,25(OH)_2D_3$ levels in lactating rats, but the drug has no effect in nonlactating controls [113]. Increased vitamin D levels are also found in women during pregnancy and lactation [32,114,115]. The negative calcium balance and transient decrease in bone mineral density observed in lacatating women may be related to increased $1,25(OH)_2D_3$. A study of rural Mexican women found that women who were lactating had higher $1,25(OH)_2D_3$ than age matched nonlactating women [116]. In support of these studies, a patient with hypoparathyroidism was found to require less exogenous vitamin D supplementation during lactation than at other times [117]. Conversely, a study of postpartum women, followed for 18 months, failed to find a relationship between vitamin D levels and serum prolactin, estradiol, lactation status, or PTHrP [118]. Another study of pregnant women did not find a relationship between PRL levels and $1,25(OH)_2D_3$ [119]. The rise in PRL associated with lactation may, however, lead to a release of PTHrP. Other than in lactating females, PRL most probably has no effect on plasma $1,25(OH)_2D_3$ levels or calcium metabolism, as patients with hyperprolactinemia due to functioning pituitary adenomas had $25OHD_3$ and $1,25(OH)_2D_3$ levels in the normal range versus age-matched controls, as well as similar serum calcium, phosphate, and PTH levels [32,120–124].

Although PRL seems to play a role in vitamin D metabolism in birds, this effect is likely species-specific. The majority of animal and clinical data suggest its positive effect on $1,25(OH)_2D_3$ production is limited to states of physiological elevation in PRL, such as lactation. For a more detailed discussion of the effects of lactation on vitamin D metabolism the reader is referred to Chapter 51.

E. Insulin

This section will be limited to the effect of insulin on vitamin D metabolism. The immunomodulatory role of vitamin D and the effect of vitamin D on insulin release will not be addressed (see Chapter 99). Patients with insulin-dependent diabetes mellitus suffer from a number of disturbances in bone mineral metabolism, including alterations in vitamin D metabolism [125]. Work in streptozotocin- and alloxan-induced diabetic rats by Schneider and co-workers [126,127] first suggested a role for insulin in the production of $1,25(OH)_2D_3$. This experimental diabetic model is associated with a reduction in duodenal calcium absorption, calcium binding protein, and total and ionized calcium levels [126,127]. Treatment with $1,25(OH)_2D_3$ but not $25OHD_3$ corrects subnormal calcium absorption in these rats [128], as does insulin replacement [129]. These data suggest that a lack of insulin in diabetes impairs 1α-hydroxylation. Further support of this concept emerged after finding that $1,25(OH)_2D_3$ levels in streptozotocin-induced diabetic rats were depressed to one-eighth the level in control rats and were returned to control values with insulin treatment [130]. No changes in serum $25OHD_3$ were found, suggesting either decreased 1α-hydroxylation of $25OHD_3$ or increased catabolism of $1,25(OH)_2D_3$. It is possible that a change in PTH is responsible for decreased conversion of $25OHD_3$. It has been shown that PTH levels increase in diabetic rats, probably secondary to calcium malabsorption resulting from decreased $1,25(OH)_2D_3$ [131–134]; however, some studies have reported low PTH levels in streptozotocin-induced diabetic rats [135]. To further localize the site at which insulin is proposed to act, Spencer *et al.* [136] studied the conversion of ^{3}H-$25OHD_3$ to ^{3}H-$1,25(OH)_2D_3$, as well as the metabolic clearance of ^{3}H-$1,25(OH)_2D_3$ in control, streptozotocin-induced diabetic and insulin-treated streptozotocin-diabetic rats. The results showed that the metabolic clearance was not increased in diabetic rats and that the *in vivo* conversion of ^{3}H-$25OHD_3$ to ^{3}H-$1,25(OH)_2D_3$ was reduced by 60% in diabetic rats, normalizing with insulin therapy. No intrinsic intestinal mucosal defect in the incorporation of ^{3}H-$1,25(OH)_2D_3$ was evident [136]. These results further support the notion that a lack of insulin impairs 1α-hydroxylation. The osteopenia seen in streptozotocin-induced diabetic rats [134,135] is also significantly attenuated by treatment with $25OHD_3$, suggesting that vitamin D deficiency contributes to this bone loss [137].

Other studies in diabetic animals have demonstrated a decrease in 1α-hydroxylase activity and an increase in 24-hydroxylase activity [138,139]. Another study in the diabetic animal model found that insulin deficiency directly inhibited 25-hydroxylase activity despite finding normal levels of serum $25OHD_3$ [140]. Insulin may also play an important role in the stimulation of renal $1,25(OH)_2D_3$ synthesis in response to phosphate deprivation. In streptozotocin-diabetic rats, $1,25(OH)_2D_3$ increased only slightly following phosphate deprivation, as compared with a marked response in rats replaced with insulin [141]. Decreased serum vitamin D–binding protein (DBP) has also been reported in experimentally induced diabetic rats, resulting in decreased total $1,25(OH)_2D_3$ and normal free $1,25(OH)_2D_3$ [142].

In vitro evidence suggests a permissive role of insulin in vitamin D metabolism. Primary cultures of chick kidney cells in serum-free medium respond to PTH with increased production of $1,25(OH)_2D_3$ only when exposed to insulin [143]. Confirming *in vitro* work, Wongsurawat *et al.* [133], using a renal slice technique, showed reduced $1,25(OH)_2D_3$ and increased $24,25(OH)_2D_3$ levels in streptozotocin-induced diabetic rats, which reversed with insulin therapy. PTH levels were found to be higher in diabetic rats relative to controls [133]. Renal resistance to PTH has been suggested as the mechanism by which 1α-hydroxylase is depressed in these rats [144]. However Wongsurawat *et al.* showed a normal cAMP response to PTH in streptozotocin-induced diabetes [133], which makes this theory unlikely. There is also *in vitro* data to suggest that insulin increases the capacity of PTH to increase in the activity of 24-hydroxylase [145].

Clinical studies have shown decreased $1,25(OH)_2D_3$ concentrations with increased $24,25(OH)_2D_3$ and normal $25OHD_3$ levels in insulin-dependent diabetic children [146,147] and in poorly controlled African diabetics [148]. However, no abnormalities of calcium or vitamin D metabolism have been found in adult insulin-dependent diabetics [149].

In summary, diabetic animals show abnormalities in vitamin D metabolism with depressed levels of $1,25(OH)_2D_3$, increased $24,25(OH)_2D_3$, and normal levels of $25OHD_3$. In human studies findings have been inconsistent, which may be due to variations among the study populations. Alternatively, the animal model of diabetes may differ from diabetes in the clinical setting with regard to changes in vitamin D.

F. Sex Steroids

1. ESTRADIOL

The possible role of estrogen in vitamin D metabolism was first demonstrated in avian studies involving egg-laying Japanese quail [150]. Increased estradiol, either via ovulation [150] or exogenous

administration [151], resulted in enhanced production of $1,25(OH)_2D_3$. In females $24,25(OH)_2D_3$ was stimulated, but in males the concentration of this metabolite was reduced. Further studies supported the notion that estradiol might be a regulator of vitamin D. Castillo *et al.* [152] administered 5 mg of estradiol to mature male quail and found that it markedly stimulated 1α-hydroxylase and suppressed 24-hydroxylase activity 24 hours after administration. In castrated male chickens, estradiol injections stimulated $1,25(OH)_2D_3$ production *in vitro,* but only in the presence of testosterone or progesterone [153]. If all three hormones were present, they acted in a synergistic manner to stimulate $1,25(OH)_2D_3$ synthesis [153]. Similarly, the injection of stilbestrol, an estrogenic drug, into immature male chickens stimulated 1α-hydroxylase and suppressed $24,25(OH)_2D_3$ production in chick kidney homogenates [154]. However, there have been some contradictory *in vitro* studies that suggest that estradiol may not exert a stimulatory effect on the hydroxylase enzymes [155,156]. Thus, the *in vivo* stimulation noted in the above studies may occur via indirect means, possibly via PTH stimulation, as discussed below.

In mammalian studies, female rats treated with estradiol benzoate daily for 8 days were found to have increased $1,25(OH)_2D_3$ concentrations in plasma, gut mucosa, and kidneys [157]. Others have not found a rise in $1,25(OH)_2D_3$ levels in rats treated with estradiol, but have recorded increased *in vivo* intestinal absorption of calcium [158–160]. In keeping with these findings, another study in rats found that estradiol's ability to increase intestinal calcium absorption was independent of $1,25(OH)_2D_3$ [161]. A study by Criddle *et al.* [162] suggests the decrease in renal calcium excretion associated with estrogen replacement is independent of $1,25(OH)_2D_3$ and may be dependent on increased expression of calbindin D28k protein, located in the distal renal tubal. Furthermore, Liel *et al*. [163] found that the administration of estrogen to ovariectomized rats was found to increase intestinal calcium absorption by increasing the activity of duodenal vitamin D receptors (VDRs), rather than increasing levels $1,25(OH)_2D_3$. However, in a study by Ash and Goldin [164], both young and old ovariectomized rats administered ^3H-$25OHD_3$ had reduced ^3H-$1,25(OH)_2D_3$ production, which was increased with estradiol replacement. In the same study, parathyroidectomy eliminated estradiol's therapeutic effect on ^3H-$1,25(OH)_2D_3$ recovery, which implicates PTH. Estradiol may either act directly on the parathyroid gland or act by decreasing bone resorption and lowering serum calcium, thus triggering PTH secretion [164]. Despite these data, these findings have not been consistently demonstrated in ovariectomized rats by others [158,165]. Of note, recent *in vivo* data also demonstrate that estrogen increases the expression of the vitamin D receptor (VDR) in bone [166].

Clinically, plasma $1,25(OH)_2D_3$ levels are elevated in human pregnancy and remain high postpartum in lactating women [114,115] (see Chapter 51). Levels of $25OHD_3$ in pregnant women are similar to those of controls [167]. However, another study did find a 39% increase in $25OHD_3$ levels in premenopausal women receiving oral contraceptives compared with nonusers [168]. Despite an expected increase in DBP, free $1,25(OH)_2D_3$ levels are also elevated in the pregnant state [169]. Low [170,171] and normal levels [172] of $1,25(OH)_2D_3$ have been reported in early postmenopausal women. The increased bone resorption associated with the postmenopaual period leads to a slight elevation of serum calcium, which decreases PTH secretion, and subsequently reduces renal 1α-hydroxylase activation and $1,25(OH)_2D_3$ production. This ultimately results in decreased calcium absorption and negative calcium balance [173] (see Chapter 67). The fall in calcium absorption seen during this period, however, appears to be only partially explained by this fall in $1,25(OH)_2D_3$ levels [174]. An alternative explanation for the decline in calcitriol is that estrogen deficiency may alter the responsivness of PTH to changes in calcium. However, in a study of 16 women who were rendered estrogen deficient via administration of a GnRH analogue, no change in the ability of PTH to respond to changes in calcium or stimulate $1,25(OH)_2D_3$ was observed [175]. Serum levels of $25OHD_3$ have been reported as unchanged [170,172] or increased [176] in various studies.

Recent studies in postmenopausal women have demonstrated increased $1,25(OH)_2D_3$ levels after both short-term and long-term estrogen therapy [171,177–179]. In women with postmenopausal osteoporosis, estrogen replacement results in decreased bone resorption that lowers serum calcium, which subsequently stimulates PTH and renal 1α-hydroxylase leading to increased $1,25(OH)_2D_3$ production. The latter increases intestinal calcium absorption [180]. However, Stock *et al.* [181], using sensitive assays for PTH, failed to show a rise in PTH levels accompanying the elevated $1,25(OH)_2D_3$ in postmenopausal women treated with estradiol. In fact 2 weeks of treatment with estradiol decreased PTH levels. This suggests that the estrogen effect on vitamin D metabolism may not only be secondary to a change in PTH [181].

Estradiol-induced elevation of $1,25(OH)_2D_3$ may occur as a result of increased DBP levels. A higher concentration of DBP would increase total $1,25(OH)_2D_3$, but leave free $1,25(OH)_2D_3$ unchanged [182,183]. However, as in pregnancy, free levels of $1,25(OH)_2D_3$

were shown to be elevated in response to estradiol treatment [184]. Although the evidence for a direct effect of estradiol on vitamin D metabolism in mammals is inconclusive, the majority of studies have found a positive effect of estrogen on $1,25(OH)_2D_3$ levels. This effect may be indirect as estrogen alters intestinal calcium absorption, bone resorption, and PTH levels which increases, in turn, $1,25(OH)_2D_3$ levels.

2. TESTOSTERONE

Evidence for a role for testosterone in vitamin D metabolism was first demonstrated in avian studies. An interdependence and synergism between testosterone and estradiol were observed during the *in vitro* stimulation of 1α-hydroxylase in castrated male chickens [153]. Castillo *et al.* [152] also demonstrated that testosterone alone administered to the male quail suppressed 24-hydroxylase but produced little change in 1α-hydroxylase. Again the data among species are not uniform. Hypoandrogenemia as a result of orchiectomy in rats has produced mixed findings, with either no change in total or free vitamin D metabolites [185] or decreased $1,25(OH)_2D_3$ and DBP with normal free $1,25(OH)_2D_3$ levels [186,187]. Castrated male guinea pigs demonstrated a 50% decline in 1α-hydroxylase activity, which was reversed with testosterone replacement. Ovariectomized female guinea pigs also responded to testosterone therapy with a 50% increase in 1α-hydroxylase activity. Both groups, however, had similar serum levels of $1,25(OH)_2D_3$, DBP, and free $1,25(OH)_2D_3$ levels versus controls [188]. Of note, testosterone administration to sexually immature, vitamin D_3 replete male chicks was shown to decrease circulating levels of $1,25(OH)_2D_3$, while intestine and bone concentrations were significantly increased [189]. There are limited data on the effect of testosterone on $25OHD_3$ levels. One study demonstrated an elevation in serum $25OHD_3$ levels following androgen treatment in ultraviolet irradiated rats compared to those that did not receive testosterone [190].

Studies in humans have been focused on hypogonadal men. Hagenfeldt *et al.* [191] studied hypogonadal men before and after treatment with testosterone enanthate every 3–4 weeks, for varying lengths of time. They found that basal serum $1,25(OH)_2D_3$, DBP and free $1,25(OH)_2D_3$ concentrations were similar to those of controls; however, testosterone treatment still increased total $1,25(OH)_2D_3$ and free $1,25(OH)_2D_3$ significantly. In contrast, Morley *et al.* [192] looked at the effects of testosterone replacement therapy in elderly hypogonadal males (mean age 77.6 ± 2.3 years) and found no effect on PTH or serum vitamin D metabolites pre- or posttreatment. Studies in hypogonadal men with osteoporosis have found both normal [193,194] and low [195] serum $1,25(OH)_2D_3$ levels. In the latter study by Francis *et al.* [195], testosterone replacement therapy increased both total and free $1,25(OH)_2D_3$ levels. However, patients treated with orchidectomy for prostate cancer were found to have no changes in total $1,25(OH)_2D_3$ or DBP concentrations [196]. In studies involving pubertal boys, results have been equivocal. Krabbe *et al.* [197] found no changes in the serum levels of vitamin D metabolites before and after peak pubertal testosterone surges, whereas Aksnes *et al.* [198] did find an increase in vitamin D.

It is interesting to note that androgens have been shown to regulate vitamin D receptors in epithelial and stromal cells of the human prostate cells [199]. Moreover, genetic polymorphisms in the vitamin D receptor have been shown to be associated with prostate cancer [200]. Please refer to Chapters 94 and 68, respectively, for detailed discussions of these topics.

In summary, it is unlikely that testosterone is a major controlling factor in vitamin D metabolism, but it may play a minor role in overall vitamin D homeostasis. Hypogonadal men, especially those at risk for osteoporosis, should be monitored for vitamin D deficiency and receive supplementation if necessary.

3. PROGESTERONE

Work by Tanaka *et al.* [153] demonstrated that in castrated male chickens progesterone, like testosterone, supported the stimulation of 1α-hydroxylase by estradiol. As previously mentioned, further marked stimulation of 1α-hydroxylase activity occurred with combined progesterone, testosterone, and estradiol treatment [153], demonstrating pronounced synergy among the sex steroids. In Japanese female quail treated with progesterone *in vitro*, renal production of $1,25(OH)_2D_3$ was stimulated; however, this was significantly less than that of estradiol [151]. In the same study, immature male quail treated with progesterone had increased $24,25(OH)_2D_3$ production [151]. Similar findings were recorded by Castillo *et al.* [152]. Unlike in birds, treatment of ovariectomized rats with progesterone led to an increase in $25OHD_3$, while $1,25(OH)_2D_3$ levels were similar to controls [159].

In postmenopausal women, progesterone in combination with estrogen was found to lower estradiol-stimulated increases in total and free vitamin D levels [201], and norethisterone treatment caused a slight decrease in free and total vitamin D [183]. No effects on vitamin D metabolites following medroxyprogesterone therapy were seen in male patients treated for glucocorticoid-induced osteoporosis [202].

Although the role of progesterone in vitamin D metabolism has been less extensively investigated than

the other sex steroids, the available literature suggests that it likely has a minor function, if any.

G. Thyroid Hormone

The effect of thyroid hormone on vitamin D metabolism is of particular interest given the concern over the impact of this hormone on bone metabolism in general. In an extensive review on bone and mineral metabolism in thyroid disease, Auwerx and Bouillon [203] describe the changes encountered in this disease. In hyperthyroidism, excess thyroid hormone stimulates bone resorption [203–206], which increases serum calcium and phosphate concentration with resultant suppression of PTH [205,207–209] and a decrease in $1,25(OH)_2D_3$ production. This leads to lower intestinal calcium absorption [210,211], which Peerenboom *et al*. [211] had earlier demonstrated to be reversible after treatment of the thyroid abnormality. Another possible factor contributing to low plasma $1,25(OH)_2D_3$ levels found in hyperthyroidism is that of enhanced metabolic clearance of $1,25(OH)_2D_3$. Karsenty *et al.* [212] studied seven hyperthyroid patients and found increased $1,25(OH)_2D_3$ clearance after administration of tritiated $1,25(OH)_2D_3$.

Hyperthyroidism has been reported to be associated with either unchanged [211,213–215] or decreased [216,217] levels of $25OHD_3$. A recent study found that 68% of men and 29% of women who were undergoing subtotal thyroidectomy for Grave's disease had vitamin D deficiency, defined as $25OHD_3 < 25$ nmol/l [218]. In fact, despite normal bone mass and markers of bone turnover, $25OHD_3$ and $1,25(OH)_2D_3$ levels have been shown to be persistently reduced in euthyroid patients who were 6 years post treatment of hyperthyroidism [219]. Dietary vitamin D intake and exposure to sunlight may contribute to these differences in $25OHD_3$. Circulating levels of $24,25(OH)_2D_3$ are generally increased in hyperthyroid patients [214,215]. Hypothyroidism is associated with decreased bone turnover [220] and low serum calcium levels that activate PTH, which, in turn, enhances 1α-hydroxylase activity [209,213,221]. Finally, serum-binding protein, DBP, does not appear to be affected by thyroid status [213].

Similar to human hyperthyroidism, daily injections of L-thyroxine to rats decreased $1,25(OH)_2D_3$ and increased $24,25(OH)_2D_3$ and $25OHD_3$ levels [222]. Hypothyroidism in the rat induced by the drug propylthiouracil, however, produces confusing results in that it is associated with low calcium and phosphate levels and decreased $1,25(OH)_2D_3$ concentration [223].

There is some *in vitro* evidence to suggest that thyroid hormone directly affects renal $25OHD_3$ metabolism. Kano and Jones [224] found that thyroxine, triiodothyronine, and thyrotropin (TSH) decreased $1,25(OH)_2D_3$ synthesis in perfused rat kidneys from vitamin D–deplete rats, whereas $24,25(OH)_2D_3$ synthesis was increased in kidneys from vitamin D–replete rats. Miller and Ghazarian [225] also demonstrated a 50% reduction in 1α-hydroxylase activity in vitamin D–deficient chicks and stimulation of 24-hydroxylase activity in vitamin D–replete chicks following thyroxine administration.

The majority of evidence suggests that thyroid hormone indirectly affects vitamin D metabolism via alterations in serum calcium, phosphate, and PTH. A direct action of thyroid hormone on renal hydroxylase activity, however, has been suggested by *in vitro* work. Again, the clinical significance in vitamin D–replete states is unknown.

H. Prostaglandins

The effect of prostaglandins on vitamin D metabolism has been investigated *in vitro* and *in vivo.* Work by Trechsel *et al.* [226] in primary chick kidney cell cultures found that the addition of prostaglandin E_2 (PGE_2) and prostaglandin F_{2a} (PGF_{2a}) stimulated 1α-hydroxylase activity in a dose-dependent manner. PGE_2 also significantly decreased 24-hydroxylase activity [226]. A proposed mechanism of action is that prostaglandins act through an increase in cAMP [226,227], but as discussed below this has not been proven.

Further *in vitro* work by Wark *et al.* [228] in isolated renal tubules, prepared from vitamin D–deficient chicks, showed that the addition of PGE_2 to the tubule incubation medium in the presence of $1,25(OH)_2D_3$ increased $1,25(OH)_2D_3$ production. Acetylsalicylic acid, which inhibits prostaglandin synthesis, decreased the prostaglandin content of the tubule medium and subsequently inhibited $1,25(OH)_2D_3$ production. Frusemide raised prostaglandin content in a dose-dependent manner, which led to a significant increase in $1,25(OH)_2D_3$ production and decreased $24,25(OH)_2D_3$ synthesis [228]. Kurose *et al.* [229] found similar results *in vitro.*

In vivo work involving vitamin D–deficient TPTX rats by Yamada *et al.* [230] found that, after an intraarterial infusion of PGE_2, $1,25(OH)_2D_3$ production was significantly stimulated. No changes in plasma calcium or phosphate levels or urinary cAMP excretion were observed, suggesting that the effects of prostaglandins are independent of the cAMP system [230].

In a further rat study, the effects of PGE_2 on the actions of PTH and calcitonin and the conversion of ^3H-$25OHD_3$ to ^3H-$1,25(OH)_2D_3$ were investigated. PGE_2 inhibited calcitonin stimulation of $1,25(OH)_2D_3$ but had no effect on PTH-stimulated $1,25(OH)_2D_3$ production. This suggests that PGE_2 may modulate the actions of calcitonin, but not those of PTH on 1α-hydroxylase [231]. Reduced plasma levels of $25OHD_3$ and $1,25(OH)_2D_3$ were also found following the administration of indomethacin, a potent inhibitor of prostaglandin synthesis, to pregnant rabbits [232].

In contrast to the above findings, a lack of effect of prostaglandin *in vivo* has been shown by Katz *et al.* [233], who administered PGE_2 by subcutaneous injection daily for 3 weeks to rats. They found no change in $1,25(OH)_2D_3$ or PTH levels, although a significant increase in bone mass in PGE_2-treated rats was demonstrated [233]. Furthermore, long-term subcutaneous administration of indomethacin failed to alter $1,25(OH)_2D_3$ or affect histomorphometric indices of bone formation and resorption [234].

Limited clinical data are available to assess the role of PGE_2 in humans. In one study, elevated levels of PGE_2 were believed to be the cause of hypercalcemia and enhanced 1α-hydroxylase activity in children with Bartter's syndrome [235]. Another study of children with idiopathic hypercalcuria found a positive correlation between $1,25(OH)_2D_3$ and prostaglandin E2 activity [236].

Overall, although it would seem that prostaglandins stimulate 1α-hydroxylase *in vitro*, variable results *in vivo* create doubt over the exact contribution that prostaglandins make in vitamin D metabolism. Again, the problem of which particular animal species is used may confound the results.

I. Tumor Necrosis Factor-α and Interferon-γ

The majority of information regarding the effects of particular cytokines on vitamin D metabolism has been derived from *in vitro* studies. Pryke *et al.* [237] examined the effects of tumor necrosis factor-α (TNFα) on 1α-hydroxylase activity in cultured alveolar macrophages. Incubation for 6 days with 50 IU TNFα resulted in an average fourfold increase in $1,25(OH)_2D_3$ production. An increase in 1α-hydroxylase activity was maximally reached at 72 hours. This finding may account for the "spontaneous" 1α-hydroxylase activity of sarcoid macrophages encountered in sarcoidosis [237] (see Chapter 79). A further study by Bickle *et al.* [238] provided evidence that TNFα stimulates $1,25(OH)_2D_3$ production in human keratinocytes. In preconfluent cells, TNFα stimulates $1,25(OH)_2D_3$ production; however, this ceased once the keratinocytes achieved confluence [238]. TNFα has also been shown to induce 1α-hydroxylase activity in human endothelial cells [239]. No effect of TNFα has been shown on bone cell lines, but TNFα was shown to inhibit vitamin D–receptor number in osteoblastic cells [240]. TNFα, via activation of NF-kappaB, has also been shown to decrease osteoblast transcriptional responsiveness to $1,25(OH)_2D_3$ [241]. This may be one of the mechanisms by which TNFα contributes to bone loss in disease states such as postmenopausal osteoporosis and inflammatory arthritis. However the predominant role of TNFα may be independent of $1,25(OH)_2D_3$ and result from osteoclastic effects on bone resorption.

There are also some data on interferon-γ (IFN-γ) and its role in vitamin D metabolism. Cultured normal human pulmonary alveolar macrophages in the presence of IFN-γ increased $1,25(OH)_2D_3$ production in a dose-dependent manner [242]. Bickle *et al.* [243] provided confirmatory findings showing that IFN-γ stimulated preconfluent keratinocytes to produce $1,25(OH)_2D_3$. Bone marrow-derived macrophages were also demonstrated to respond to IFN-γ with enhanced $1,25(OH)_2D_3$ production [244]. Because lung T lymphocytes from sarcoidosis patients produce IFN-γ, this may play a role in extrarenal $1,25(OH)_2D_3$ production *in vivo* in sarcoidosis [245].

Little is known about the *in vivo* effect of IFN-γ on vitamin D metabolism. Mann *et al.* [246] studied the effect of IFN-γ on bone mineral metabolism in rats and reported no changes in serum ionized calcium, PTH, or $1,25(OH)_2D_3$ levels, whereas IFN-γ had a significant osteopenic effect.

Human studies on the effects of these cytokines are lacking, and the underlying disease for which these factors are therapeutically administered may influence the results (e.g., chronic active hepatitis and malignancy).

In summary, aside from the possibility that TNFα and IFN-γ may play a role in extrarenal $1,25(OH)_2D_3$ production in certain disease states, like sarcoidosis, it is at present unclear if TNFα and IFN-γ physiologically influence vitamin D metabolism. The reader should refer to Chapter 79 for further information on extra-renal 1α-hydroxylase activity in diseases such as sarcoidosis.

III. DRUG EFFECTS ON VITAMIN D METABOLISM

The effects of drugs on vitamin D metabolism are summarized in Table II.

A. Anticonvulsants

Anticonvulsants have long been recognized to cause a number of alterations in bone mineral metabolism. In 1968 Kruse [247] first reported osteomalacia resulting from the use of anticonvulsants, and since then there have been numerous documentations of this finding [248–250]. Despite this, the effect of these agents on vitamin D metabolism remains controversial. Numerous animal and clinical studies have yielded conflicting results.

Animal studies have demonstrated enhanced metabolism [251] and biliary excretion [252] of vitamin D. Although Hahn *et al.* [253] showed an initial increase in $25OHD_3$ after phenobarbital treatment in the rat, this was followed by a subsequent decline in levels. However, Ohta *et al.* [254] reported no effect of low-dose phenytoin treatment on serum $25OHD_3$ or $1,25(OH)_2D_3$ under conditions where the rats were fed a vitamin D–supplemented diet. More recent animal data revealed that administration of phenytoin to growing rats for 5 weeks led to a significant decrease in osteocalcin levels, while there were not significant changes in serum calcium, pyridinoline, $25OHD_3$ or PTH compared with vehicle-treated rats [255]. This same study showed decreased trabecular bone volume and trabecular thickness, without significant change in osteoid thickness in phenytoin-treated animals. This may be modified by the administation of vitamin D and/or analogs [255].

A number of biochemical changes have been demonstrated in humans after anticonvulsant use. Serum calcium is reduced [249,256–258], which leads to the development of secondary hyperparathyroidism [249,258,264,266]. However, serum phosphate levels have been reported to be unchanged [249,258,262, 264,266]. Levels of $1,25(OH)_2D_3$ have been reported to be high [267,273,274], normal [265,267,269], or low [266], while $24,25(OH)_2D_3$ levels have been shown to be decreased [274,275] following long-term anticonvulsant use. An increase in $1,25(OH)_2D_3$ concentration may be secondary to increased PTH secretion. Levels of DBP are reported as unchanged [266], and intestinal absorption of vitamin D is not altered by anticonvulsant drugs [249].

The overwhelming majority of reports demonstrate low serum levels of $25OHD_3$ [249,258–268]; however, there are some studies in which $25OHD_3$ levels are unchanged [250,269–272]. These conflicting data have been thought to be due to differences in study design and in particular, differences in the ambulatory status of subjects [276]. One might hypothesize that the variation in vitamin D levels may be more related to exposure to ultraviolet light than to the direct effect of these agents on vitamin D metabolism. To control for this potential important confounder, more recent studies have attempted to characterize the skeletal health of ambulatory patients. A recent study of 30 ambulatory adult patients treated with phenytoin, carbamazepine or valproate found significantly lower $25OHD_3$ levels than age- and sex-matched controls [277]. The decrease in $25OHD_3$ was independent of anticonvulsant type. In contrast, a study of 18 ambulatory pediatric patients on valproate or carbamezapine found normal levels of vitamin D and PTH [278]. Another study of 60 pediatric patients receiving carbamazapine also found evidence of normal vitamin D metabolism [279].

Anticonvulsants are unlikely to have uniform effects on vitamin D metabolism. Phenobarbital, phenytoin, carbamazepine, and primidone are well-known inducers of hepatic cytochrome P450 enzymes [280–282]. The observed reduction in $25OHD_3$ levels with these agents is thought to arise from their enhancing the hepatic breakdown of vitamin D into inactive polar metabolites other than $25OHD_3$ [252,260,291]. Hahn *et al.* [260] demonstrated, both *in vivo* in humans and *in vitro* in rat liver, that phenobarbital stimulated the conversion of vitamin D to more polar metabolites. Serum $25OHD_3$ levels are clearly decreased in patients receiving phenobarbital [259–262,268] and phenytoin [259,261,262,265,268,274,283]. Conflicting evidence, however, exits regarding the effect of carbamazepine on $25OHD_3$ levels. Some reports show no change [276,284], and some show decreased levels [259,285,286] with carbamazepine. The newer anticonvulsants such as valproate, lamotrigine, clonazapam, gabapentin, topamirate, and ethosuximide are noninducers of cytochrome P450 enzymes. No reduction of $25OHD_3$ concentration has been reported with the use of sodium valproate [259,261]. Yet, osteoporosis has been reported in children on chronic valproic acid therapy [287,288], and long-term use of valproate and lamotragine has been shown to be associated with reduced bone formation [289]. Of note, a recent study of 71 patients on chronic anticonvulsant therapy found no significant difference in $25OHD_3$ levels between patients on inducers versus noninducers; despite this, patients on inducers did have lower BMD than patients on noninducers [290].

Various factors that have been shown to be associated with more severe changes in vitamin D metabolism include: polytherapy [256,259,263,268], larger total daily dose [256,262,265,268], duration of therapy [262], and female sex [266]. Treatment of anticonvulsant-induced alterations of vitamin D with replacement therapy of 400–4000 IU/day of vitamin D_3 has been shown to be effective in normalizing parameters of mineral metabolism and improving bone

mass [249,292]. In addition, treatment of anticonvulsant osteomalacia with small doses of oral $25OHD_3$ has also proved effective [263].

In summary, the overall results demonstrate that most anticonvulsants increase the metabolism of $25OHD_3$, and therefore it is recommended that vitamin D metabolites be consistently monitored. For patients on anticonvulsant therapy for longer than 6 months, the prophylactic use of vitamin D should be considered. Those especially at risk include long-term institutionalized patients, those with reduced ultraviolet light exposure, those with poor dietary intake of vitamin D, and those receiving long-term treatment with multiple anticonvulsant drugs [292].

B. Corticosteroids

Corticosteroid use is among the most important causes of secondary osteoporosis (see Chapter 73). Pharmacological levels of corticosteroids impair the intestinal absorption of calcium [293–296] and induce hypercalciuria [297,298]. Both lead to a state of secondary hyperparathyroidism [298–301]. Hyperparathyroidism, however, does not appear to explain the observed effects of corticosteroids on the skeleton. The histology of bone exposed to glucocorticoids reveals decreased bone remodeling, while that of bone exposed to hyperparathyroidism shows increased bone remodeling [302]. The evidence against PTH having a significant putative role in the development of glucocorticoid-induced osteoporosis is detailed in a recent review by Rubin and Bilezekian [303]. In this section, we focus on potential actions of corticosteroids on vitamin D metabolism. There are multiple other pathways postulated for the effect of these agents on bone, most notably the decrease in number and function of osteoblasts, i.e. increased apoptosis, changes in synthesis and binding of skeletal growth factors, and enhanced activity of 11-beta hydroxysteroid dehydrogenase type 1 in osteoblasts [304].

Levels of $25OHD_3$ were initially shown to be low in glucocorticoid-treated men. Avioli and co-workers [305] reported evidence of impaired hepatic conversion of vitamin D to $25OHD_3$. Furthermore, Klein *et al.* [295] demonstrated low $25OHD_3$ levels following high dose prednisone therapy, although in patients receiving low or alternate day doses $25OHD_3$ levels were unchanged. Similar findings were reported by Seeman *et al.* [299] in patients receiving high dose glucocorticoids for the treatment of connective tissue disorders. These findings are plausible in light of the fact that corticosteroids are known to induce hepatic microsomal oxidase enzymes [280], and hence chronic glucocorticoid use could produce effects similar to chronic anticonvulsant use (see Section III.A above).

However, other studies have failed to show a decrease in $25OHD_3$ concentrations. No differences in serum $25OHD_3$ levels have been shown in patients with corticosteroid-induced osteopenia [300,306,307] or in healthy [295] or diseased subjects treated with corticosteroids [308–310]. Patients treated with inhaled steroids for asthma also fail to demonstrate any alterations in $25OHD_3$, $1,25(OH)_2D_3$, or PTH levels [311,312]. Furthermore, patients with Cushing's disease were also found to have normal levels of $25OHD_3$ [313,314]. Similarly, in animal studies, no effect on the conversion of vitamin D to $25OHD_3$ or $25OHD_3$ to $1,25(OH)_2D_3$ has been reported [296,315,316].

Carre *et al.* [317] suggested that use of prednisolone may enhance the inactivation of $1,25(OH)_2D_3$ to more polar biologically inactive metabolites at the tissue level in rats. The same study showed no alteration in the conversion of ^3H-$25OHD_3$ to ^3H-$1,25(OH)_2D_3$ or ^3H-$24,25(OH)_2D_3$. This is in accordance with the findings of Favus *et al.* [316], who demonstrated normal conversion of tritiated $25OHD_3$ to $1,25(OH)_2D_3$ and normal subcellular localization of $1,25(OH)_2D_3$ in the intestinal mucosa of glucocorticoid-treated rats.

In children treated with glucocorticoids for glomerulonephritis, $1,25(OH)_2D_3$ concentrations have been shown to be reduced [318]. However, these children had proteinuria, which could decrease the levels of DBP. Adolescents with systemic lupus erythematosus also had low $1,25(OH)_2D_3$ levels following treatment with glucocorticoids [319]. However, the effect of corticosteroids on $1,25(OH)_2D_3$ levels remains controversial. Seeman *et al.* [299] reported no changes in $1,25(OH)_2D_3$ levels in 14 patients with either endogenous or exogenous glucocorticoid excess. They also showed no differences in the production or metabolic clearance rate of $1,25(OH)_2D_3$ [299]. Others have confirmed these findings of unchanged $1,25(OH)_2D_3$ levels [293,307–309,315]. However, certain studies have shown increased $1,25(OH)_2D_3$ values following subacute prednisone use [294,320–322], and in patients with Cushing's disease $1,25(OH)_2D_3$ levels are in the normal range but decrease following remission. This suggests that higher, though normal, values of $1,25(OH)_2D_3$ are present in the untreated state [313].

Increased levels of $1,25(OH)_2D_3$ may be due to the state of secondary hyperparathyroidism produced by glucocorticoids. Cortisol has been shown to stimulate PTH secretion by rat parathyroid glands *in vitro* [323]. *In vivo* this has been demonstrated both in rats [324] and in humans following acute and chronic administration of glucocorticoids [298–301]. Elevated PTH is not a consistent finding, however, as normal PTH

levels have been reported in both endogenous [313] as well as exogenous [294,299,307–309] glucocorticoid excess. Studies have also demonstrated no differences in levels of DBP [293] or concentrations of $24,25(OH)_2D_3$ [294,307–309].

The exact effect that corticosteroids have on vitamin D metabolism remains controversial. The conflicting evidence may reflect the differences in experimental conditions including variations in dosages, dietary intake, and/or sunlight exposure, and very importantly the effect of the underlying disease for which the glucocorticoids were given. Another explanation might be variation in genetic susceptibility to the effects of glucocorticoids among individuals. The current literature, however, does not support an association between vitamin D receptor gene polymorphisms and corticosteroid-induced osteoporosis [325]. Please refer to Chapter 73 for a detailed discussion of glucocorticoids and vitamin D.

C. Ethanol

It is well known that chronic alchoholics suffer from bone disease, and abnormalities in vitamin D metabolism have been well described. Despite this, the most common histomorphometric abnormality seen in these patients is osteoporosis, rather than osteomalacia [326]. Serum levels of $25OHD_3$ have been reported to be low [327–333] or normal [334–336] among alcoholic patients. Serum $1,25(OH)_2D_3$ has also been shown to be reduced [332,337,338] or unchanged [334]. This seems to be due to a combination of factors. First, many alcoholics have an inadequate dietary supply of vitamin D. A cross-sectional study of 181 male alcoholics revealed a small but significant association between bone mass and $25OHD_3$; however, a marked association between nutritional status and bone mass was observed [339]. Second, many have insufficient exposure to the ultraviolet light of the sun for adequate synthesis of vitamin D. Malabsorption [340] and increased biliary excretion of $25OHD_3$ [341] are other possible factors involved. However, intestinal absorption of vitamin D has been shown to be normal in patients with alcoholic liver disease [336,342]. Induction by alcohol of the cytochrome P450 system may also occur with subsequent increase in the degradation of vitamin D metabolites in the liver [343]. Ethanol may also inhibit hydroxylase activity in the kidney [344] or liver. However, hepatic hydroxylation was normal in cirrhotic alcoholics [336,343,345]. Decreased DBP may lead to low levels of vitamin D metabolites in patients with cirrhotic liver disease [346,347]. It is important to note that acute ethanol ingestion has not been shown to cause changes in vitamin D metabolism [348].

One might hypothesize that PTH mediates the effect of ethanol on vitamin D metabolism. However, human and animal data on the effects of ethanol on PTH are conflicting. Acute ethanol loading in rats has produced both elevated [349] and suppressed [350] serum levels of PTH, whereas alcohol has been shown to stimulate PTH release from bovine parathyroid cells *in vitro* [351]. In humans, acute alcohol ingestion produces unchanged [352] or increased PTH [351] levels. Serum levels of PTH in chronic alcoholics have been normal [345,353–355], decreased [339], or increased [328,332,334,356], the latter probably secondary to diminished intestinal absorption of calcium [357–360]. Increased PTH secretion leads to inhibition of tubular reabsorption of phosphate and lower serum phosphate levels [361]. However, $1,25(OH)_2D_3$ levels are not increased despite increased PTH and low phosphate, which suggests a direct inhibition of 1α-hydroxylase by ethanol [362]. The decreased PTH levels reported in some human studies may be related to hypomagnesemia [363].

In contrast to human studies, ethanol in rats seems to increase the production of $25OHD_3$ by the liver and lowers $1,25(OH)_2D_3$ levels [364–366]. This is due to the presence of both mitochondrial and microsomal hydroxylases in the rat, as opposed to only mitochondrial enzymes in humans [367,368]. Ethanol inhibits mitochondrial enzymes, but induces microsomal ones. This might lead to increased $25OHD_3$ in rats by microsomal induction and to decreased $25OHD_3$ in humans by mitochondrial suppression [369]. Thus, the rat does not appear to be an appropriate model for assessing changes in vitamin D metabolism induced by ethanol.

In summary, in chronic alcoholics, the circulating concentrations of vitamin D metabolites likely depend on the overall nutritional and health status of the individual [370]. A well-functioning alcoholic with satisfactory dietary ingestion and adequate sunlight exposure might be expected to have normal levels of circulating $25OHD_3$ or $1,25(OH)_2D_3$. In contrast, nonfunctioning alcoholics with poor nutrition and reduced sunlight exposure are likely to have low $25OHD_3$ and $1,25(OH)_2D_3$ levels [370].

D. Ketoconazole

The antifungal agent ketoconazole has been shown to inhibit cytochrome P450-dependent enzymes [371], and thus might be expected to alter vitamin D metabolism. In *in vitro* data demonstrated a dose-dependent reduction in 24-hydroxylase activity [372].

Additional *in vitro* data revealed that both ketoconazole and a similar antifungal, miconazole, behave as competitive inhibitors of 1α-hydroxylation of $25OHD_3$ [373].

Clinically, a reduction in $1,25(OH)_2D_3$ was demonstrated in healthy men treated for 1 week with ketoconazole [374]. No changes in $25OHD_3$, PTH, or serum calcium or phosphate levels were shown, suggesting a direct inhibitory effect of the drug on renal 1α-hydroxylase activity [374]. Subsequently, Glass and Eil [375] treated patients with primary hyperparathyroidism and hypercalcemia for one week with ketoconazole. The treatment produced a reduction in $1,25(OH)_2D_3$ levels. Serum total calcium but not serum ionized calcium levels fell, with no changes in $25OHD_3$, PTH, or serum phosphate [375]. A slightly longer study of two weeks duration, also in hyperparathyroid patients, confirmed low $1,25(OH)_2D_3$ levels but also demonstrated a nonsignificant fall in $25OHD_3$ [376]. This raises the possibility that the 25-hydroxylase enzyme is also inhibited, as it is a cytochrome P450-dependent enzyme. Perhaps studies of a longer duration will demonstrate significant falls in $25OHD_3$ as well. Finally, ketoconazole has been shown to be effective in decreasing serum $1,25(OH)_2D_3$ and calcium concentrations, both in cultured pulmonary alveolar macrophages taken from patients with sarcoidosis [377] and *in vivo* in sarcoid patients [377,378]. This suggests that ketoconazole also inhibits the extrarenal production of $1,25(OH)_2D_3$ known to occur in sarcoidosis (see Chapter 79).

Current data on the effects of ketoconazole on vitamin D metabolism suggest that it decreases $1,25(OH)_2D_3$ levels by directly inhibiting 1α-hydroxylase activity. The effects of ketoconazole on 25-hydroxylase activity remain to be determined. During treatment with ketoconazole for any of its indications, susceptible individuals should be monitored and supplemented with vitamin D, if necessary. Of note, combination therapy with ketoconazole and vitamin D analogs enhances the inhibitory effects of vitamin D on prostate cancer cell growth [379]. See Chapter 94 for a discussion of vitamin D and prostate cancer.

E. Hypolipidemics

Drugs that inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) are the most commonly prescribed agents for the treatment hypercholesterolemia. Because HMG-CoA reductase inhibitors (or STATINs, as they are also known) are potent inhibitors of cholesterol synthesis, they may impact vitamin D production because cholesterol is the vitamin D_3 precursor. Ismail *et al.* [380] studied 40 hypercholesterolemic patients treated for 24 weeks with one of the selective HMG-CoA reductase inhibitors, pravastatin (40–80 mg daily). Results showed that although levels of total and low density lipoprotein (LDL) cholesterol were significantly reduced, no changes in $25OHD_3$, $1,25(OH)_2D_3$, or PTH levels were observed [380]. The capacity of the skin to synthesize vitamin D_3 after ultraviolet light exposure also showed no changes after three months of pravastatin therapy [381]. Lack of effect of HMG-CoA reductase inhibitors on vitamin D metabolism has been confirmed by others [382], and indeed combination therapy using both pravastatin (40 mg/day) and the bile acid sequestrant cholestyramine (24 g/day) failed to affect levels of vitamin D metabolites or PTH [380]. Although statins have a theoretic potential to alter vitamin D metabolism, the literature does not seem to show that these agents reduce or increase vitamin D levels. This is intriguing in light of recent literature suggesting that statins may have an anabolic effect on bone [383] and may be protective against fracture [384].

Absorption of vitamin D from the gut requires the presence of bile acid. Moreover, vitamin D is excreted in the bile [385], and some degree of enterohepatic circulation of vitamin D occurs [386,387]. Thus, it stands to reason that cholestyramine might adversely affect vitamin D absorption. There have been reports in rats of decreased vitamin D absorption following treatment with cholestyramine [388]. Moreover, there are isolated case reports of osteomalacia associated with long-term cholestyramine use, which were attributed to cholestyramine-induced vitamin D deficiency [389,390]. Compston and Thompson [391] also reported decreased levels of $25OHD_3$ and reduced intestinal absorption of vitamin D in patients with primary biliary cirrhosis treated with cholestyramine for greater than two years. However, a large double-blind randomized trial of patients treated with cholestyramine (24 g/day) for four months showed similar levels of vitamin D metabolites, PTH, and serum calcium and phosphate compared with placebo-treated patients [392]. Ismail *et al.* [392] showed equivalent findings after six months of therapy [392], as did long-term studies using another bile acid sequestrant, colestipol, for the treatment of children with familial hypercholesterolemia [393,394].

Ezetimibe, a novel cholesterol absorption inhibitor, has been shown not to inhibit the absorption of vitamin D across the intestinal wall in rodents [295]. The effect of this agent on vitamin D absorption, calcium metabolism, or bone mass has not been reported in humans. A single report of the effect of fibrates on vitamin D metabolism showed a decline in $25OHD_3$ and a rise in $1,25(OH)_2D_3$ levels [396].

It seems, therefore, that vitamin D is unaffected by the statin drugs and short-term cholestyramine treatment.

Long-term cholestyramine therapy may affect $25OHD_3$, and levels should be routinely monitored in susceptible individuals and vitamin D supplementation provided, if necessary. The effect of cholestyramine on bone mass and fracture has not been investigated. Research into the effects of ezetimibe on vitamin D metabolism in humans is warranted.

F. Bisphosphonates

Bisphosphonates are widely used for the treatment of osteoporosis, Paget's bone disease, and hypercalcemia of malignancy. By inhibiting bone resorption [397,398], the agents cause an increase in the calcium mineral content of bone and a decrease in serum calcium levels [399]. This fall in serum calcium results in the stimulation of PTH secretion. PTH reduces the renal tubular reabsorption of phosphate, which reduces serum phosphate levels. Both increased PTH and low phosphate levels can lead to increased $1,25(OH)_2D_3$ production [400–402].

The first generation bisphosphonate, ethane-1-hydroxy-l,1-diphosphate (EHDP), also known as *etidronate*, was initially shown to cause a reduction of $1,25(OH)_2D_3$ levels at high doses *in vivo* [403,404]. This effect was probably an indirect one as EHDP failed to stimulate or inhibit 1α- or 24-hydroxylase in primary chick kidney cell cultures [10]. Later, evidence emerged to suggest that bisphosphonates indirectly stimulate 1α-hydroxylase at least in part by some unknown humoral factor. An experimental bisphosphonate compound, YM175, was shown in TPTX and sham-operated rats to increase $1,25(OH)_2D_3$ and 1α-hydroxylase in a dose-dependent manner; however, no increase in 1α-hydroxylase activity was demonstrated *in vitro* [405].

In clinical studies, Paget's disease patients treated with etidronate had higher $1,25(OH)_2D_3$ levels without significant changes in serum PTH, ionized calcium, or phosphate [66,406]. Lawson-Matthew *et al.* [407] found increased $1,25(OH)_2D_3$ concentrations following short-term oral etidronate treatment but decreased $1,25(OH)_2D_3$ levels after high-dose intravenous etidronate. The effects of etidronate are thus most probably dose-related. In rats low-dose etidronate inhibits bone resorption and calcium release from bone and stimulates dietary calcium absorption. The lower calcium concentration, in turn, probably either directly or indirectly, via PTH, mediates 1α-hydroxylase activation. However, high-dose etidronate inhibits bone formation as well as resorption, and decreased calcium absorption from the gut is found [399]. Paget's disease patients treated with another bisphosphonate, aminohydroxy-propylidene bisphosphonate (APD), known as *pamidronate*, also had elevated $1,25(OH)_2D_3$ levels following short-term intravenous [408,409] and oral [410] administration. PTH rose to twice pretreatment levels in a response to a fall in ionized calcium levels [408,410]. $24,25(OH)_2D_3$ concentration declined, but $25OHD_3$ remained unchanged [408]. Concentrations of $1,25-(OH)_2D_3$ [411,412], as well as PTH [412–414], were also elevated after treatment with pamidronate in patients with tumor-associated hypercalcemia.

The third generation bisphosphonate 4-amino-hydroxybutylidene-l,1-bisphosphonate, or alendronate, is a 100- to 500-fold more potent inhibitor of bone resorption than is etidronate [415]. Postmenopausal women treated with alendronate showed initial rises in $1,25(OH)_2D_3$ and PTH that normalized after chronic administration of the drug [401,416,417], probably because of inhibition of bone resorption and decreased serum calcium concentrations. $1,25(OH)_2D_3$ and PTH levels also rose following intravenous alendronate infusion for the treatment of hypercalcemia of malignancy [412].

Bisphosphonates also appear to have an effect on $25(OH)D_3$ levels. A randomized controlled trial of 20 breast cancer patients treated with clodronate versus calcitonin revealed decreased $25(OH)D_3$ levels in addition to decreased calcium levels and increased PTH and $1,25(OH)_2D_3$ levels in the clodronate treated subjects [418].

In general, bisphosphonates do appear to influence vitamin D metabolism. The most common finding is an elevation in $1,25(OH)_2D_3$ levels, which is most likely due to secondary alterations in serum-ionized calcium, phosphate, and PTH levels. These alterations generally appear to be short-term, but may theoretically also confer some initial benefit in regard to an anabolic action of the elevated PTH levels. It is advisable to rule out vitamin D deficiency prior to initiation of bisphosphonate therapy and to supplement all patients with calcium and vitamin D.

G. Thiazide Diuretics

Clinically, the use of thiazide diuretics has been associated with favorable effects on bone mineral density [419–421] and hip fracture rate [423–425]. These beneficial effects may be related to its ability to decrease PTH-stimulated bone resorption and decrease bone remodeling [422].

Thiazide use leads to a number of alterations in bone mineral metabolism. Thiazides are well known to decrease urinary calcium excretion [420,425–427], with resultant increases in serum calcium concentration [426,428]. This leads to reduced PTH levels [420,422], which in turn decrease $1,25(OH)_2D_3$

synthesis [420,422,429,430] and leads to a reduction in intestinal calcium absorption [420].

Clinical studies on the effect of hydrochlorothiazide on vitamin D metabolism have shown a consistent decrease in $1,25OH_2D_3$ levels. The administration of 50 mg/day of hydrochlorothiazide to postmenopausal women significantly decreased $1,25(OH)_2D_3$ and increased $25OHD_3$ and $24,25(OH)_2D_3$ levels [420]. Similar findings of low $1,25(OH)_2D_3$ levels were reported by Sowers and co-workers [429]. Riis and Christiansen [431] conducted a double-blind, long-term controlled trial in early postmenopausal women and demonstrated a trend toward lower $1,25(OH)_2D_3$ with a significantly elevated $24,25(OH)_2D_3$ concentration [431].

No evidence of a direct effect on the synthesis or degradation of $1,25(OH)_2D_3$ by thiazides is currently available. It thus seems that changes in vitamin D metabolites found after the use of thiazide diuretics are most likely secondary to alterations in serum calcium concentrations and PTH levels.

H. Calcium Channel Blockers

Calcium channel blockers, of which nifedipine, verapamil, and diltiazem are the most well recognized and frequently prescribed, are chemically dissimilar. As such they have different effects on PTH secretion. *In vitro* experiments show that verapamil both increases and decreases PTH secretion, depending on extracellular calcium concentrations [432–434]. Diltiazem inhibited PTH release in bovine parathyroid cells by 40% and in human parathyroid cells by 20% [435]. A similar inhibition of PTH secretion was seen with nitrendipine, an analog of nifedipine [436].

It has been suggested that in certain circumstances calcium channel blockers can act as calcium agonists, thus leading to an increase rather than a decrease in intracellular calcium concentration with associated inhibition of PTH secretion [435]. Verapamil has been reported to inhibit PTH secretion from rat parathyroid glands *in vitro* [437] and from goat parathyroid glands perfused *in vivo* [434]. In conflicting *in vivo* animal studies, verapamil stimulated PTH secretion in rats [438,439], whereas $1,25(OH)_2D_3$ levels were decreased [438].

Clinical studies, however, have shown no change in PTH levels using either verapamil [440] or nifedipine [441]. Only a short-term (three-day) study with diltiazem reported decreased PTH levels with normal ionized calcium and phosphate concentrations [435]. Studies evaluating vitamin D metabolites showed that 16 weeks of diltiazem administration had no effect on either PTH or $1,25(OH)_2D_3$ levels [442]. Long-term use of nifedipine also failed to alter PTH or $25OHD_3$ levels or to affect serum parameters of bone turnover or bone mineral density in a group of males treated with calcium channel blockers for coronary heart disease [433]. Finally, calcium channel blockers are known inhibitors of hepatic microsomal cytochrome P450-dependent enzymes, and thus have the potential to cause alterations in vitamin D metabolites [434]. Despite possible interactions with PTH release, all past and recent clinical data have failed to demonstrate any effect on vitamin D metabolites, and thus calcium channel blockers cannot be considered to have a major influence on vitamin D metabolism.

I. Heparin

Long-term use of heparin has been associated with the development of osteopenia in humans [435–438] and in rats [439–441]. Chronic heparin use is most frequently encountered in pregnant women with venous thrombosis. Pronounced bone loss and low $1,25(OH)_2D_3$ levels have been seen in such patients, while serum $25OHD_3$ and $24,25(OH)_2D_3$ levels and calcium and phosphate concentrations have been unchanged [442,443].

Heparin decreases bone formation in cultured fetal rat calvaria [444,445] and stimulates bone resorption by increasing the number and activity of osteoclasts *in vitro* [446]. Mutoh *et al.* [439] treated four-week-old vitamin D–deficient rats with heparin (2000 lU/day). Significant bone loss developed after two weeks, which peaked at four weeks. No change in serum total or ionized calcium was observed, but a significant elevation of serum PTH was seen. Furthermore, $1,25(OH)_2D_3$ levels were decreased by 54% versus controls [439]. Although in the Mutoh *et al*. study no change in serum ionized calcium was demonstrated, this is presumed to be the mechanism by which heparin increases serum PTH. Heparin has been reported to have a high affinity for calcium ions [447]. This may lead to lower calcium levels, which would stimulate PTH release. However, this is a doubtful mechanism for heparin-induced osteopenia, as calcium salts of heparin are as effective in inducing osteoporosis as the corresponding sodium salts [448]. The reasons for the low $1,25(OH)_2D_3$ values with heparin are entirely speculative, but may involve direct inhibition of the 1α-hydroxylase system, with the low $1,25(OH)_2D_3$ levels in turn influencing receptors on the parathyroid gland, which induces PTH stimulation.

The use of low molecular weight heparins (LMWH) in the treatment of thrombotic disorders has steadily grown over the past decade. The long-term effect of

these agents on bone is yet to be determined. *In vitro* data suggest that LMWHs inhibit osteoblast growth [449]. Delayed fracture repair has also been reported in rabbits treated with the LMWH, enoxaparin [450]. A prospective study of 16 pregnant women who received 19–32 weeks of enoxaparin revealed a significant decrease in bone mineral density of the proximal femur at six months postpartum [451]. The effect of LMWH on vitamin D metabolism has not been studied.

J. Cimetidine

Cimetidine, a histamine H_2 receptor antagonist, is frequently used for the treatment of peptic ulcer disease. *In vitro* studies have demonstrated histamine H_2 receptors in both normal and adenomatous parathyroid gland tissue [452], and stimulation of these receptors by histamine increases PTH release [453]. Cimetidine has been shown to decrease serum PTH in patients with either parathyroid adenoma [454,455] or secondary hyperparathyroidism due to chronic renal insufficiency [456,457]. However, there have been equivocal findings regarding the effects on serum calcium accompanying the changes in PTH, with both low [454] and unchanged levels [455,457,458] being found.

Cimetidine was also shown to significantly decrease net intestinal calcium transport either secondary to its effect on PTH or via changes in vitamin D [459]. Cimetidine is an inhibitor of microsomal drug metabolism [460,461], and thus one might expect this agent to inhibit hepatic vitamin D 25-hydroxylase, a cytochrome P450-dependent enzyme.

Animal studies have shown a dose-dependent decrease in 25-hydroxylase activity in the presence of increasing concentrations of cimetidine [462]. Cimetidine was also shown to reduce $25OHD_3$ levels in hens [463].

In humans, short-term use of cimetidine (800 mg/day for four weeks) did not decrease the level of $25OHD_3$ but prevented the expected seasonal rise in $25OHD_3$ [404]. After cessation of cimetidine therapy, levels rose significantly. Levels of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ were not affected, and serum calcium and phosphate concentrations remained normal [464].

A review of the literature up till the present shows that cimetidine has multiple effects on calciotropic hormones. PTH levels are decreased, and $25OHD_3$ is reduced by inhibition of hepatic 25-hydroxylase activity. Despite low PTH levels, however, no changes in serum $1,25(OH)_2D_3$ or serum calcium or phosphate concentrations are evident. Monitoring of $25OHD_3$ levels is indicated only in susceptible individuals, such as those with hepatic insufficiency, poor nutrition, or the elderly. Other H_2 receptor antagonists, such as ranitidine, have less effect on hepatic drug metabolism, [461] and thus are less likely to interfere with vitamin D metabolism. No studies employing ranitidine or other H_2 receptor antagonists and their effects on vitamin D metabolism are currently available. Data on the effect of cimetidine on bone density and fracture incidence has either not been explored or is unknown.

K. Aluminum

Chronic use of dialysate or total parenteral nutrition (TPN) containing aluminum is known to cause metabolic bone disease. Aluminum containing TPN has also been associated with low levels of $1,25(OH)_2D_3$ [466]. Casein hydrolysate, which is used as a protein source in some TPN solutions, was identified as containing substantial amounts of aluminum [467]. These patients also develop a low-turnover osteomalacia with aluminum accumulation in bone [468]. Patients undergoing hemodialysis with water containing high levels of aluminum also have a high incidence of aluminum bone disease [469,470]. Aluminum content in bone is elevated and correlates positively with the development of osteomalacia [471–475]. Patients with renal failure are also susceptible to aluminum accumulation in bone [474,476]. There is some evidence that aluminum may act indirectly on bone by suppressing PTH release. Hemodialysis patients with osteomalacia have demonstrated lower mean PTH values than hemodialysis patients with normal $25OHD_3$ levels [473,475].

In rats, aluminum was shown to accumulate in parathyroid tissue [477], and reports show that aluminum impairs PTH release *in vitro* [478]. This may lead to low $1,25(OH)_2D_3$ production. High-dose aluminum injections have also caused osteomalacia [474,479] and lowered PTH levels in rats [479]. However, in another similar experiment in rats using equivalent doses of aluminum, no skeletal changes or alterations in serum vitamin D metabolites were observed [480]. In dogs, serum calcium increased and serum phosphate and PTH did not change significantly following five weeks of parenteral aluminum administration daily [481]. $25OHD_3$ was normal, but a marked decline in $1,25(OH)_2D_3$ was demonstrated. Renal function also declined, and this may account for the changes in $1,25(OH)_2D_3$. However, the reduction in $1,25(OH)_2D_3$ occurred prior to the appearance of renal impairment, suggesting a direct inhibitory effect on the synthesis of $1,25(OH)_2D_3$ [482].

Opposite effects on vitamin D metabolites seem to occur following the oral ingestion of aluminum hydroxide as an antacid. Increased values of $1,25(OH)_2D_3$ may occur with hypophosphatemia induced by oral

aluminum salts, which in turn increases 1α-hydroxylase activity in the kidney [483]. Aluminum is absorbed from the gut and deposits in bone in patients both with [484,485] and without [486] renal impairment. Daylong intake by a group of postmenopausal women resulted in a fall in serum phosphate, which correlated significantly with a rise in $1,25(OH)_2D_3$ levels. Total and ionized calcium and PTH levels were unchanged [487]. This study was very short in duration, however, and longer studies would be required to assess the effects of oral aluminum on vitamin D metabolism. Nevertheless, chronic use of high doses of aluminum containing antacids has been reported to induce osteomalacia and rickets [488–431].

L. Antituberculous Agents

Anecdotal case reports of rifampicin (rifampin)-induced osteomalacia [492] led to further investigation of the effect of antituberculous agents on vitamin D metabolism. Studies by Brodie and colleagues [493] initially suggested that the frequently-used first-line antituberculous drugs rifampicin and isoniazid, alone or in combination, could affect vitamin D metabolism. In the first study, short-term use of rifampicin (600 mg/day for two weeks) in healthy subjects reduced plasma $25OHD_3$ levels by as much as 70%, whereas $1,25(OH)_2D_3$ and PTH remained unchanged [493]. Rifampicin is a known hepatic enzyme-inducer [494], and the enzyme 25-hydroxylase is a cytochrome P450-dependent enzyme located in the liver [495]. It is most likely, therefore, that the decreased levels of $25OHD_3$ represent increased hepatic metabolism of $25OHD_3$ by 25-hydroxylase [493]. Short-term use of isoniazid (300 mg/day) in healthy subjects also produced a decrease in $25OHD_3$ and $1,25OH_2D_3$ levels, accompanied by a fall in serum calcium and phosphate and rise in PTH [344]. Inhibition by isoniazid of hepatic enzyme activity [496] could explain the decreased levels of $25OHD_3$ and $1,25(OH)_2D_3$, as hepatic 25-hydroxylase and renal 1α-hydroxylase are both cytochrome P450-dependent enzyme systems [497]. A further study using both rifampicin and isoniazid also led to decreased $25OHD_3$ and $1,25(OH)_2D_3$ levels together with raised PTH [498]. Similar short-term effects of rifampicin and isoniazid have been reported by others [499,500].

Despite evidence of short-term derangements in vitamin D metabolism, it seems that long-term studies reveal no significant effects [501,502]. One study reported that treatment of tuberculous patients with both rifampicin and isoniazid for nine months produced no significant alterations in $25OHD_3$ and $l,25(OH)_2D_3$ levels [502]. A more recent study found that tuberculous patients had low baseline $25OHD_3$, and PTH levels and high urinary calcium and $1,25(OH)_2D_3$ levels, presumably due to granulomatous synthesis of 1α-hydroxylase. After treatment with isoniazid and rifampin for nine months, these parameters normalized [503].

Thus, overall it seems that only in the short-term are patients treated with these agents susceptible to vitamin D deficiency. Patients in developing countries with insufficient vitamin D intake and others at risk for vitamin D deficiency may require vitamin D supplementation, but it is unlikely that antituberculous drugs contribute to the development of overt metabolic bone disease. In fact, as suggested by Martinez *et al.* [503], long-term antituberculous therapy may lead to a correction of the abnormalities of vitamin D and calcium metabolism seen in these patients as it treats the underlying condition.

M. Caffeine

The first mention of a potential effect of caffeine on bone metabolism is found in a study by Daniell [504], who noted a higher caffeine intake in osteoporotic patients compared to age-matched controls. Heaney and Recker [505], in studies of perimenopausal women, were the first to show a negative calcium balance in association with caffeine intake. Additional controlled trials of caffeine use indicate that in individuals ingesting inadequate dietary calcium, caffeine ingestion leads to a small negative calcium balance via a small but significant decrease in calcium absorption efficiency [506]. Barger-Lux and Heaney have estimated that this small effect on calcium balance can be offset by the addition of 1–2 tablespoons of milk per cup of coffee [507].

The more recent literature examining the effects of caffeine on the skeleton is conflicting. The Framingham Osteoporosis Study failed to show an affect of caffeine intake on bone mass in elderly men and women [508]. Rico *et al*. failed to show a relationship between caffeine intake on bone mass as measured by quantitiative phalangeal bone ultrasound in 93 healthy postmenopausal women. [509]. Similarly, Lloyd *et al.* [510] did not find an association between dietary caffeine intake and total body or hip BMD. However, another study found that elderly women who consumed >300 mg/d of caffeine had higher rates of bone loss than women with an intake of <300 mg/d [511]. These conflicting reports may be due to differences in determination of caffeine intake and methods of assessing bone density [509].

Caffeine has been reported to enhance hepatic microsomal drug metabolism in rats and mice [512,513].

An inhibitory effect on the conversion of $25OHD_3$ to $1,25(OH)_2D_3$ was reported in isolated renal tubules from vitamin D–deficient chicks [514]. Studies in rats following chronic caffeine administration showed normal serum calcium but increased urinary excretion, and intestinal endogenous excretion of calcium, as well, increased intestinal absorption of calcium [515]. Yeh and Aloia [516] studied serial changes of serum calcium, PTH, $1,25(OH)_2D_3$, and calcium balance in young and old adult rats following daily caffeine administration for four weeks. In young rats, urinary calcium excretion increased and serum calcium decreased initially, but then returned to control levels. Serum PTH and $1,25(OH)_2D_3$ increased after two weeks, and intestinal absorption of calcium remained unchanged. In adult rats similar changes occurred except that $1,25(OH)_2D_3$ levels were similar to those in controls [516]. One drawback to this study is that the caffeine content that these rats received is the equivalent of 16 cups of coffee each day, which would be rather excessive for human consumption. A further study demonstrated no effect on bone histomorphometry after administration of caffeine to rats [517].

Thus, caffeine seems to have some effect on calcium homeostasis in humans and animals. Its impact is likely more substantial in patients who are already at nutritional risk, such as the elderly. A clear effect on vitamin D metabolism has not been demonstrated.

N. Theophylline

Theophylline, a once common treatment for reactive airway disease, has been shown to have an impact on vitamin D metabolism. An *in vitro* study showed an inhibitory effect of theophylline on the conversion of $25OHD_3$ to $1,25(OH)_2D_3$ [514]. This occurred despite an increase in renal tubule cAMP levels, which have been shown to enhance $1,25(OH)_2D_3$ formation *in vivo* [19]. Furthermore, there are reports of enhanced 24-hydroxylase activity by aminophylline in normal birds [518] and rats [519]. In rat studies, the effect of long-term constant subcutaneous theophylline infusion was assessed. Increased urinary calcium excretion was demonstrated together with a reduction in total body calcium. Serum $25OHD_3$ was decreased, but no changes in $1,25(OH)_2D_3$ or PTH levels were observed [520]. One possible explanation for the effects on $25OHD_3$ is that theophylline, an agent that has been previously demonstrated to induce hepatic microsomal enzymes [513,521], enhances conversion of $25OHD_3$ to other metabolites.

In humans, theophylline increased phosphate and calcium urinary excretion in healthy males [522]. McPherson *et al.* [523] found that patients hospitalized for theophylline toxicity had hypercalcemia that normalized after theophylline was discontinued. PTH levels were unchanged, which implies that theophylline may act by enhancing the action of available PTH.

Although animal data suggest that theophylline may alter vitamin D metabolism and clinical studies demonstrate a change in calcium balance, a direct effect on vitamin D has not been demonstrated in humans.

O. Immunosuppressants

Potent immunosuppressants, which are widely used in the management of transplant patients and patients suffering from autoimmune conditions, have altered the prognosis of these disorders. This section will not address the role of vitamin D as an immune modulator (see Chapter 36) or its use in immune related disorders (see Chapters 98 and 99).

The T-cell-specific immunosuppressant cyclosporin A (CsA) produces a high turnover osteopenia in the rat [524–526]. In both the rat and mouse, CsA has been shown to stimulate $1,25(OH)_2D_3$ production in the absence of any changes in serum-ionized calcium, phosphate, or PTH levels [525–527]. A significant increase in 24-hydroxylase activity has been seen in kidney homogenates from rats following 14 days of oral CsA treatment (15 mg/kg). Furthermore, a significant increase in 1α-hydroxylase activity was demonstrated in mice treated with 30–50 mg/kg CsA for three days, which was shown to be due to renal and not extrarenal stimulation of 1α-hydroxylase [527]. There was no evidence of increased metabolic clearance of $1,25(OH)_2D_3$ in CsA-treated rats [527]. Young rats responded to CsA with greater $1,25(OH)_2D_3$ production than older ones [528], and $1,25(OH)_2D_3$ levels normalized following cessation of CsA treatment [526]. Grenet *et al.* [529] recently reported that CaA induced an 85% decrease in the vitamin D–binding proteins, calbindin –D28k, a 40% decrease in the vitamin D receptor and a 69% decrease in the 24-hydroxylase enzyme, while $1,25(OH)_2D_3$ was increased. These findings provide further support for the evidence that CsA significantly impairs the vitamin D activation pathway. Studies in Rowett-nude T-cell-deficient rats also showed a similar increase in $1,25(OH)_2D_3$ production in response to treatment with CsA. This suggests that CsA affects vitamin D independent of the presence of T lymphocytes [530]. Cyclosporin G (CsG), an equipotent immunosuppressive molecular analog of CsA that is less nephrotoxic, [531,532] also increased $1,25(OH)_2D_3$ levels in rats, independent of changes in

ionized calcium or PTH levels, but to a lesser degree than CsA [533–535].

The fungal macrolide tacrolimus (FK506) is another potent immunosuppressive that has a similar action to CsA [536]. In the rat, FK506 appears as deleterious as CsA, in that it too produces severe high turnover osteopenia; however, unlike CsA, it has not been shown to have an effect on $1,25(OH)_2D_3$ production [533,535]. Azathioprine, a thioguanine derivative of mercaptopurine, acts as a purine antagonist and is an effective antiproliferative agent. It, too, failed to influence $1,25(OH)_2D_3$, PTH, or ionized calcium levels in the rat [537]. Finally, administration to rats of another immunosuppressant, rapamycin (sirolimus), caused either no change [538] or low $1,25(OH)_2D_3$ levels [539] with normal PTH or phosphate and ionized calcium concentrations. No reports of effects on vitamin D by any of the other immunosuppressants are known.

Calcineurin inhibitors are most widely used in the posttransplantation setting and thus clinical data on their effect on vitamin D metabolism are difficult to interpret, as the underlying disease process and renal impairment induced by these agents interfere with vitamin D metabolism. Moreover, multiple drugs including corticosteroids, which inhibit $1,25(OH)_2D_3$, are given concurrently. A recent prospective study of 57 patients post kidney transplant found a high prevalence osteomalacia on bone biopsy despite normal levels of calcidiol and calcitriol. The authors theorize that these findings suggest a state of vitamin D resistance [540]. In addition, posttransplantation, the use of CsA [541–545], or azathioprine [543–548] does not appear to result in any changes in $1,25(OH)_2D_3$ levels compared to control subjects.

These agents have also been evaluated in the nontransplant setting. Long-term CsA treatment of patients with multiple sclerosis revealed similar $1,25(OH)_2D_3$, $25OHD_3$, ionized calcium, and phosphate levels compared to those treated with azathioprine, although the latter group had lower PTH levels [549]. Patients with primary biliary cirrhosis treated for one year with CsA also showed no evidence of any changes in vitamin D metabolites or PTH levels [550]. Thus, it would seem that only experimentally, in rats and mice, does CsA affect vitamin D and that immunosuppressants, collectively, do not significantly affect vitamin D metabolism in the clinical setting.

Nevertheless, the use of calcitriol has been shown to be beneficial in patients receiving these agents. A recent prospective study of the effects of calcitriol in 53 patients post heart transplantation on high-dose tacrolimus, found, at 12 months, that femoral neck BMD stabilized and lumbar spine BMD increased significantly in patients treated with calcitriol, while femoral neck BMD decreased and spine BMD stabilized in placebo-treated subjects. Both groups received calcium supplementation and treatment of hypogonadism [551]. Thus, because of the overall effect on bone loss, it is advocated that patients treated with calcineurin inhibitors be supplemented with vitamin D analogs and oral daily calcium [552,553].

P. Fluoride

Fluoride is no longer used as a treatment for osteoporosis; however, it is commonly found in drinking water at varying concentrations. Although studies in rats have found reduced calcium and phosphate levels when fluoride was added to drinking water at the 2.0 mM level, no changes in either serum $25OHD_3$ or $1,25(OH)_2D_3$ concentrations were reported [554]. In human studies, no changes in serum PTH, $1,25(OH)_2D_3$, $25OHD_3$, or calcium and phosphate levels were found after one to two years of fluoride treatment for osteoporosis in both men and women [555,556]. Thus, these data suggest that fluoride does not interfere with vitamin D metabolism.

Q. Olestra

Olestra, formerly known as *sucrose polyester* (SPE), is a nonabsorbable mixture of hexa-, hepta-, and octa-carbon fatty acid esters of sucrose. It is an edible material that can be incorporated into the diet as a fat substitute. Olestra has physical properties similar to those of conventional dietary fats [557,558]; however, it is not absorbed [559] or hydrolyzed by gastric lipases [560]. As dietary vitamin D is absorbed from the intestine in association with dietary fats [561] and has an enterohepatic circulation, these processes may be altered by the presence of nondigestible lipid. Although there have been reports of reduced absorption of the fat-soluble vitamins A and E [557,558,562,563], all human studies have thus far shown no significant effects on serum 25OHD levels [562–565] or dietary vitamin D absorption [564]. A recent post-marketing surveillance study did not show an association between olestra consumption and concentrations of fat-soluble vitamins [566]. A 20-month feeding study in dogs also showed no effects on vitamin D status following Olestra ingestion [567]. Since photoinduced cutaneous synthesis of vitamin D is the major factor determining vitamin D status [568–570], Olestra ingestion would not be expected to adversely affect vitamin D nutritional status.

R. Orlistat

Orlistat is a gastric and pancreatic lipase inhibitor, which is widely used in the management of obesity. Given its inhibition of absorption of lipids from the gastrointestinal tract, it has the potential to inhibit the absorption of fat soluble vitamins. A small prospective study found a significant decrease in vitamin D levels after one month of therapy with orlistat in adolescents, despite the use of a multivitamin [571]. However, a randomized, double-blinded study found no significant difference in vitamin D levels between orlistat users and controls [572]. Additional studies examining the effect of this agent on vitamin D metabolism are warranted. In the meantime, it may be prudent to monitor vitamin D levels in patients using this drug.

S. Lithium

Lithium is a monovalent cation and is widely used in the management of bipolar affective disorders. It is well known to have an array of endocrine-related side effects, including the alteration of systemic calcium metabolism. In humans, lithium has been shown to lower serum phosphate [573–575] and reduce urinary calcium excretion [575–580]. This occurs as a result of increased tubular reabsorption of calcium, and it results in hypercalcemia [576–578,581–585]. Increases in PTH levels and parathyroid volume have been attributed to lithium therapy [574,575,577,581–585,587]. Drug withdrawal reverses these effects [506]. *In vitro* evidence has shown that lithium stimulates the release of PTH from human parathyroid tissue [512]. Parathyroid adenoma has been reported in several patients with lithium-induced hyperparathyroidism [588], although another possible mechanism by which lithium may elevate PTH is secondary to lithium-induced nephropathy [589,590]. However, serum creatinine levels were found to be normal in a patient with increased PTH [506]. Renal tubular acidosis has also been associated with lithium use [586].

There is limited knowledge about the effects of lithium on vitamin D as most metabolic studies following lithium administration in humans and animals have not measured vitamin D metabolites. One study found elevated PTH levels, yet normal $1,25(OH)_2D_3$ levels after either short-term (mean, 1.7 months) or long-term (mean, 103 months) lithium carbonate administration [508]. Another study of 10 patients treated for one month with lithium carbonate noted elevated serum PTH and reduced $1,25(OH)_2D_3$ levels, although $25OHD_3$ and serum calcium levels remained unchanged [591]. This is surprising as the elevation of PTH levels would be expected to increase rather than decrease $1,25(OH)_2D_3$ levels. The authors hypothesize that lithium may act by inhibiting renal 1α-hydroxylase. The sample size and mean serum lithium levels were similar in both of the above studies; thus, the conflicting observations may relate to differences in the patient populations.

Although lithium use can affect serum calcium, phosphate, and PTH levels, a direct effect on vitamin D metabolism has not been demonstrated thus far.

IV. CONCLUSION

The effects of various endogenous and exogenous hormones and drugs on the metabolism of vitamin D are complex. PTH and PTHrP are clearly potent stimulators of $1,25(OH)_2D_3$ production. Past and current literature suggest that growth hormone, via IGF-1, and estrogen increase production of $1,25(OH)_2D_3$. Long-term use of most anticonvulsants leads to increased $25OHD_3$ catabolism which is likely the main cause of the low bone mass observed in these patients. A strong association exists between the use of corticosteroids and immunosuppressants and the development of significant bone loss; however, the effect of these agents on vitamin D metabolism in humans is less clear. Although statins do not appear to effect vitamin D metabolism, long term use of cholestyramine may decrease $25OHD_3$ levels. Parenteral aluminum appears to decrease $1,25(OH)_2D_3$ levels, while its oral use results in hypophosphatemia and increased $1,25(OH)_2D_3$ levels. The evidence on the effect of ethanol and commonly-used agents such as heparin, lithium, theophylline, and cimetidine on vitamin D metabolism is inconclusive. More careful screening of susceptible individuals, such as those with poor nutritional status or who lack exposure to ultraviolet light, is likely indicated. Further attention must be directed towards new drugs, such as ezetimibe and low molecular weight heparin, which have the potential to influence vitamin D absorption and metabolism, respectively.

References

1. Garabedian M, Holick MF, DeLuca HF, Boyle IT 1976 Control of 25-hydroxycholecalciferol metabolism by parathyroid glands. Proc Natl Acad Sci USA **69**:1673–1676.
2. Fraser DR, Kodicek E 1973 Regulation of 25-hydroxycholecalciferol-1-hydroxylase activity in kidney by parathyroid hormone. Nature (New Biol) **241**:163–166.
3. Henry HL, Midgett RJ, Norman AW 1974 Regulation of 25-hydroxyvitamin D_3-l-hydroxylase *in vivo*. J Biol Chem **249**:7584–7592.

4. Henry HL 1979 Regulation of the hydroxylation of 25-hydroxyvitamin D_3 *in vivo* and in primary cultures of chick kidney cells. J Biol Chem **254**:2722–2729.
5. Booth BE, Tsai HC, Morris RC Jr 1977 Parathyroidectomy reduces 25-hydroxyvitamin D_3-1a-hydroxylase activity in the hypocalcemic vitamin D–deficient chick. J Clin Invest **60**:1314–1320.
6. Mitlak BH, Williams DC, Bryant HU, Paul DC, Neer RM 1992 Intermittent administration of bovine PTH(l-34) increases serum 1,25-dihydroxyvitamin D concentrations and spinal bone density in senile (23 month) rats. J Bone Miner Res **7**:479–484.
7. Shigematsu T, Horiuchi N, Ogura Y, Miyahara T, Suda T 1986 Human parathyroid hormone inhibits renal 24-hydroxylase activity of 25-hydroxyvitamin D_3 by a mechanism involving adenosine 3′,5′-monophosphate in rats. Endocrinology **118**:1583–1589.
8. Rasmussen H, Wong M, Bikle D, Goodman DBP 1972 Hormonal control of the renal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol. J Clin Invest **51**:2502–2504.
9. Larkins RG, MacAuley SJ, Rapoport A, Mertin TJ, Tulloch BR, Byfield PGH, Matthews EW, Maclntyre I 1974 Effects of nucleotides, hormones, ions, and 1,25-dihydroxycholecalciferol on 1,25-dihydroxycholecalciferol production in isolated chick renal tubules. Clin Sci Mol Med **46**:569–582.
10. Trechsel U, Bonjour J-P, Fleisch H 1979 Regulation of the metabolism of 25-hydroxyvitamin D_3 in primary cultures of chick kidney cells. J Clin Invest **64**:206–217.
11. Juan D, DeLuca HF 1977 The regulation of 24,25-dihydroxyvitamin D_3 production in cultures of monkey kidney cells. Endocrinology **101**:1184–1193.
12. Armbrecht HJ, Wongsurawat N, Zenser T, Davis BB 1982 Differential effects of parathyroid hormone on the renal 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 production of young and adult rats. Endocrinology **111**: 1339–1344.
13. Armbrecht HJ, Wongsurawat N, Zenser TV, Davis BB 1984 Effect of PTH and $1,25(OH)_2D_3$ on renal $25(OH)D_3$ metabolism, adenylate cyclase, and protein kinase. Am J Physiol **246**:E102–E107.
14. Rost CR, Bickle DD, Kaplan RA 1981 *In vitro* stimulation of 25-hydroxycholecalciferol 1a-hydroxylation by parathyroid hormone in chick kidney slices: Evidence for a role for adenosine 3′,5′-monophosphate. Endocrinology **108**: 1002–1006.
15. Fukase M, Birge SJ Jr, Rifas L, Avioli LV, Chase LR 1982 Regulation of 25 hydroxyvitamin D_3 1-hydroxylase in serum-free monolayer culture of mouse kidney. Endocrinology **110**:1073–1075.
16. Murer H, Werner A, Reshkin S, Wuarin F, Biber J 1991 Cellular mechanisms in proximal tubular reabsorption of phosphate. Am J Physiol **260**:C885–C899.
17. Ro H-K, Tembe V, Krug T, Yang P-YJ, Bushinsky DA, Favus MJ 1990 Acidosis inhibits $1,25(OH)_2D_3$ but not cAMP production in response to parathyroid hormone in the rat. J Bone Miner Res **5**:273–278.
18. Chase LR, Aurbach GD 1967 Parathyroid function and the renal excretion of 3′,5′-adenylic acid. Proc Natl Acad Sci **58**:518–525.
19. Horiuchi N, Suda T, Takahashi H, Shimazawa E, Ogata E 1977 *In vivo* evidence for the intermediary role of 3′,5′-cyclic AMP in parathyroid hormone-induced stimulation of 1a,25-dihydroxyvitamin D_3 synthesis in rats. Endocrinology **101**:969–974.
20. Armbrecht HJ, Forte LR, Wongsurawat N, Zenser TV, Davis BB 1984 Forskolin increases 1,25-dihydroxyvitamin D_3 production by rat renal slices *in vitro*. Endocrinology **114**: 644–649.
21. Henry HL 1985 Parathyroid hormone modulation of 25-hydroxyvitamin D_3 metabolism by cultured chick kidney cells is mimicked and enhanced by forskolin. Endocrinology **116**:503–510.
22. Seamon KB, Daly JW 1982 Forskolin: A unique diterpene activator of cyclic AMP generating systems. J Cyclic Nucleotide Res **71**:201.
23. Kong XF, Zhu KH, Peo YL, Jackson DM, Holcih MF 1999 Molecular cloning, characterization, and promotor analysis of human 25-hydroxyvitamin D_3-1-alpha-hydroxylase gene. PNAS **36**:6988–6993.
24. Murayama A, Takeyama K, Kitanaka S, Kodera Y, Kawaguchi Y, Hosoya T, Kato S 1999 Positive and negative regulations of the renal 25-hydroxyvitamin D_3 1-alpha-hydroxylase gene by parathyroid hormone, calcitonin and 1 alpha,$25(OH)_2D_3$ in intact animals. Endocrinology **140**:2224–2231.
25. Shinki T, Jin CH, Nishimura A, Nagai Y, Ohyama Y, Noshiro M, Okuda K, Suda T 1992 Parathyroid hormone inhibits 25-hydroxyvitamin D_3-24-hydroxylase mRNA expression stimulated by 1a,25-dihydroxyvitamin D_3 in rat kidney but not in intestine. J Biol Chem **267**:13757–13762.
26. Zierold C, Mings JA, DeLuca HF 2001 Parathyroid hormone regulates 25-hydroxyvitamin D_3-24-hydroxylase mRNA by altering its stability. PNAS **98**:13572–13576.
27. Janulis M, Tembe V, Favus MJ 1992 Role of protein kinase C in parathyroid hormone stimulation of renal 1,25-dihydroxyvitamin D_3 secretion. J Clin Invest **90**:2278–2283.
28. Ro HK, Tembe V, Favus MJ 1992 Evidence that activation of protein kinase-C can stimulate 1,25-dihydroxyvitamin D_3 secretion by rat proximal tubules. Endocrinology **131**: 1424–1428.
29. Friedlander J, Janulis M, Tembe V, Ro HK, Wong M-S, Favus MJ 1994 Loss of parathyroid hormone-stimulated 1,25-dihydroxyvitamin D_3 production in aging does not involve protein kinase A or C pathways. J Bone Miner Res **9**:339–345.
30. Kaplan RK, Haussler MR, Deftos LJ, Bone H, Pak CYC 1977 The role of 1a,25-dihydroxyvitamin D in the mediation of intestinal hyperabsorption of calcium in primary hyperparathyroidism and absorptive hypercalciuria. J Clin Invest **59**:756–760.
31. Mawer EB, Backhouse J, Hill LF, Lumb GA, De Silva P, Taylor CM, Stanbury SW 1975 Vitamin D metabolism and parathyroid function in man. Clin Sci Mol Med **48**:349–365.
32. Brown DJ, Spanos E, Maclntyre I 1980 Role of pituitary hormone in regulating renal vitamin D metabolism in man. Br Med J **1**:277–278.
33. Nakayama K, Fukumoto S, Takeda S, Takeuchi Y, Ishikawa T, Miura M, Hata K, Hane M, Tamura Y, Tanaka Y, Kitaoka M, Obara T, Ogata E, Matsumoto T 1996 Differences in bone and vitamin D metabolism between primary hyperparathyroidism and malignancy-associated hypercalcemia. J Clin Endocrinol Metab **81**:607–611.
34. Bilezikian JP, Canfield RE, Jacobs TP, Polay JS, D'Adamo AP, Eisman JA, DeLuca HF 1978 Response of 1a,25-dihydroxyvitamin D_3 to hypocalcemia in human subjects. N Engl J Med **299**:437–441.
35. Rosen JF, Fleischman AR, Finberg L, Eisman J, DeLuca HF 1977 1,25-Dihydroxycholecalciferol: Its use in the long-term management of idiopathic hypoparathyroidism in children. J Clin Endocrinol Metab **45**:457–468.

36. Kooh SW, Fraser D, DeLuca HF, Holick MF, Belsey RE, Clark MB, Murray TM 1975 Treatment of hypoparathyroidism and pseudohypoparathyroidism with metabolites of vitamin D: Evidence for impaired conversion 25-hydroxyvitamin D to 1a,25-dihydroxyvitamin D. N Engl J Med **293**:840–844.
37. Davies M, Taylor CM, Hill LF, Stanbury SW 1977 1,25-Dihydroxycholecalciferol in hypoparathyroidism. Lancet **1**:55–59.
38. Drezner MK, Neelon FA, Haussler M, McPherson HT, Lebovitz HE 1976 1,25-Dihydroxycholecalciferol deficiency: The probable cause of hypocalcemia and metabolic bone disease in pseudohypoparathyroidism. J Clin Endocrinol Metab **42**:621–628.
39. Brancaccio D, Cozzolino M, Gorio A, Di Giulio AM, Gallieni M 2002 Bone disease in uremic patients: advances in PTH suppression. J Nephrol **15**(Suppl 6):S86–S93.
40. Bilezikian JP 1990 Parathyroid hormone-related peptide in sickness and in health. N Engl J Med **322**:1151–1153.
41. Orloff JJ, Kats Y, Urena P, Schipani E, Vasavada RC, Philbrick WM, Behal A, Abou-Samra A-B, Segre GV, Juppner H 1995 Further evidence for a novel receptor for amino-terminal parathyroid hormone-related protein on keratinocytes and squamous carcinoma cell lines. Endocrinology **136**:3016–3023.
42. Strewler GJ, Stern PH, Jacobs JW, Eveloff J, Klein RF, Leung SC, Rosenblatt M, Nissenson RA 1987 Parathyroid hormone-like protein from human renal carcinoma cells. Structural and functional homology with parathyroid hormone. J Clin Invest **80**:1803–1807.
43. Yates AJP, Gutierrez GE, Smolens P, Travis PS, Katz MS, Auf-demorte TB, Boyce BF, Hymer TK, Poser JW, Mundy GR 1988 Effects of a synthetic peptide of a parathyroid hormone-related protein on calcium homeostasis, renal tubular calcium reabsorption, and bone metabolism *in vivo* and *in vitro* in rodents. J Clin Invest **81**:932–938.
44. Stewart AF, Horst R, Deftos LJ, Cadman EC, Lang R, Broadus AE 1980 Biochemical evaluation of patients with cancer-associated hypercalcemia. N Engl J Med **303**: 1377–1383.
45. Ralston SH, Cowan RA, Robertson RA, Gardner MD, Boyle IT 1984 Circulating vitamin D metabolites and hypercalcemia of malignancy. Acta Endocrinol **106**:556–563.
46. Syed MA, Horowitz MJ, Tedesco MB, Garcia-Ocana A, Wisniewksi SR, Stewart AF 2001 Parathyroid hormone related protein (1-36) stimulates renal tubular reabsorption in normal human volunteers: implications for the pathogenesis of humoral hypercalcemia of malignancy. J Clin Endocrinol Metab **55**:219–227.
47. Horowitz MJ, Tedesco MB, Sereika SM, Hollis BW, Garcia-Ocana A, Stewart AF 2003 Direct comparison of sustained infusion of human parathyroid-related protein versus hPTH on serum calcium, plasma 1,25 dihydroxyvitamin D concentration and calcium excretion in healthy volunteers. J Clin Endocrinol Metab **88**:1603–1609.
48. Stewler GJ 2000 The physiology of parathyroid hormone-related protein. N Engl J Med **342**:177–185.
49. Insogna KL, Stewart AF, Vignery AMC, Weir EC, Namnum PA, Baron RE, Kirkwood JM, Deftos LM, Broadus AE 1984 Biochemical and histomorphometric characterization of a rat model for humoral hypercalcemia of malignancy. Endocrinology **114**:888–896.
50. Horiuchi N, Caulfield MP, Fisher JE, Goldman ME, McKee RL, Reagan JE, Levy JJ, Nutt RF, Rodan SB, Schofield TL, Clemens TL, Rosenblatt M 1987 Similarity of synthetic peptide from human tumor to parathyroid hormone *in vivo* and *in vitro*. Science **238**:1566–1568.
51. Walker AT, Stewart AF, Korn EA, Shiratori T, Mitnick MA, Carpenter TO 1990 Effect of parathyroid hormone-like peptides on 25-hydroxyvitamin D-1a-hydroxylase activity in rodents. Am J Physiol **258**:E297–E303.
52. Michigami T, Yamato H, Suzuki H, Nagai-Itagaki Y, Sato K 2001 Conflicting actions of parathyroid hormone-related protein and serum calcium as regulators of 25-hydroxyvitamin D_3-1α-hydroxylase expression in a nude rat model of humoral hypercalcemia of malignancy. J Endocrinol **171**:249–257.
53. Fukumoto S, Matsumoto T, Yamoto H, Kawashima H, Ueyama Y, Tamaoki N, Ogata E 1989 Suppression of serum 1,25-dihydroxyvitamin D in humoral hypercalcemia of malignancy is caused by an elaboration of a factor that inhibits renal 1,25-dihydroxyvitamin D_3 production. Endocrinology **124**:2057–2062.
54. Schilling T, Pecherstorfer M, Blind E, Leidig G, Ziegler R, Raue F 1993 Parathyroid hormone-related protein (PTHrP) does not regulate 1,25-dihydroxyvitamin D serum levels in hypercalcemia of malignancy. J Clin Endocrinol Metab **76**:801–803.
55. Everhart-Caye M, Inzucchi SE, Guinness-Henry J, Mitnick MA, Stewart AF 1996 Parathyroid hormone (PTH)-related protein(l-36) is equipotent to PTH(l-34) in humans. J Clin Endocrinol Metab **81**:199–208.
56. Fraher LJ, Hodsman AB, Jonas K, Saunders D, Rose CI, Henderson JE, Hendy GN, Goltzman D 1992 A comparison of the *in vivo* biochemical responses to exogenous parathyroid hormone-(1-34) [PTH-(1-34)] and PTH-related peptide-(l-34) in man. J Clin Endocrinol Metab **75**: 417–423.
57. Horowitz MJ, Tedesco MB, Sereika SM, Hollis BW, Garcia-Ocana A, Stewart AF 2003 Direct comparison of sustained infusion of human parathyroid-related protein versus hPTH on serum calcium, plasma 1,25 dihydroxyvitmain D concentration and calcium excretion in healthy volunteers. J Clin Endocrinol Metab **88**:1603–1609.
58. Mundy GR 1988 Hypercalcemia of malignancy revisited. J Clin Invest **82**:1–6.
59. Kovac CS, Kronenberg HM 1997 Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation. Endocr Rev **18**:832–872.
60. Mather KJ, Chik CL, Corenblum B 1999 Maintenance of serum calcium by parathyroid-related peptide during lactation in a hypoparathyroid patient. J Clin Endocr Metab **84**:424–426.
61. Galante L, Colston KW, Macauley SJ, Maclntyre I 1972 Effect of calcitonin on vitamin D metabolism. Nature **238**:271–273.
62. Lorenc R, Tanaka Y, DeLuca HF, Jones G 1977 Lack of effect of calcitonin on the regulation of vitamin D metabolism in the rat. Endocrinology **100**:468–472.
63. Horiuchi N, Takahashi H, Matsumoto T, Takahashi N, Shimazawa E, Suda T, Ogata E 1979 Salmon calcitonin-induced stimulation of 1a,25-dihydroxycholecalciferol synthesis in rats involving a mechanism independent of adenosine 3′,5′-cyclic monophosphate. Biochem J **184**:269–275.
64. Jaeger P, Jones W, Clemens TL, Hayslett JP 1986 Evidence that calcitonin stimulates 1,25-dihydroxyvitamin D production and intestinal absorption of calcium *in vivo*. J Clin Invest **78**:456–461.

65. Shinki T, Ueno Y, DeLuca HF, Suda T 1999 Calcitonin is a major regulator for the expression of renal 25-hydroxyvitamin D_3-1alpha-hydroxylase gene in normocalcemic rats. Proc Natl Acad Sci USA **96**:8253–8258.
66. Beckman MJ, Goff JP, Reinhardt TA, Beitz DC, Horst RL 1994 *In vivo* regulation of rat intestinal 24-hydroxylase: Potential new role of calcitonin. Endocrinology **135**: 1951–1955.
67. Armbrecht HJ, Wongsurawat N, Paschal RE 1987 Effect of age on renal responsiveness to parathyroid hormone and calcitonin in rats. J Endocrinol **114**:173–178.
68. Kawashima H, Torikai S, Kurokawa K 1981 Calcitonin selectively stimulates 25-hydroxyvitamin D_3-1a-hydroxylase in proximal straight tubule of rat kidney. Nature **291**: 327–329.
69. Emmertsen K, Melsen F, Mosekilde L, Lund B, Lund B, Sorensen OH, Nielsen HE, Solling H, Hansen HH 1981 Altered vitamin D metabolism and bone remodeling in patients with medullary thyroid carcinoma and hypercalcitonemia. Metab Bone Dis Related Res **4**:17–23.
70. Aloia JF, Vaswani A, Kapoor A, Yeh JK, Cohn SH 1985 Treatment of osteoporosis with calcitonin, with and without growth hormone. Metabolism **34**:124–129.
71. Hartwell D, Hassager C, Overgaard K, Riis BJ, Podenphant J, Christiansen C 1990 Vitamin D metabolism in osteoporotic women during treatment with estrogen, and anabolic steroid, or calcitonin. Acta Endocrinol **122**:715–721.
72. Thamsborg G, Jensen JEB, Kollerup G, Hauge EM, Melsen F, Sorensen OH 1996 Effect of nasal salmon calcitonin on bone remodeling and bone mass in postmenopausal women. Bone **18**:207–212.
73. Thamsborg G, Storm TL, Daugaard H, Schiffer S, Sorensen OH 1991 Circulating levels of calciotropic hormones during treatment with nasal salmon calcitonin. Acta Endocrinol **125**:127–131.
74. Nunziata V, Giannattasio R, Di Giovanni G, Lettera AM, Nunziata CA 1993 Vitamin D status in Paget's bone disease. Effect of calcitonin therapy. Clin Orthop Related Res **293**:366–371.
75. Devlin RD, Gutteridge DH, Prince RL, Retallack RW, Worth GK 1990 Alterations in vitamin D metabolites during treatment of Paget's disease of bone with calcitonin or etidronate. J Bone Miner Res **5**:1121–1126.
76. Mainoya JR 1975 Effects of bovine growth hormone, human placental lactogen, and ovine prolactin on intestinal fluid and ion transport in the rat. Endocrinology **96**:1165.
77. Henneman PH, Forbes AP, Moldawer M, Dimpsey EF, Carrol EL 1962 Effects of human growth hormone in man. J Clin Invest **39**:1223.
78. Finkelstein JD, Schachter D 1962 Active transport of calcium by intestine: Effects of hypophysectomy and growth hormone. Am J Physiol **203**:873.
79. Spanos E, Barret D, Maclntyre I, Pike JW, Safilian EF, Haussler MR 1978 Effect of growth hormone on vitamin D metabolism. Nature **273**:246–247.
80. Fontaine O, Pavlovitch H, Balsan S 1978 25-Hydroxycholecalciferol metabolism in hypophysectomized rats. Endocrinology **102**:1822–1826.
81. Spencer EM, Tobiassen O 1981 The mechanism of the action of growth hormone on vitamin D metabolism in the rat. Endocrinology **108**:1064–1070.
82. Pahuja DN, DeLuca HF 1981 Role of the hypophysis in the regulation of vitamin D metabolism. Mol Cell Endocrinol **23**:345–350.
83. Gray RW, Garthwaite TL 1985 Activation of renal 1,25-dihydroxyvitamin D_3 synthesis by phosphate deprivation: Evidence for a role for growth hormone. Endocrinology **116**:189–193.
84. Denis I, Thomasset M, Pointillart A 1994 Influence of exogenous porcine growth hormone on vitamin D metabolism and calcium and phosphorus absorption in intact pigs. Calcif Tissue Int **54**:489–492.
84. Wu S, Grieff M, Brown AJ 1997 Regulation of renal vitamin D-24-hydroxylase by phosphate: effects of hypophysectomy, growth hormone, and insulin-like growth factor I. Biochem Biophys Res Commun; **233**:813–817.
85. Eskildsen PC, Lund B, Sorensen OH, Lund B, Bishop JE, Norman AW 1979 Acromegaly and vitamin D metabolism: Effect of bromocriptine treatment. Endocrinology **49**:484–486.
85. Lund B, Eskildsen PC, Lund B, Norman AW, Sorensen OH 1981 Calcium and vitamin D metabolism in acromegaly. Acta Endocrinol **96**:444–450.
86. Bijlsma JWJ, Nortier JWR, Duursma SA, Croughs RJM, Bosch R, Thijssen JHH 1983 Changes in bone metabolism during treatment of acromegaly. Acta Endocrinol **104**:153–159.
87. Brixen K, Nielsen HK, Bouillon R, Flyvberg A, Mosekilde L 1992 Effects of short-term growth hormone treatment on PTH, calcitriol, thyroid hormones, insulin, and glucagon. Acta Endocrinol **127**:331–336.
88. Marcus R, Butterfield G, Holloway L, Gilliland L, Baylink DJ, Hintz RL, Sherman BM 1990 Effects of short-term administration of recombinant human growth hormone to elderly people. J Clin Endocrinol Metab **70**:519–527.
89. Harbison MD, Gertner JM 1990 Permissive action of growth hormone on the renal response to dietary phosphorus deprivation. J Clin Endocrinol Metab **70**:1035–1040.
90. Chipman JJ, Zerwekh J, Nicar M, Marks J, Pak CYC 1980 Effect of growth hormone administration: Reciprocal changes in serum 1a,25-dihydroxyvitamin D and intestinal calcium absorption. J Clin Endocrinol Metab **51**:321–324.
91. Burstein S, Chen IW, Tsang RC 1983 Effects of growth hormone replacement therapy on 1,25-dihydroxyvitamin D and calcium metabolism. J Clin Endocrinol Metab **56**:1246–1251.
92. Gertner JM, Horst RL, Broadus AE, Rasmussen H, Genel M 1979 Parathyroid function and vitamin D metabolism during human growth hormone replacement. J Clin Endocrinol Metab **49**:185–188.
93. Panczyk-Tomaszewska M, Ziolkowska H, Debinski A, Sieniawska M 2000 Vitamin D metabolite requirements in dialysed children receiving recombinant human growth hormone. Nephrol Dial Transplant **15**:375–378.
94. Beshyah SA, Thomas E, Kyd P, Sharp P, Fairney A, Johnson DG 1994 The effect of growth hormone replacement therapy in hypopituitary adults on calcium and bone metabolism. Clin Endocrinol **40**:383–391.
95. Wei S, Tanaka H, Kubo T, Ono T, Kanzaki S, Seino Y 1997 Growth hormone increases 1,25 dihydroxyvitamin D levels and decreases 24,25-dihyroxyvitamin D levels in children with growth hormone deficiency. Eur J Endocrinol **136**:30–32.
96. Bianda T, Hussain MA, Glatz Y, Bouillon R, Froesch ER, Schmid C 1997 Effects of short-term insulin-like growth factor–1 or growth hormone on bone turnover, renal phosphate reabsorption, and 1,25 dihydroxyvitamin D_3 production in healthy man. J Intern Med **241**:143–150.
97. Wright NM, Papadea N, Wentz B, Hollis B, Bell NH 1997 Increased serum 1,24 dihydroxyvitamin D after growth hormone administration is not parathyroid hormone medicated. Calcif Tissue Int **61**:101–103.

98. Bickle DD, Spencer EM, Burke WH, Rost CR 1980 Prolactin but not growth hormone stimulates 1,25-dihydroxyvitamin D_3 production by chick renal preparations *in vitro*. Endocrinology **107**:81–84.
99. Hammerman MR, Gavin III JR 1986 Binding of IGF-I and IGF-I-stimulated phosphorylation in canine renal basolateral membrane. Am J Physiol **251**:E32–E41.
100. Condamine L, Vztovsnik F, Friedlander G, Menaa C, Garabedian M 1994 Local action of phosphate depletion and insulin-like growth factor 1 on *in vitro* production of 1,25-dihydroxyvitamin D by cultured mammalian kidney cells. J Clin Invest **94**:1673–1679.
101. Halloran BP, Spencer EM 1988 Dietary phosphate and 1,25-dihydroxyvitamin D metabolism: Influence of insulin-like growth factor 1. Endocrinology **123**:1225–1229.
102. Caverzasio J, Motessuit C, Bonjour JP 1990 Stimulatory effect of insulin-like growth factor-1 on renal Pi transport and plasma 1,25-dihydroxyvitamin D_3. Endocrinology **127**: 453–459.
103. Gray RW 1987 Evidence that somatomedins mediate the effect of hypophosphatemia to increase serum 1,25-dihydroxyvitamin D_3 levels in rats. Endocrinology **121**:504–512.
104. Nesbitt T, Drezner MK 1993 Insulin-like growth factor-1 regulation of renal 25-hydroxyvitamin D-1-hydroxylase activity. Endocrinology **132**:133–138.
105. Spanos E, Pike JW, Haussler MR, Colston KW, Evans IMA, Goldner AM, McCain TA, Maclntyre I 1976 Circulating 1a,25-dihydroxyvitamin D in the chicken: Enhancement by injection of prolactin and during egg laying. Life Sci **19**:1751–1756.
106. Spanos E, Brown DJ, Stevenson JC, Maclntyre I 1981 Stimulation of 1,25-dihydroxycholecalciferol production by prolactin and related peptides in intact renal cell preparations *in vitro*. Biochim Biophys Acta **672**:7–15.
107. Spanos E, Colston KW, Evans IMS, Galante LS, Macauley SJ, Maclntyre I 1976 Effect of prolactin on vitamin D metabolism. Mol Cell Endocrinol **5**:163–167.
108. Matsumoto T, Horiuchi N, Suda T, Takahashi H, Shimazawa E, Ogata E 1979 Failure to demonstrate stimulatory effect of prolactin on vitamin D metabolism in vitamin-D–deficient rats. Metabolism **28**:925–927.
109. Kenny AD, Baksi SN, Galli-Galardo SM 1977 Vitamin D metabolism in amphibia and fish. Fed Proc **36**:1097.
110. Mainoya JR 1975 Further studies on the action of prolactin on fluid and ion absorption by the rat jejunum. Endocrinology **96**:1158–1164.
111. Reichlin S 1974 Neuroendocrinology: Lactation. In: William RH (ed) Textbook of Endocrinology. Saunders: Philadelphia, Pennsylvania, pp. 800–801.
112. Boass A, Toverud SA, McCain TA, Pike JW, Haussler MR 1977 Elevated serum levels of 1a,25-dihydroxycholecalciferol in lactating rats. Nature **267**:630–632.
113. Maclntyre I, Colston KW, Szelke M, Spanos E 1978 A survey of the hormonal factors that control calcium metabolism. Ann NY Acad Sci **307**:345–355.
114. Kumar R, Cohen WR, Silva P, Epstein FH 1979 Elevated 1,25-dihydroxyvitamin D plasma levels in normal human pregnancy and lactation. J Clin Invest **63**:342–344.
115. Lund B, Seines A 1979 Plasma 1,25-dihydroxyvitamin D levels in pregnancy and lactation. Acta Endocrinol **92**:330–335.
116. DeSantiago S, Alonso L, Halhali A, Larrea F, Isiard F, Bourges H 2002 Negative calcium balance during lactation in rural Mexican women. Am J Clin Nutr **76**: 845–851.
117. Cundy T, Raining SA, Guilland-Cumming DF, Butler J, Kanis JA 1987 Remission of hypoparathyroidism during lactation: Evidence for a physiological role for prolactin in the regulation of vitamin D metabolism. Clin Endocrinol **26**:667–674.
118. Sowers M, Zhang D, Hollis BW, Shapiro B, Janney CA, Crutchfield M, Schork MA, Stanczyk F, Randolph J 1998 Role of calcitropic hormones in calcium mobilization of lactation. Am J Clin Nutr **67**:284–291.
119. Ardawi MS, Nasrat HA, BA'Aqueel HS 1997 Calcium-regulating hormones and parathyroid-related peptide in normal human pregnancy and postpartum. A longitudinal study. Eur J Endocrinol **137**:402–409.
120. Kumar R, Abboud CF, Riggs BL 1980 The effect of elevated prolactin levels on plasma 1,25-dihydroxyvitamin D and intestinal absorption of calcium. Mayo Clin Proc **55**:51–53.
121. Adams ND, Garthwaite TL, Gray RW, Hagen TC, Lemann J Jr 1979 The interrelationships among prolactin, 1,25-dihydroxyvitamin D, and parathyroid hormone in humans. J Clin Endocrinol Metab **49**:628–630.
122. Klibanski A, Neer R, Beitins I, Ridgeway E, McArthur J 1981 Decreased bone density in hyperprolactinemic women. N Engl J Med **303**:1511.
123. Schlechte JA, Sherman B, Martin R 1983 Bone density in amenorrheic women with and without hyperprolactinemia. J Clin Endocrinol Metab **56**:1120–1123.
124. Koppelman MCS, Kurtz DW, Morrish KA, Bou E, Susser JK, Shapiro JR, Loriaux DL 1984 Vertebral body bone mineral content in hyperprolactinemic women. J Clin Endocrinol Metab; **59**:1050–1053.
125. Bouillon R 1991 Diabetic bone disease. Calcif Tissue Int **49**:155–160.
126. Schneider LE, Schedl HP 1972 Diabetes and intestinal calcium absorption in the rat. Am J Physiol **223**:1319–1323.
127. Schneider LE, Wilson HD, Schedl HP 1974 Effects of alloxan diabetes on duodenal calcium-binding protein in the rat. Am J Physiol **227**:832–838.
128. Schneider LE, Omdahl EJ, Schedl HP 1976 Effects of vitamin D and its metabolites on calcium transport in the diabetic rat. Endocrinology **99**:793–799.
129. Schneider LE, Nowosielski LM, Schedl HP 1977 Insulin-treatment of diabetic rats: Effects on duodenal calcium absorption. Endocrinology **100**:67–73.
130. Schneider LE, Schedl HP, McCain T, Haussler MR 1977 Experimental diabetes reduces circulating 1,25-dihydroxyvitamin D in the rat. Science **196**:1452–1454.
131. Schneider LE, Hargis EK, Schedl HP, Williams GA 1974 Parathyroid function in the alloxan diabetic rat. Endocrinology **95**:749.
132. Schedl HP, Heath H, Wenger J 1978 Serum calcitonin and parathyroid hormone in experimental diabetes: Effects of insulin treatment. Endocrinology **103**:1368–1373.
133. Wongsurawat N, Armbrecht HJ, Zenser TV, Davis BB, Thomas ML, Forte LR 1983 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 production by isolated renal slices is modulated by diabetes and insulin in the rat. Diabetes **32**:302–306.
134. Glajchen N, Epstein S, Thomas S, Fallen M, Chakrabarti S 1988 Bone mineral metabolism in experimental diabetes mellitus: Osteocalcin as a measure of bone remodeling. Endocrinology **123**:290–295.
135. Shires R, Teitelbaum SL, Bergfeld MA, Fallen MD, Slatopolsky E, Avioli LV 1981 The effect of streptozotocin-induced chronic diabetes mellitus on bone and mineral homeostasis in the rat. J Clin Lab Med **97**:231–240.

136. Spencer EM, Khalil M, Tobiassen O 1980 Experimental diabetes in the rat causes an insulin-reversible decrease in renal 25-hydroxyvitamin D_3-1a-hydroxylase activity. Endocrinology **107**:300–305.
137. Takeshita N, Yoshino T, Mutoh S, Yamaguchi I 1994 Possible involvement of vitamin D_3-deficiency and relatively enhanced bone resorption in the development of bone loss in streptozotocin-induced diabetic rats. Life Sci **55**:291–299.
138. Hough S, Fausto A, Sonn Y, Dong Jo OK, Birge SJ, Avioli LV 1983 Vitamin D metabolism in the chronic streptozotocin-induced diabetic rat. Endocrinology **113**:790–796.
139. Epstein S, Takizawa M, Stein B, Katz IA, Joffe II, Romero DF, Liang XG, Ke HZ, Jee WSS, Jacobs TW, Berlin J 1994 The effect of cyclosporin A on bone mineral metabolism in experimental diabetes mellitus in the rat. J Bone Miner Res **9**:557–566.
140. Sulimovici S, Roginsky MS 1980 Hepatic metabolism of vitamin D_3 in streptozotocin-induced diabetic rat. Acta Endocrinol **93**:346–350.
141. Matsumoto T, Kawanobe Y, Ezawa I, Shibuya N, Hata K, Ogata E 1986 Role of insulin in the increase in serum 1,25-dihydroxyvitamin D concentrations in response to phosphorus deprivation in streptozotocin-induced diabetic rats. Endocrinology **118**:1440–1444.
142. Nyomba B, Bouillon R, Lissens W, Van Baelen H, De Moor P 1985 1,25-Dihydroxyvitamin D and vitamin D–binding protein are both decreased in streptozotocin-diabetic rats. Endocrinology **116**:2483–2488.
143. Henry HL 1981 Insulin permits parathyroid hormone stimulation of 1,25-dihydroxyvitamin D_3 production in cultured kidney cells. Endocrinology **108**:733–735.
144. Sulimovici S, Roginsky MS, Susser F 1981 Nephrogenous cyclic AMP in streptozotocin-induced diabetic rat. Biochem Biophys Res Commun **100**:471–477.
145. Ambrecht HJ, Wongsurawat VJ, Hodam TL, Wongsurawat N 1996 Insulin markedly potentiates the capacity of parathyroid hormone to increase expression of 25-hydroxyvitamin D_3 24-hydroxylase in rat osteoblastic cells in the presence of 1,25 dihydroxyvitamin D_3. FEBS Lett **393**:77–80.
146. Gertner J, Horst R, Tamborlane W 1979 Mineral metabolism and vitamin D status in juvenile diabetics: Changes following normalization of plasma glucose with a portable infusion pump. Diabetes **28**:354.
147. Frazer TE, White NH, Hough S, Santiago JV, McGee BR, Bryce G, Mallon J, Avioli LV 1981 Alterations in circulating vitamin D metabolites in the young insulin–dependent diabetic. J Clin Endocrinol Metab **53**:1154–1159.
148. Nyomba BL, Bouillon R, Bidingija M, Kandjingu K, De Moor P 1986 Vitamin D metabolites and their binding protein in adult diabetic patients. Diabetes **35**:911–915.
149. Heath III H, Lambert PW, Service FJ, Arnaud SB 1979 Calcium homeostasis in diabetes mellitus. J Clin Endocrinol Metab **49**:462–466.
150. Kenny AD 1976 Vitamin D metabolism: Physiological regulation in egg-laying Japanese quail. Am J Physiol **230**:1609–1615.
151. Baksi SN, Kenny AD 1977 Vitamin D_3 metabolism in immature Japanese quail: Effect of ovarian hormones. Endocrinology **101**:1216–1220.
152. Castillo L, Tanaka Y, DeLuca HF, Sunde ML 1977 The stimulation of 25-hydroxyvitamin D_3-1-a-hydroxylase by estrogen. Arch Biochem Biophys **179**:211–217.
153. Tanaka Y, Castillo L, Wineland MJ, DeLuca HF 1978 Synergistic effect of progesterone, testosterone, and estradiol in the stimulation of chick renal 25-hydroxyvitamin D_3-1a-hydroxylase. Endocrinology **103**:2035–2039.
154. Pike JW, Spanos E, Colston KW, Maclntyre I, Haussler MR 1978 Influence of estrogen on renal vitamin D hydroxylases and serum 1a,25$(OH)_2D_3$ in chicks. Am J Physiol **235**: E338–E343.
155. Trechsel U, Bonjour J-P, Fleisch H 1979 Regulation of the metabolism of 25-hydroxyvitamin D_3 in primary cultures of chick kidney cells. J Clin Invest **64**:206–217.
156. Henry HL 1981 25$(OH)D_3$ metabolism in kidney cell cultures: Lack of direct effect of estradiol. Am J Physiol **240**:E119–E124.
157. Baksi SN, Kenny AD 1978 Does estradiol stimulate *in vivo* production of 1,25-dihydroxyvitamin D_3 in the rat. Life Sci **22**:787–792.
158. Kalu DN, Liu CC, Hardin RR, Hollis BW 1989 The aged rat model of ovarian hormone deficiency bone loss. Endocrinology **124**:7–16.
159. Kalu DN, Salerno E, Liu CC, Echon R, Ray M, Garza-Zapata M, Hollis BW 1991 A comparative study of the actions of tamoxifen, estrogen, and progesterone in the oophorectomized rat. Bone Miner **15**:109–124.
160. Arjmandi BH, Hollis BW, Kalu DN 1994 *In vivo* effect of 17/3-estradiol on intestinal calcium absorption in rats. Bone Miner **26**:181–189.
161. Colin EM, Van Den Bemd GJ, Van Aken M Christakos S, De Jonge HR, Deluca HF, Prahl JM, Birkenhager JC, Buurman CJ, Pols HA, Van Leeuwen JP 1999 Evidence for involvement of 17 beta-estradiol in intestinal calcium absorption independent of 1,25 dihydroxyvitamin D3 level in the rat. J Bone Miner Res **14**:57–64.
162. Criddle RA, Zheng MH, Dick IM, Callus B, Prince RL 1997 Estrogen responsiveness of renal calbindin-D28k gene expression in rat kidney. J Cell Biochem **65**:340–348.
163. Liel Y, Shany S, Smirnoff P, Schwartz B 1999 Estrogen increases 1,25 dihydroxyvitamin D receptors expression and bioresponse in the rat duodenal mucosa. Endocrinology **140**: 280–285.
164. Ash SL, Goldin BR 1988 Effects of age and estrogen on renal vitamin D metabolism in the female rat. Am J Clin Nutr **47**:694–699.
165. Ismail F, Epstein S, Fallon MD, Thomas SB, Reinhardt TA 1988 Serum bone gla protein and the vitamin D endocrine system in the oophorectomized rat. Endocrinology **122**:624–630.
166. Duque G, Abdaimi KE, Macoritto M, Miller MM, Kremer R 2002 Estrogens regulate expression and response of 1,25-dihydroxyvitamin D3 receptors in bone cells. Changes with aging and hormone deprivation. Biochem Biophys Res Commun **299**:446–454.
167. Turton CWG, Stamp TCB, Stanley P, Maxwell JD 1977 Altered vitamin D metabolism in pregnancy. Lancet **1**:222–224.
168. Harris SS, Dawson-Hughes B 1998 The association of oral contraceptive use with plasma 25-hydroxyvitamin D levels. J Am Coll Nutr **17**:282–284.
169. Bickle DD, Gee E, Halloran B, Haddad JG 1984 Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. J Clin Invest **74**:1966–1971.
170. Gallagher JC, Riggs BL, Eisman J, Hamstra A, Arnaud SB, DeLuca HF 1979 Intestinal calcium absorption and serum vitamin D metabolites in normal subjects and osteoporotic patients. J Clin Invest **64**:729–736.

171. Lund B, Sorensen OH, Lund B, Agner E 1982 Serum 1,25-dihydroxyvitamin D in normal subjects and in patients with post-menopausal osteopenia. Influence of age, renal function, and oestrogen therapy. Horm Metab Res **14**:271–274.
172. Falch JA, Oftebro H, Haug E 1987 Early postmenopausal bone loss is not associated with a decrease in circulating levels of 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, or vitamin D–binding protein. J Clin Endocrinol Metab **64**:836–841.
173. Gallagher JC 1990 The pathogenesis of osteoporosis. Bone Miner **9**:215–227.
174. Wishart JM, Scopacasa F, Horowitz M, Morris HA, Need AG, Clifton PM, Nordin BE 2000 Effect of perimenopause on calcium absorption: a longitudinal study. Climacteric **3**:102–108.
175. Finkelstein JS, Scheonfeld DA 1999 Effects of gonadal suppression on the regulation of parathyroid hormone and 1,25 dihydroxyvitamin D secretion in women. J Clin Endocrinol Metab **84**:2151–2161.
176. Lore F, Di Cairano G, Signorini AM, Caniggia A 1981 Serum levels of 25-hydroxyvitamin D in postmenopausal osteoporosis. Calcif Tissue Int **33**:467–470.
177. van Hoof HJC, van der Mooren MJ, Swinkels LMJW, Rolland R, Benraad THJ 1994 Hormone replacement therapy increases serum 1,25-dihydroxyvitamin D: A two-year prospective study. Calcif Tissue Int **55**:417–419.
178. van Hoff HJ, van der Mooren MJ, Swinkels LM, Sweep CG, Merkus JM, Benraad TJ 1999 Female sex hormone replacement therapy increases free 1,25 dihydroxyvitamin D3: a one-year perspective study. Clin Endocrinol **50**:511–516.
179. Jeikkinen A, Parviainen MT, Tuppurainen MT, Niskanen L, Komulainen MH, Saarikoski S 1998 Effects of postmenopausal hormone replacement therapy with and without vitamin D3 on circulating levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D. Calcif Tissue Int **62**:26–30.
180. Gallagher JC, Riggs BL, DeLuca HF 1980 Effect of estrogen on calcium absorption and serum vitamin D metabolites in postmenopausal osteoporosis. J Clin Endocrinol Metab **51**:1359–1364.
181. Stock JL, Coderre JA, Mallette LE 1985 Effects of short course of estrogen on mineral metabolism in postmenopausal women. J Clin Endocrinol Metab **61**:595–600.
182. Bouillon R, Van Assche FA, Van Baelen H, Heyns W, De Moor P 1981 Influence of the vitamin D–binding protein on the serum concentration of 1,25-dihydroxyvitamin D_3. Significance of the free 1,25-dihydroxyvitamin D_3 concentration. J Clin Invest **67**:589–596.
183. Selby PL, Peacock M, Barkworth SA, Brown WB, Taylor GA 1985 Early effects of ethinyloestradiol and norethisterone treatment in postmenopausal women on bone resorption and calcium hormones. Clin Sci **69**:265–271.
184. Cheesma C, Grant BF, Marcus R 1989 Effects of estrogen on circulating "free" and total 1,25-dihydroxyvitamin D and on the parathyroid-vitamin D axis in postmenopausal women. J Clin Invest **83**:537–542.
185. Vanderschueren D, Van Herck E, Suiker AMH, Visser WJ, Schot LPC, Bouillon R 1992 Bone and mineral metabolism in aged male rats: Short- and long-term effects of androgen deficiency. Endocrinology **130**(No. 5):2906–2916.
186. Bouillon R, Vandoren G, Van Baelen H, De Moor P 1978 Immunochemical measurement of the vitamin D-binding protein in rat serum. Endocrinology **102**:1710–1715.
187. Nyomba BL, Bouillon R, DeMoor P 1987 Evidence for an interaction of insulin and sex steroids in the regulation of vitamin D metabolism in the rat. J Endocrinol **115**:295–301.
188. Hagenfeldt Y, Eriksson H, Bjorkhem I 1989 Stimulatory effect of testosterone on renal 25-hydroxyvitamin D_3 1a-hydroxylase in guinea pig. Biochim Biophys Acta **1002**:84–88.
189. Otremski I, Lev-Ran M, Salama R, Edelstein S 1997 The metabolism of vitamin D3 in response to testosterone. Calcif Tissue Int **60**:485–487.
190. Ohata M, Sakagami Y, Fujita T 1977 Elevation of serum 25-hydroxycalciferol levels in androgen-treated and ultraviolet-irradiated rats. Endocrinol Jpn **24**:519–521.
191. Hagenfeldt Y, Linde K, Sjoberg HE, Zumkeller W, Arver S 1992 Testosterone increases serum 1,25-dihydroxyvitamin D and insulin-like growth factor-1 in hypogonadal men. Int J Androl **15**:93–102.
192. Morley JE, Perry III HM, Kaiser FE, Kraenzle D, Jensen J, Houston K, Mattammal M, Perry HM Jr 1993 Effects of testosterone replacement therapy in old hypogonadal males: A preliminary study. J Am Geriatr Soc **41**:149–152.
193. Jackson JA, Kleerekoper M, Parfitt M, Sudhaker Rao D, Villanueva AR, Frame B 1987 Bone histomorphometry in hypogonadal and eugonadal men with spinal osteoporosis. J Clin Endocrinol Metab **65**:53–58.
194. Finkelstein JS, Klibanski A, Neer RM, Doppelt SH, Rosenthal DI, Segre GV, Crowley WFJ 1989 Increase in bone density during treatment of men with idiopathic hypogonadotropic hypogonadism. J Clin Endocrinol Metab **69**(No. 4):776–783.
195. Francis RM, Peacock M, Aaron JE, Selby PL, Taylor GA, Thompson J, Marshall DH, Horsman A 1986 Osteoporosis in hypogonadal men: Role of decreased plasma 1,25-dihydroxyvitamin D, calcium malabsorption, and low bone formation. Bone **7**:261–268.
196. Hagenfeldt Y, Carlstrom K, Berlin T, Stege R 1991 Effects of orchidectomy and different modes of high-dose estrogen treatment on circulating "free" and total 1,25-dihydroxyvitamin D in patients with prostatic cancer. J Steroid Biochem Mol Biol **39**:155–159.
197. Krabbe S, Hummer L, Christiansen C 1986 Serum levels of vitamin D metabolites and testosterone in male puberty. J Clin Endocrinol Metab **62**:503–507.
198. Aksnes L, Aarskog D 1982 Plasma concentrations of vitamin D metabolites in puberty: Effect of sexual maturation and implications for growth. J Clin Endocrinol Metab **55**:94–101.
199. Leman ES, DeMiguel F, Gao AC, Getzenberg RH 2003 Regulation of androgen and vitamin D receptors by 1,25-dihydroxyvitamin D3 in human prostate epithelia and stromal cell. J Urol **170**: 235–240.
200. Ingles SA, Ross RK, Yu MC, Irvine RA, Pera G, Haile RW 1997 Association of prostate cancer risk with genetic polymorphisms of the vitamin D receptor and androgen receptor. J Natl Cancer Inst **89**:166–170.
201. Bickle DD, Halloran BP, Harris ST, Potale AA 1992 Progestin antagonism of estrogen stimulated 1,25-dihydroxyvitamin D levels. J Clin Endocrinol Metab **72**:519–523.
202. Grecu EO, Simmons R, Baylink DJ, Haloran BP, Spencer ME 1991 Effects of medroxyprogesterone acetate on some parameters of calcium metabolism in patients with glucocorticoid-induced osteoporosis. Bone Miner **13**:153–161.
203. Auwerx J, Bouillon R 1986 Mineral and bone metabolism in thyroid disease: A review. Q J Med **60**:737–752.
204. Mundy GR, Shapiro JL, Bandelin JG, Canalis EM, Raisz LG 1976 Direct stimulation of bone resorption by thyroid hormones. J Clin Invest **58**:529–534.
205. Mosekilde L, Melsen F, Bagger JP, Myhre-Jensen O, Sorensen NS 1977 Bone changes in hyperthyroidism: Interrelationships between bone morphometry, thyroid

function, and calcium-phosphorus metabolism. Acta Endocrinol **85**:515–525.
206. Bayley TA, Harrison JE, McNeill KG, Mernagh JR 1980 Effect of thyrotoxicosis and its treatment on bone mineral and muscle mass. J Clin Endocrinol Metab **50**:916–922.
207. Mosekilde L, Christensen MS 1977 Decreased parathyroid function in hyperthyroidism: Interrelationships between serum parathyroid hormone, calcium-phosphorus metabolism, and thyroid function. Acta Endocrinol **84**:566–575.
208. Burman KD, Monchik JM, Earll JM, Wartofsky L 1976 Ionized and total serum calcium and parathyroid hormone in hyperthyroidism. Ann Int Med **84**:668–671.
209. Bouillon R, De Moor P 1973 Parathyroid function in patients with hyper- or hypothyroidism. J Clin Endocrinol Metab **38**:999–1004.
210. Shafer RB, Gregory DH 1972 Calcium malabsorption in hyperthyroidism. Gastroenterology **63**:235–239.
211. Peerenboom H, Keck E, Kruskemper HL, Strohmeyer G 1984 The defect of intestinal calcium transport in hyperthyroidism and its response to therapy. J Clin Endocrinol Metab **59**:936–940.
212. Karsenty G, Bouchard P, Ulmann A, Schaison G 1985 Elevated metabolic clearance rate of l,25-dihydroxyvitamin D_3 in hyperthyroidism. Acta Endocrinol **110**:70–74.
213. Bouillon R, Muls E, De Moor P 1980 Influence of thyroid function on the serum concentration of 1,25-dihydroxyvitamin D_3. J Clin Endocrinol Metab **51**:793–797.
214. Jastrup B, Mosekilde L, Melsen F, Lund B, Lund B, Sorensen OH 1982 Serum levels of vitamin D metabolites and bone remodeling in hyperthyroidism. Metabolism **31**:126–132.
215. Macfarlane IA, Mawer EB, Berry J, Hann J 1982 Vitamin D metabolism in hyperthyroidism. Clin Endocrinol **17**: 51–59.
216. Velentzas C, Oreopoulos DG, From G, Porret B, Rapoport A 1977 Vitamin D levels in thyrotoxicosis. Lancet **1**:370–371.
217. Mosekilde L, Lund B, Sorensen OH, Christensen MS, Melsen F 1977 Serum-25-hydroxycholecalciferol in hyperthyroidism. Lancet **1**:806–807.
218. Yamashita H, Noguchi S, Murakami T, *et al*. 2000 Calcium and its regulating hormones in patients with Graves disease: sex differences and relation to postoperative tetany. Eur J Surg **166**: 924–8.
219. Langdahl BL, Loft AG, Eriksen EF, Mosekilde L, Charles P 1996 Bone mass, bone turnover, calcium homeostasis, and body composition in surgically and radioiodine-treated former hyperthyroid patients. Thyroid **6**:169–175.
220. Krane SM, Brownell GL, Stanbury JB, Corrigan H 1956 The effect of thyroid disease on calcium metabolism in man. J Clin Invest **35**:874–897.
221. Bijlsma JWJ, Duursma SA, Roelofs JMM, der Kinderen PJ 1983 Thyroid function and bone turnover. Acta Endocrinol **104**:42–49.
222. Weisman Y, Eisenberg Z, Lubelski R, Spirer Z, Edelstein S, Hare 11 A 1981 Decreased 1,25-dihydroxycholecalciferol and increased 25-hydroxy- and 24,25-dihydroxycholecalciferol in tissues of rats treated with thyroxine. Calcif Tissue Int **33**:445–447.
223. Pahuja DN, De Luca HF 1982 Thyroid hormone and vitamin D metabolism in the rat. Arch Biochem Biophys **213**:293–298.
224. Kano K, Jones G 1984 Direct *in vitro* effect of thyroid hormones on 25-hydroxyvitamin D_3 metabolism in the perfused rat kidney. Endocrinology **114**:330–336.
225. Miller ML, Ghazarian JG 1981 Differential response of kidney mitochondrial calcium-regulating mixed functional oxidase to thyrotoxicosis. J Biol Chem **256**:5643–5645.
226. Trechsel U, Taylor CM, Bonjour J-P, Fleisch H 1980 Influence of prostaglandins and of cyclic nucleotides on the metabolism of 25-hydroxyvitamin D_3 in primary chick kidney cell culture. Biochem Biophys Res Commun **93**: 1210–1216.
227. Biddulph DM, Currie MG, Wrenn RW 1979 Effects of interactions of parathyroid hormone and prostaglandins on adenosine 3′,5′-monophosphate concentrations in isolated renal tubules. Endocrinology **104**:1164–1171.
228. Wark JD, Larkins RG, Eisman JA, Wilson KR 1981 Regulation of 25-hydroxyvitamin D-1a-hydroxylase in chick-isolated renal tubules: Effects of prostaglandin E_2, frusemide and acetylsalicyclic acid. Clin Sci **61**:53–59.
229. Kurose H, Sonn YM, Jafari A, Birge SJ, Avioli LV 1985 Effects of prostaglandin E_2 and indomethacin on 25-hydroxyvitamin D_3-1a-hydroxylase activity in isolated kidney cells of normal and streptozotocin-induced diabetic rats. Calcif Tissue Int **37**:625–629.
230. Yamada M, Matsumoto T, Takahashi N, Suda T, Ogata E 1983 Stimulatory effect of prostaglandin E_2 on 1a,25-dihydroxyvitamin D_3 synthesis in rats. Biochem J **216**:237–240.
231. Yamada M, Matsumoto T, Su K-W, Ogata E 1985 Inhibition of prostaglandin E_2 of renal effects of calcitonin in rats. Endocrinology **116**:693–697.
232. Sedrani SH, El-Banna AA 1987 Effect of indomethacin on plasma levels of vitamin D metabolites, oestradiol, and progesterone in rabbits during early pregnancy. Comp Biochem Physiol **87**A:635–639.
233. Katz IA, Jee WSS, Joffe II, Stein B, Takizawa M, Jacobs TW, Setterberg R, Lin BY, Tang LY, Ke HZ, Zeng QQ, Berlin JA, Epstein S 1992 Prostaglandin E_2 alleviates cyclosporin A-induced bone loss in the rat. J Bone Miner Res **7**:1191–1200.
234. Boiskin I, Epstein S, Ismail F, Fallen MD, Levy W 1988 Long-term administration of prostaglandin inhibitors *in vivo* fail to influence cartilage and bone mineral metabolism in the rat. Bone Miner **4**:27–36.
235. de Rovetto CR, Welch TR, Hug G, Clark KE, Bergstrom W 1989 Hypercalciuria with Bartter syndrome: Evidence for an abnormality of vitamin D metabolism. J Pediatr **115**:397–404.
236. Hasanoglu A, Ercan ZS, Buyan N, Memioglu N, Hasanoglu E 1997 Parathyroid, 1,25 dihydroxyvitamin D and prostaglandin E correlation in children with idiopathich hypercalcuria. Prostaglandins Leukot Essent Fatty Acids **56**:235–237.
237. Pryke AM, Duggan C, White CP, Posen S, Mason RS 1990 Tumor necrosis factor-alpha induces vitamin D-1-hydroxylase activity in normal human alveolar macrophages. J Cell Physiol **142**:652–656.
238. Bickle DD, Pillai S, Gee E, Hincenbergs M 1991 Tumor necrosis factor-a regulation of 1,25-dihydroxyvitamin D production by human keratinocytes. Endocrinology **129**: 33–38.
239. Zehnder D, Bland R, Chana RS, Wheeler DC, Howie AJ, Williams M, Stewart PM, Hewison M 2002 Synthesis of 1,25 dihydroxyvitamin D3 by human endothelial cells is regulated by inflammatory cytokines: an novel autocrine determinant of vascular cell adhension. J Am Soc Nephrol **13**:621–629.
240. Mayur N, Lewis S, Catherwood BD, Nanes MS 1993 Tumor necrosis factor *a* decreases 1,25-dihydroxyvitamin D_3 receptors in osteoblastic ROS 17/2.8 cells. J Bone Miner Res **8**:997–1003.
241. Farmer PK, He X, Schmitz ML, Rubin J, Nanes MS 2000 Inhibitory effect of NF-κB on 1,25-dihydroxyvitamin D3 and retinoid X receptor function. Am J Physiol Endocrinol Metab **279**:E213–E220.

242. Koeffler HP, Reichel H, Bishop JE, Norman AW 1989 Gamma Interferon stimulates production of 1,25-dihydroxyvitamin D_3 by normal human macrophages. Biochem Biophys Res Commun **127**:596–603.
243. Bickle DD, Pillai S, Gee E, Hincenbergs M 1989 Regulation of 1,25-dihydroxyvitamin D production in human keratinocytes by interferon-y. Endocrinology **124**:655–660.
244. Reichel H, Koeffler HP, Norman AW 1987 Synthesis *in vitro* of 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 by interferon-y-stimulated normal human bone marrow and alveolar macrophages. J Biol Chem **262**:10931–10937.
245. Robinson BWS, McLemore TL, Crystal RG 1985 Gamma interferon is spontaneously released by alveolar macrophages and lung T lymphocytes in patients with pulmonary sarcoidosis. J Clin Invest **75**:1488–1495.
246. Mann GN, Jacobs TW, Buchinsky FJ, Armstrong EC, Li M, Ke HZ, Ma YF, Jee WSS, Epstein S 1994 Interferon-gamma causes loss of bone volume *in vivo* and fails to ameliorate cyclosporin A-induced osteopenia. Endocrinology **135**: 1077–1083.
247. Kruse R 1968 Osteopathien bei antiepileptisher langzeittherapie. I fonatsschrift fur Kinderheilkd **116**:378–381.
248. Ashworth B, Horn DB 1977 Evidence of osteomalacia in an outpatient group of adult epileptics. Epilepsia **18**:37–43.
249. Hahn TJ, Halstead LR 1979 Anticonvulsant drug-induced osteomalacia: Alterations in mineral metabolism and response to vitamin D_3 administration. Calcif Tissue Int **27**:13–18.
250. Lau KHW, Nakade O, Barr B, Taylor AK, Hochin K, Baylink DJ 1995 Phenytoin increases markers of osteogenesis for the human species *in vitro* and *in vivo*. J Clin Endocrinol Metab **80**:2347–2353.
251. Hahn TJ, Scharp CR, Avioli LV 1974 Effect of phenobarbital administration on the subcellular distribution of vitamin D_3-^{3}H in rat liver. Endocrinology **94**:1489–1495.
252. Silver J, Neale G, Thompson GR 1974 Effect of phenobarbitone treatment on vitamin D metabolism in mammals. Clin Sci Mol Med **46**:433–448.
253. Hahn TJ, Halstead LR, Scharp CR, Haddad JG Jr 1975 Enhanced biotransformation and biologic efficacy of vitamin D following phenobarbital administration in the rat. Clin Res **23**:111A.
254. Ohta T, Wergedal JE, Gruber HE, Baylink DJ, Lau KHW 1995 Low-dose phenytoin is an osteogenic agent in the rat. Calcif Tissue Int **56**:42–48.
255. Onodera K, Takahashi A, Mayanagi H, Wakabayashi H, Kamei J, Shi H 2001 Phenytoin-induced bone loss and its prevention with alfacalcidiol or calcitriol in growing rats. Calcif Tissue Int **69**:109–116.
256. Richens A, Rowe DJF 1970 Disturbance of calcium metabolism by anticonvulsant drugs. Br Med J **4**:73–76.
257. Hunter J, Maxwell JD, Stewart DA, Parsons V, Williams R 1971 Altered calcium metabolism in epileptic children on anticonvulsants. Br Med J **4**:202–204.
258. Weinstein RS, Bryce GF, Sappington LJ, King DW, Gallagher BB 1984 Decreased serum-ionized calcium and normal vitamin D metabolite levels with anticonvulsant drug treatment. J Clin Endocrinol Metab **58**:1003–1009.
259. Gough H, Goggin T, Bissessar A, Baker M, Crowley M, Callaghan N 1986 A comparative study of the relative influence of different anticonvulsant drugs, UV exposure, and diet on vitamin D and calcium metabolism in out-patients with epilepsy. Q J Med **59**:569–577.
260. Hahn TJ, Birge SJ, Scharp CR, Avioli LV 1972 Phenobarbital-induced alterations in vitamin D metabolism. J Clin Invest **51**:741–748.
261. Davie MWJ, Emberson CE, Lawson DEM, Roberts GE, Barnes JLC, Barnes ND, Heeley AF 1983 Low plasma 25-hydroxyvitamin D and serum calcium levels in institutionalized epileptic subjects: Associated risk factors, consequences, and response to treatment with vitamin D. Q J Med **205**:79–91.
262. Hahn TJ, Hendin BA, Scharp CR, Haddad JG 1972 Effect of chronic anticonvulsant therapy on serum 25-hydroxycalciferol levels in adults. N Engl J Med **287**:900–904.
263. Stamp TCB, Round JM, Rowe DJF, Haddad JG 1972 Plasma levels and therapeutic effect of 25-hydroxycholecalciferol in epileptic patients taking anticonvulsant drugs. Br Med J **4**:9–12.
264. Bouillon R, Reynaert J, Claes JH, Lissens W, De Moor P 1975 The effect of anticonvulsant therapy on serum levels of 25-hydroxyvitamin D, calcium, and parathyroid hormone. J Clin Endocrinol Metab **41**:1130–1135.
265. Gascon-Barre M, Villeneuve J-P, Lebrun L-H 1984 Effect of increasing doses of phenytoin on the plasma 25-hydroxyvitamin D and 25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D concentrations. J Am College Nutr **3**:45–50.
266. Valimaki M, Tiihonen M, Laitinen K, Tahtela R, Karkkainen M, Lamberg-Allardt C, Makela P, Tunninen R 1994 Bone mineral density measured by dual-energy X-ray absorptiometry and novel markers of bone formation and resorption in patients on antiepileptic drugs. J Bone Miner Res **9**:631–637.
267. Jubiz W, Haussler MR, McCain TA, Tolman KG 1977 Plasma 1,25-dihydroxyvitamin D levels in patients receiving anticonvulsant drugs. J Clin Endocrinol Metab **44**:617–621.
268. Hahn TJ, Hendin BA, Scharp CR, Boisseau VC, Haddad JG Jr 1975 Serum 25-hydroxycalciferol levels and bone mass in children on chronic anticonvulsant therapy. N Engl J Med **292**:550–554.
269. Takeshita N, Seino Y, Ishida H, Seino Y, Tanaka H, Tsutsumi C, Ogata K, Kiyohara K, Kato H, Nozawa M, Akiyama Y, Kara K, Imura H 1989 Increasing circulating levels of y-carboxyglutamic acid-containing protein and decreased bone mass in children on anticonvulsant therapy. Calcif Tissue Int **44**:80–85.
270. Wark JD, Larkins RG, Perry-Keene D, Peter CT, Ross DL, Sloman JG 1979 Chronic diphenylhydantoin therapy does not reduce plasma 25-hydroxyvitamin D. Clin Endocrinol **11**:267–274.
271. Tjellesen L, Nilas L, Christiansen C 1983 Does carbamazepine cause disturbances in calcium metabolism in epileptic patients? Acta Neurol Scand **68**:13–19.
272. Filardi S, Guerreiro CA, Magna LA, Marques Neto JF 2000 Bone mineral density, vitamin D, and anticonvulsant therapy. Arq Neuropsiquiatr **58**:616–620.
273. Bell RD, Pak CYC, Zerwekh J, Barilla DE, Vasko M 1979 Effect of phenytoin on bone and vitamin D metabolism. Ann Neurol **5**:374–378.
274. Zerwekh JE, Homan R, Tindall R, Pak CYC 1982 Decreased 24,25-dihydroxyvitamin D concentration during long-term, anticonvulsant therapy in adult epileptics. Ann Neurol **12**:184–186.
275. Weisman Y, Fattal A, Eisenberg Z, Harel S, Spirer Z, Harell A 1979 Decreased serum 24,25-dihydroxy-vitamin D concentrations in children receiving chronic anticonvulsant therapy. Br Med J **2**:521–523.
276. Baer MT, Kozlowski BW, Blyler EM, Trahms Cm, Taylor ML 1997 Vitamin D, calcium, and bone status in children with developmental delay in relation to anticonvulsant use and ambulatory status. Am J Clin Nutr **65**: 1042–1051.

277. Pedrera JD, Canal ML Carvajal J, Postigo S, Villa LF, Hernandez ER, Rico H 2000 Influence of vitamin D administration on bone ultrasound measurements in patients on anticonvulsant therapy. Euro J Clin Investigat **30**: 895–905.
278. Tsukahara H, Kimura K, Todoroki Y, Ohshima Y, Hiraoka M, Shigematsu Y, Tsukahara Y, Miura M, Mayumi M 2002 Bone mineral status in ambulatory pediatric patients on long-term antiepileptic drug therapy. Pediatrics Int **44**:247.
279. Verrotti A, Greco R, Latini G, Morgese G, Chiarelli F 2002 Increased bone turnover in prebupertal, pubertal, and postpubertal patients receiving carbamazepine. Epilepsia **43**:1488–1492.
280. Conney AH 1967 Pharmacological implications of microsomal enzyme induction. Pharmacol Rev **19**:317–366.
281. Eichelbaum M, Ekbom K, Bertilsson L, Ringer-Berger VA, Rane A 1975 Plasma kinetics of carbamazepine and its epoxide metabolite in man after single and multiple doses. Eur J Clin Pharmacol **8**:337–341.
282. Perucca E 1978 Clinical consequences of microsomal enzyme-induction by antiepileptic drugs. Pharmacol Ther **2**:285–314.
283. Mosekilde L, Christensen MS, Lund B, Sorensen OH, Melsen F 1977 The interrelationships between serum 25-hydroxycholecalciferol, serum parathyroid hormone, and bone changes in anticonvulsant osteomalacia. Acta Endocrinol **84**:559–565.
284. Nielsen HE, Melsen F, Lund B, Sorensen OH, Mosekilde L 1983 Bone histomorphometry, vitamin D metabolites, and calcium phosphate metabolism in anticonvulsant treatment with carbamazepine. Calcif Tissue Int **35**(Suppl.):224.
285. Hoikka V, Alhava EM, Karjalainen P, Keranen T, Savolainen KE, Riekkinen P, Korhonen R 1984 Carbamazepine and bone mineral metabolism. Acta Neurol Scand **69**:77–80.
286. Feldkamp J, Becker A, Witte OW, Scharff D, Scherbaum WA 2000 Long-term anticonvulsant therapy leads to low bone mineral density—evidence for direct drug effects of phenytoin and carbamazepine on human osteoblast-like cells. Exp Clin Endocrinol Diabetes **108**(1):37–43.
287. Kroger H, Lotaniemi A, Vaino P, Alhava E 1992 Bone densitometry of the spine and femur in children by dual X-ray absorptiometry. Bone Miner **17**:75–85.
288. Sheth RD, Wesolowski CA, Jacob JC, Penney S, Hobbs GR, Riggs JE, Bodensteiner JB 1995 Effect of carbamazepine and valproate on bone mineral density. J Pediatr **127**:256–262.
289. Guo CY, Ronen GM, Atkinson SA 2001 Long-term valproate and lamotrigine treatment may be a marker for reduced growth and bone mass in children with epilepsy. Epilepsia **42**:1141–1147.
290. Farhat G, Yamout B, Mikati MA, Demirjian S, Sawaya R, El-Hajj Fuleihan G 2002 Effect of antiepileptic drug on bone density in ambulatory patients. Neurology **58**:1348–1353.
291. Dent CE, Richens A, Rowe DJF, Stamp TCB 1970 Osteomalacia with long-term anticonvulsant therapy in epilepsy. Br Med J **4**:69–72.
292. Collins N, Maher J, Cole M, Baker M, Callaghan N 1991 A prospective study to evaluate the dose of vitamin D required to correct low 25-hydroxyvitamin D levels, calcium, and alkaline phosphatase in patients at risk of developing antiepileptic drug-induced osteomalacia. Q J Med **286**:113–122.
293. Morris HA, Need AG, O'Loughlin PD, Horowitz M, Bridges A, Nordin BEC 1990 Malabsorption of calcium in corticosteroid-induced osteoporosis. Calcif Tissue Int **46**:305–308.
294. Hahn TJ, Halstead LR, Baran DT 1981 Effects of short-term glucocorticoid administration on intestinal calcium absorption and circulating vitamin D metabolite concentrations in man. J Clin Endocrinol Metab **52**:111–115.
295. Klein RG, Arnaud SB, Gallagher JC, DeLuca HF, Riggs BL 1977 Intestinal calcium absorption in exogenous hypercortisonism. J Clin Invest **60**:253–259.
296. Kimberg DV, Baerg RD, Gershon E, Graudusius RT 1971 Effect of cortisone treatment on the active transport of calcium by the small intestine. J Clin Invest **50**:1309–1321.
297. Adams JS, Wahl TO, Lukert BP 1981 Effect of hydrochlorothiazide and dietary sodium restriction on calcium metabolism in corticosteroid treated patients. Metabolism **30**:217–221.
298. Suzuki Y, Ichikawa Y, Saito E, Homma M 1983 Importance of increased urinary calcium excretion in the development of secondary hyperparathyroidism of patients under glucocorticoid therapy. Metabolism **32**:151–156.
299. Seeman E, Kumar R, Hunder G, Scott M, Heath III H, Riggs BL 1980 Production, degradation, and circulating levels of 1,25-dihydroxyvitamin D in health and in glucocorticoid excess. J Clin Invest **66**:664–669.
300. Hahn TJ, Halstead LR, Teitelbaum SL, Hahn BH 1979 Altered mineral metabolism in glucocorticoid-induced osteopenia. J Clin Invest **64**:655.
301. Lukert BP, Adams JS 1976 Calcium and phosphorus homeostasis in man. Arch Intern Med **136**:1249–1253.
302. Carbonare LD, Arlot ME, Chavassieux PM, *et al*. 2001 Comparison of trabecular bone microarchitecture and remodeling in glucorticoid-induced and postmenopausal osteoporosis. J Bone Miner Res **16**:97–103.
303. Rubin M, Bilezikian JP 2002 The role of parathyroid hormone in the pathogensis of glucocorticoid-induced osteoporosis: A re-examination of the evidence. J Clin Endocrinol Metab **87**:4022–4041.
304. Canalis E 2003 Mechanisms of glucocorticoid-induced osteoporosis. Current opinions in rheumatology **15**:454–457.
305. Avioli LV, Birge SJ, Lee SW 1968 Effects of prednisone on vitamin D metabolism in man. Clin Endocrinol **28**:1341–1346.
306. Hahn TJ, Halstead LR, Haddad JG Jr 1977 Serum 25-hydroxyvitamin D concentrations in patients receiving chronic corticosteroid therapy. J Clin Lab Med **90**:399–404.
307. LoCascio V, Bonucci E, Imbimbo B, Ballanti P, Adami S, Milani S, Tartarotti D, DellaRocca C 1990 Bone loss in response to long-term glucocorticoid therapy. Bone Miner **8**:39–51.
308. Prummel MF, Wiersinga WM, Lips P, Sanders GTP, Sauerwein HP 1991 The course of biochemical parameters of bone turnover during treatment with corticosteroids. J Clin Endocrinol Metab **72**:382–386.
309. Zerwekh JE, Emkey RD, Harris ED Jr 1984 Low-dose prednisone therapy in rheumatoid arthritis: Effect on vitamin D metabolism. Arth Rheum **27**:1050–1052.
310. Slovik DM, Neer RM, Ohman JL, Lowell FC, Clark MB, Segre GV, Potts JT Jr 1980 Parathyroid hormone and 25-hydroxyvitamin D levels in glucocorticoid-treated patients. Clin Endocrinol **12**:243–248.
311. Hodsman AB, Toogood JH, Jennings B, Fraher LJ, Baskerville JC 1991 Differential effects of inhaled budesonide and oral prednisolone on serum osteocalcin. J Clin Endocrinol Metab **72**:530–540.
312. Jennings BH, Andersson KE, Johansson SA 1991 The assessment of the systemic effects of inhaled glucocorticosteroids. Eur J Clin Pharmacol **41**:11–16.
313. Findling JW, Adams ND, Lemann J Jr, Gray RW, Thomas CJ, Tyrrell JB 1982 Vitamin D metabolites and parathyroid hormone in Cushing's syndrome: Relationship to calcium

and phosphorus homeostasis. J Clin Endocrinol Metab **54**:1039–1044.
314. Aloia JF, Roginsky M, Ellis K, Shukla K, Chon S 1974 Skeletal metabolism and body composition in Cushing's syndrome. J Clin Endocrinol Metab **39**:981–985.
315. Jowell PS, Epstein S, Fallen MD, Reinhardt TA, Ismail F 1987 1,25-Dihydroxyvitamin D_3 modulates glucocorticoid-induced alteration in serum bone gla protein and bone histomorphometry. Endocrinology **120**:531–536.
316. Favus MJ, Kimberg DV, Millar GN, Gershon E 1973 Effects of cortisone administration on the metabolism and localization of 25-hydroxycholecalciferol in the rat. J Clin Invest **52**:1328–1335.
317. Carre M, Ayigbede O, Miravet L, Rasmussen H 1974 The effect of prednisolone upon the metabolism and action of 25-hydroxy- and 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA **71**:2996–3000.
318. Chesney RW, Hamstra AJ, Mazess RB, DeLuca HF, O'Reagan S 1978 Reduction of serum-l,25-dihydroxy-vitamin-D_3 in children receiving glucocorticoids. Lancet **2**:1123–1125.
319. O'Regan S, Chesney RW, Hamstra A, Eisman JA, O'Gorman AM, DeLuca HF 1979 Reduced serum 1,25-$(OH)_2$ vitamin D_3 levels in prednisone-treated adolescents with systemic lupus erythematosus. Acta Paediatr Scand **68**:109–111.
320. Bikle DD, Halloran B, Fong L, Steinbach L, Shellito J 1993 Elevated 1,25-dihydroxyvitamin D levels in patients with chronic obstructive pulmonary disease treated with prednisone. J Clin Endocrinol Metab **76**:456–461.
321. Lukert BP, Stanbury SW, Mawer EB 1973 Vitamin D and intestinal transport of calcium: Effects of prednisolone. Endocrinology **93**:718–722.
322. Braun JJ, Juttmann JR, Visser TJ, Birkenhager JC 1982 Short-term effect of prednisone on serum 1,25-dihydroxy-vitamin D in normal individuals and in hyper- and hypoparathyroidism. Clin Endocrinol **17**:21–28.
323. Au WYW 1976 Cortisol stimulation of parathyroid hormone secretion by rat parathyroid glands in organ culture. Science **193**:1015–1017.
324. Williams GA, Peterson WC, Bowser EN, Henderson WJ, Hargis GK, Martinez NJ 1974 Interrelationship of parathyroid and adrenocortical function in calcium homeostasis in the rat. Endocrinology **95**:707–712.
325. Ho YV, Briganti EM, Duan Y, Buchanan R, Hall S, Seeman E 1999 Polymorphism of the vitamin D receptor gene and corticosteroid-related osteoporosis. Osteoporos Int **9**:134–138.
326. Lindholm J, Steiniche T, Rasmussen E, Thamsborg G, Nielson IO 1991 Bone disorder in men with chronic alchoholism: a reversible disease. J Clin Endocrinol Metab **73**:118–124.
327. Bjorneboe GEA, Johnsen J, Bjorneboe A, Rousseau B, Pedersen JI, Norum KR, Morland J, Drevon CA 1986 Effect of alcohol consumption on serum concentration of 25-hydroxyvitamin D_3, retinol, and retinol-binding protein. Am J Clin Nutr **44**:678–682.
328. Feitelberg S, Epstein S, Ismail F, D'Amanda C 1987 Deranged bone mineral metabolism in chronic alcoholism. Metabolism **36**:322–326.
329. Mobarhan SA, Russell RM, Recker RR, Posner DB, Iber FL, Miller P 1984 Metabolic bone disease in alcoholic cirrhosis: A comparison of the effect of vitamin D_2, 25-hydroxyvitamin D, or supportive treatment. Hepatology **4**:266–273.
330. Bjorneboe GA, Johnsen J, Bjorneboe A, Morland J, Drevon C 1987 Effect of heavy alcohol consumption on serum concentrations of fat-soluble vitamins and selenium. Alcohol and Alcoholism (Suppl. 1):533–537.
331. Peris P, Pares A, Guanabens N, Del Rio L, Pons F, Jesus M, De Osaba M, Monegal A, Caballeria J, Rodes J, Munoz-Gomez J 1994 Bone mass improves in alcoholics after two years of abstinence. J Bone Miner Res **9**:1607–1612.
332. Lalor BC, France MW, Powell D, Adams PH, Counihan TB 1986 Bone and mineral metabolism and chronic alcohol abuse. Q J Med **59**:497–511.
333. Verbanck M, Verbanck J, Brauman J, Mullier JP 1977 Bone histology and 25-OH vitamin D levels in alcoholics without cirrhosis. Calcif Tissue Res **22**(Suppl.):538–541.
334. Laitinen K, Lamberg-Allardt C, Tunninen R, Karonen S-L, Ylikahri R, Valimaki M 1991 Effects of three weeks' moderate alcohol intake on bone and mineral metabolism in normal men. Bone Miner **13**:139–151.
335. Pepersack T, Fuss M, Otero J, Bergmann P, Valsamis J, Corvilain J 1992 Longitudinal study of bone metabolism after ethanol withdrawal in alcoholic patients. J Bone Miner Res **7**:383–387.
336. Barragry JM, Long RG, France MW, Wills MR, Boucher BJ, Sherlock S 1979 Intestinal absorption of cholecalciferol in alcoholic liver disease and primary biliary cirrhosis. Gut **20**:559–564.
337. Lindholm J, Steiniche T, Rasmussen E, Thamsborg G, Nielsen IO, Brockstedt-Rasmussen H, Storm T, Hyldstrup L, Schou C 1991 Bone disorder in men with chronic alcoholism: A reversible disease? J Clin Endocrinol Metab **73**:118–124.
338. Bjorneboe GE, Bjorneboe A, Johnsen J, Skylv N, Oftebro N, Gautvik KM, Hoiseth A, Morland J, Drevon CA 1988 Calcium status and calcium-regulating hormones in alcoholics. Alcohol Clin Exp Res **12**:229–232.
339. Santolaria F, Gonzalez-Reimers E, Perez-Manzano JL, Milena A, Gomez-Rodriguez MA, Gonzalez-Diaz A, de la Vega MJ, Martinez-Riera A 2000 Osteopenia assessed by body composition analysis is related to malnutrition in alcoholic patients. Alcohol **22**:147–157.
340. Meyer M, Wechsler S, Shibolet S, Jedwab M, Harell A, Edelsteim S 1978 Malabsorption of vitamin D in man and rat with liver cirrhosis. J Mol Med **3**:29–37.
341. Gascon-Barre M, Joly J-G 1981 The biliary excretion of [^{3}H]-25-hydroxyvitamin D_3 following chronic ethanol administration in the rat. Life Sci **28**:279–286.
342. Lund B, Sorensen OH, Hilden M, Lund B 1977 The hepatic conversion of vitamin D in alcoholics with varying degrees of liver affection. Acta Med Scand **202**:221–224.
343. Avioli LV, Lee SW, McDonald JE, Lund J, DeLuca HF 1967 Metabolism of D_3-^{3}H in human subjects. Distribution to blood, bile, feces, and urine. J Clin Invest **46**:983–992.
344. Brodie MJ, Boobis AR, Hillyard CJ, Abeyasekera G, Maclntyre I, Park BK 1981 Effect of isoniazid on vitamin D metabolism and hepatic monooxygenase activity. Clin Pharmacol Ther **30**:363–367.
345. Posner DB, Russell RM, Absood S, Connor TB, Davis C, Martin L, Williams JB, Norris AH, Merchant C 1978 Effective 25-hydroxylation of vitamin D_2 in alcoholic cirrhosis. Gastroenterology **74**:866–870.
346. Bickle DD, Halloran BP, Gee E, Ryzen E, Haddad JG 1986 Free 25-hydroxyvitamin D levels are normal in subjects with liver disease and reduced total 25-hydroxyvitamin D levels. J Clin Invest **78**:748–752.
347. Bouillon R, Auwerx J, Dekeyser L, Fevery J, Lissens W, De Moor P 1984 Serum vitamin D metabolites and their

binding protein in patients with liver cirrhosis. J Clin Endocrinol Metab **59**:86–89.
348. Laitinen K, Lamberg C, Tunninen R, Karonen SL, Ylikahri R, Valimaki M 1991 Effects of three weeks of moderate alchohol intake on bone and mineral metabolism in normal men. Bone Miner **13**:139–151.
349. Shah JH, Bowser EN, Hargis GK, Wongsurawat N, Banerjee P, Henederson WJ, Williams GA 1978 Effect of ethanol on parathyroid hormone secretion in the rat. Metabolism **27**: 257–260.
350. Channard J, Lacour B, Drueke T, Brunois JP, Ruiz JC 1980 Effect of acute ethanol loading on parathyroid gland secretion in the rat. Adv Exp Med Biol **128**:495–504.
351. Williams GA, Bowser EN, Hargis GK, Kukreja SC, Shah JH, Vora NM, Henderson WJ 1978 Effect of ethanol on parathyroid hormone and calcitonin secretion in man. Proc Soc Exp Biol Med **159**:187–191.
352. Ljunghall S, Lundin L, Wide L 1985 Acute effects of ethanol intake on the serum concentrations of parathyroid hormone, calcium, and phosphate. Exp Clin Endocrinol **85**:365–368.
353. Bjorneboe GE, Bjorneboe A, Johnsen J, Skylv N, Oftebro H, Gautvik KM, Hoiseth A, Morland J, Drevon CA 1988 Calcium status and calcium-regulating hormones in alcoholics. Alcoholism **12**:229–232.
354. Laitinen K, Valimaki M, Lamberg-Allardt C, Kivisaari L, Lalla M, Karkkainen M, Ylikahri R 1990 Deranged vitamin D metabolism but normal bone mineral density in Finnish noncirrhotic male alcoholics. Alcoholism **14**:551–556.
355. Crilly RG, Anderson C, Hogan D, Delaquerriere-Richardson L 1988 Bone histomorphometry, bone mass, and related parameters in alcoholic males. Calcif Tissue Int **43**:269–276.
356. Bickle DD, Genant HK, Cann C, Recker RR, Halloran BP, Strewler GJ 1985 Bone disease in alcohol abuse. Ann Intern Med **103**:42–48.
357. Krawitt EL 1975 Effect of ethanol ingestion on duodenal calcium transport. J Clin Lab Med **85**:665–671.
358. Vodoz JF, Luisier M, Donath A, Courvoisier B, Garcia B 1977 Diminution de l'absorption intestinale de 47-calcium dans l'alcoolisme chronique. Schweiz Med Wochenschr **107**:1525–1529.
359. Avery DH, Overall JE, Calil HM, Hollister LE 1983 Plasma calcium and phosphate during alcohol intoxication. Alcoholics versus nonalcoholics. J Stud Alcohol **44**:205–214.
360. Krawitt EL 1973 Ethanol inhibits intestinal calcium transport in rats. Nature **243**:88–89.
361. Territo MC, Tanaka KR 1974 Hypophosphatemia in chronic alcoholism. Arch Intern Med **134**:445–447.
362. Kent JC, Devlin RD, Gutteridge DH, Retallack RW 1979 Effect of alcohol on renal vitamin D metabolism in chickens. Biochem Biophys Res Commun **89**:155–161.
363. Rude RK, Oldham SB, Sharp CF Jr, Singer FR 1978 Parathyroid hormone secretion in magnesium deficiency. J Clin Endocrinol Metab **78**:748–52.
364. Gascon-Barre M 1982 Plasma 25-hydroxyvitamin D_3 response to pharmacological dose of vitamin D_3 or 25-hydroxyvitamin D_3 during chronic ethanol administration in the rat. Horm Metab Res **14**:332–333.
365. Turner RT, Aloia RC, Segel LD, Hannon KS, Bell NH 1988 Chronic alcohol treatment results in disturbed vitamin D metabolism and skeletal abnormalities in the rat. Alcohol Clin Exp Res **12**:159–162.
366. Gascon-Barre M 1982 Interrelationships between vitamin D_3 and 25-hydroxyvitamin D_3 during chronic ethanol administration in the rat. Metabolism **31**:67–72.
367. Saarem K, Bergseth S, Oftebro H, Pedersen JI 1984 Subcellular localization of vitamin D_3 25-hydroxylase in human liver. J Biol Chem **259**:10936–10940.
368. Saarem K, Pedersen JI 1985 25-Hydroxylation of 1-hydroxyvitamin D_3 in rat and human liver. Biochim Biophys Acta **840**:117–126.
369. Laitinen K (ed) 1993 Alcohol and Bone. Yliopistopaino, Helsinki.
370. Gascon-Barre M 1985 Influence of chronic ethanol consumption on the metabolism and action of vitamin D. J Am College Nutr **4**:565–574.
371. Feldman D 1986 Ketoconazole and other imidizole derivatives as inhibitors of steroidogenesis. Endocr Rev **7**:409–420.
372. Loose DS, Kan PB, Hirst MA, Marcus RA, Feldman D 1983 Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes. J Clin Invest **71**:1495–1499.
373. Henry HL 1985 Effect of ketoconazole and miconazole on 25-hydroxyvitamin D_3 metabolism by cultured chick kidney cells. J Steroid Biochem Mol Biol **23**:991–994.
374. Glass AR, Eil C 1986 Ketoconazol-induced reduction in serum 1,25-dihydroxyvitamin D. J Clin Endocrinol Metab **63**(No. 3):766–769.
375. Glass AR, Eil C 1988 Ketoconazole-induced reduction in serum 1,25-dihydroxyvitamin D and total serum calcium in hypercalcemic rats. J Clin Endocrinol Metab **66**:934–938.
376. Riancho JA, Amado JA, Freijanes J, Otero M, Gonzalez Marcias J 1989 Ketoconazole and vitamin D metabolism in hyperparathyroidism. Horm Metab Res **21**:51.
377. Adams JS, Sharma OP, Diz MM, Endres DB 1990 Ketoconazole decreases the serum 1,25-dihydroxyvitamin D and calcium concentration in sarcoidosis-associated hypercalcemia. J Clin Endocrinol Metab **70**:1090–1095.
378. Glass AR, Cerletty JM, Elliott W, Lemann J Jr, Gray RW, Eil C 1990 Ketoconazole reduces elevated serum levels of 1,25-dihydroxyvitamin D in hypercalcemic sarcoidosis. J Endocrinol Invest **13**:407–413.
379. Peehl DM, Seto E, Hsu JY, Feldman D 2002 Preclinical activity of ketoconazole in combination with calcitriol or the vitamin D analogue EB 1089 in prostate cancer cells. J Urol **168**:1583–1588.
380. Ismail F, Corder CN, Epstein S, Barbi G, Thomas S 1990 Effects of pravastatin and cholestyramine on circulating levels of parathyroid hormone and vitamin D metabolites. Clin Ther **12**:427–430.
381. Dobs A, Levine MA, Margolis S 1991 Effects of pravastatin, a new HMG-CoA reductase inhibitor, on vitamin D synthesis in man. Metabolism **40**:524–528.
382. Montagnani M, Lore F, Di Cairano G, Gonnelli S, Ciuoli C, Montagnani A, Gennari C 1994 Effects of pravastatin treatment on vitamin D metabolites. Clin Ther **16**:824–829.
383. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Gutierrez G 1999 Stimulation of bone formation *in vitro* and in rodents by statins. Science **286**:1946–1949.
384. Meier Cr, Schlienger RG, Kraenzlin ME, Schlegel B, Jick H 2000 HMG-CoA reductase inhibitors and the risk of fracture. JAMA **282**:3205–3210. Wang PS, Solomon DH, Mogun H, Avorn J 2000 HMG-CoA reductase inhibitors and the risk of hip fracture in elderly patients. JAMA **283**:3211–3216.
385. Gascon-Barre M 1982 Biliary excretion of [^{3}H]-25-hydroxyvitamin D_3 in the vitamin D-depleted rat. Am J Physiol **242**:G522–G532.
386. Clements MR, Chalmers TM, Fraser DR 1984 Enterohepatic circulation of vitamin D: A repraisal of the hypothesis. Lancet **1**:1376–1379.

387. Kumar R, Nagubandi S, Mattox VR, Londowski JM 1980 Enterohepatic physiology of 1,25-dihydroxyvitamin D_3. J Clin Invest **65**:277–284.
388. Thompson WG, Thompson GR 1969 Effect of cholestyramine on the absorption of vitamin D_3 and calcium. Gut **10**:717–722.
389. Heaton KW, Lever JV, Barnard D 1972 Osteomalacia associated with cholestyramine therapy for posileectomy diarrhea. Gastroenterology **62**:642–646.
390. Compston JE, Horton LWL 1978 Oral 25-hydroxyvitamin D_3 in treatment of osteomalacia associated with ileal resection and cholestyramine therapy. Gastroenterology **74**:900–902.
391. Compston JE, Thompson RPH 1977 Intestinal absorption of 25-hydroxyvitamin D and osteomalacia in primary biliary cirrhosis. Lancet **1**:721–724.
392. Hoogwerf BJ, Hibbard DM, Hunninghake DB 1992 Effects of long-term cholestyramine administration on vitamin D and parathormone levels in middle-aged men with hypercholesterolemia. J Clin Lab Med **119**:407–411.
393. Schwarz KB, Goldstein PD, Witztum JL, Schonfeld G 1980 Fat-soluble vitamin concentrations in hypercholesterolemic children treated with colestipol. Pediatrics **65**:243–250.
394. Tsang RC, Roginsky MS, Mellies M, Glueck CJ, Kashyap ML 1977 Plasma 25 OH-vitamin D: Familial hypercholesterolemic children on colestipol resin. Clin Res **35**:567A.
395. van Heek M, Farley C, Compton DS, Hoos L, Davis HR 2001 Ezetimibe selectively inhibits intestinal cholesterol absorption in rodents in the presence and absence of exocrine pancreatic function. Br J Pharmacol **134**:409–417.
396. Wilczek H, Sobra J, Ceska R, Justova V, Prochazkova R, Kvasilova M, Juzova Z 1993 Lecba fibraty a metabolismus vitaminu D. Casopis Lekaru Ceskych **132**:630–632.
397. Fleisch H, Russell RGG, Francis MD 1969 Diphosphonates inhibit hydroxyapatite dissolution *in vitro* and bone resorption in tissue culture and *in vivo*. Science **165**:1262–1264.
398. Russell RG, Muhlbauer RC, Bisaz S, Williams DA, Fleisch H 1970 The influence of pyrophosphate, condensed phosphates, phosphonates, and other phosphate compounds on the dissolution of hydroxyapatite *in vitro* and on bone resorption induced by parathyroid hormone in tissue culture and in thyroparathyroidectomized rats. Calcif Tissue Res **6**:183–196.
399. Gasser AB, Morgan DB, Fleisch H, Richelle LJ 1972 The influence of two diphosphonates on calcium metabolism in the rat. Clin Sci **43**:31–45.
400. Kanis JA, Gertz BJ, Singer F, Ortolani S 1995 Rationale for the use of alendronate in osteoporosis. Osteoporosis Int **5**:1–13.
401. Rossini M, Gatti D, Zamberlan N, Braga V, Dorizzi R, Adami S 1994 Long-term effects of a treatment course with oral alendronate on postmenopausal osteoporosis. J Bone Miner Res 9(11):1833–1837.
402. O'Doherty DP, Bickerstaff DR, McCloskey EV, Hamdy NAT, Beneton MNC, Harris S, Mian M, Kanis JA 1990 Treatment of Paget's disease of bone with aminohydroxybutylidene bisphosphonate. J Bone Miner Res **5**:483–491.
403. Trechsel U, Taylor CM, Eisman JA, Bonjour JP, Fleisch H 1981 Plasma levels of vitamin D metabolites in diphosphonate-treated rats. Clin Sci **61**:471–476.
404. Hill LF, Lumb GA, Mawer EB, Stanbury SW 1973 Indirect inhibition of biosynthesis of 1,25-dihydroxycholecalciferol in rats treated with diphosphonate. Clin Sci **44**:335–347.
405. Nagao Y, Ishitobi Y, Kinoshita H, Fukushima S, Kawashima H 1991 YM175, a new bisphosphonate, increases serum 1,25-dihydroxyvitamin D in rats via stimulating renal 1-hydroxylase activity. Biochem Biophys Res Commun **180**:1172–1178.
406. Ralston SH, Boyce BF, Cowan RA, Fogelman I, Smith ML, Jenkins A, Boyle IT 1987 The effect of 1a-hydroxyvitamin D_3 on the mineralization defect in disodium-etidronate-treated Paget's disease—A double-blind, randomized clinical study. J Bone Miner Res **2**:5–12.
407. Lawson-Matthew PJ, Guilland-Cummings DF, Yates AJP, Russell RGG, Kanis JA 1988 Contrasting effects of intravenous and oral etidronate on vitamin D metabolism. Clin Sci **74**:101–106.
408. Devlin RD, Retallack RW, Fenton AJ, Grill V, Gutteridge DH, Kent GN, Prince RL, Worth GK 1994 Long-term elevation of 1,25 dihydroxyvitamin D after short-term intravenous administration of pamidronate (aminohydroxypropylidene bisphosphonate, APD) in Paget's disease of bone. J Bone Miner Res **9**:81–85.
409. Papapoulos SE, Frolich M, Mudde AH, Harinck HIJ, Berg HVD, Bijvoet OLM 1987 Serum osteocalcin in Paget's disease of bone: Basal concentrations and response to bisphosphonate treatment. J Clin Endocrinol Metab **65**:89–94.
410. Adami S, Frijlink WB, Bijvoet OLM, O'Riordan JLH, Clemens TL, Papapoulos SE 1982 Regulation of calcium absorption by 1,25-dihydroxyvitamin D—Studies of the effects of a bisphosphonate treatment. Calcif Tissue Int **34**: 317–320.
411. Body JJ, Magritte A, Seraj F, Sculier JP, Borkowski A 1989 Aminohydroxypropylidene bisphosphonate (APD) treatment for tumor-associated hypercalcemia: A randomized comparison between a three-day treatment and single 24-hour infusions. J Bone Miner Res **4**:923–928.
412. Budayr AA, Zysset E, Jenzer A, Thiebaud D, Ammann P, Rizzoli R, Jaquet-Muller F, Bonjour JP, Gertz B, Burckhardt P, Halloran BP, Nissenson RA, Strewler GJ 1994 Effects of treatment of malignancy-associated hypercalcemia on serum parathyroid hormone-related protein. J Bone Miner Res **9**:521–526.
413. Grill V, Murray RML, Ho PWM, Santamaria JD, Pitt P, Potts C, Jerums G, Martin TJ 1992 Circulating PTH and PTHrP levels before and after treatment of tumor-induced hypercalcemia with pamidronate disodium (APD). J Clin Endocrinol Metab **74**:1468–1470.
414. Fraser WD, Logue FC, Gallacher SJ, O'Reilly DSJ, Beastall GH, Ralston SH, Boyle IT 1991 Direct and indirect assessment of the parathyroid hormone response to pamidronate therapy in Paget's disease of bone and hypercalcemia of malignancy. Bone Miner **12**:113–121.
415. Sahni M, Guenther HL, Fleisch H, Collin P, Martin TJ 1993 Bisphosphonates act on rat bone resorption through the mediation of osteblasts. J Clin Invest **91**:2004–2011.
416. Chesnut CH, McClung MR, Ensrud KE, Bell NH, Genant HK, Harris ST, Singer FR, Stock JL, Yood RA, Delmas PD, Kher U, Pryor-Tillotson S, Santora II AC 1995 Alendronate treatment of the postmenopausal osteoporotic woman: Effect of multiple dosages on bone mass and bone remodeling. Am J Med **99**:144–152.
417. Harris ST, Gertz BJ, Genant HK, Eyre DR, Survill TT, Ventura JN, DeBrock J, Ricerca E, Chesnut III CH 1993 The effect of short-term treatment with alendronate on vertebral density and biochemical markers of bone remodeling in early postmenopausal women. J Clin Endocrinol Metab **76** (No. 6):1399–1406.
418. Martinez ME, Pastrana P, Snachez-Cabezudo MJ, Jariego D, Del Campo MT 1997 Effect of clodronate on calcidiol serum levels in women with breast cancer. Calcif Tissue In **61**:148–150.

419. Wasnich RD, Benfante RJ, Yano K, Heilbrun L, Vogel JM 1983 Thiazide effect on the mineral content of bone. N Engl J Med **309**:344–347.
420. Sakhaee K, Nicar MJ, Glass K, Zerwekh JE, Pak CYC 1984 Reduction in intestinal calcium absorption by hydrochorothiazide in postmenopausal osteoporosis. J Clin Endocrinol Metab **59**:1037–1043.
420. Wasnich R, Davis J, Ross P, Vogel J 1990 Effect of thiazide on rates of bone mineral loss: A longitudinal study. Br Med J **301**:1303–1305.
421. Dawson-Hughes B, Harris S 1993 Thiazides and seasonal bone changes in healthy postmenopausal women. Bone Miner **21**:41–51.
422. LaCroix AZ, Wienphal J, White LR, Wallace RB, Scherr PA, George LK, Cornoni-Huntley J, Ostfeld AM 1990 Thiazide diuretic agents and the incidence of hip fractures. N Engl J Med **322**:286–290.
423. Ray WA, Downey W, Griffin MR, Melton III LJ 1989 Long-term use of thiazide diuretics and risk of hip fracture. Lancet **1**:687–690.
424. Felson DT, Sloutskis D, Anderson JJ, Anthony JM, Kiel DP 1991 Thiazide diuretics and the risk of hip fracture. JAMA **265**:370–373.
425. Middler S, Pak CYC, Murad F, Bartter C 1973 Thiazide diuretics and calcium metabolism. Metabolism **22**:139–146.
426. Ljunghall S, Backman U, Danielson BG, Fellstrom B, Johansson G, Wikstrom B 1981 Calcium and magnesium metabolism during long-term treatment with thiazides. Scand J Urol Nephrol **15**:257–262.
427. Lamberg B-A, Kuhlback B 1959 Effect of chorothiazide and hydrochlorothiazide on the excretion of calcium in urine. Scand J Clin Lab Invest **11**:351–357.
428. Stote RM, Smith LH, Wilson DM, Dube WJ, Goldsmith RS, Arnaud CD 1972 Hydrochorothiazide effects on serum calcium and immunoreactive parathyroid hormone concentrations. Ann Intern Med **77**:587–591.
429. Sowers MR, Wallace RB, Hollis BW 1990 The relationship of 1,25-dihydroxyvitamin D and radial bone mass. Bone Miner **10**:139–148.
430. Zerwekh JE, Pak CYC 1980 Selective effects of thiazide therapy on serum 1a,25-dihydroxyvitamin D and intestinal calcium absorption in renal and absorptive hypercalciurias. Metabolism **29**:13–17.
431. Riis B, Christiansen C 1985 Actions of thiazides on vitamin D metabolism: A controlled therapeutic trial in normal women early in the postmenopause. Metabolism **34**:421–424.
432. Ramp WK, Cooper CW, Ross III AJ, Wells SA Jr 1979 Effects of calcium and cyclic nucleotides on rat calcitonin and parathyroid hormone secretion. Mol Cell Endocrinol **14**:205–215.
433. Larsson R, Akerstrom G, Gylfe E, Johansson H, Ljunghall S, Rastad J, Wallfelt C 1985 Paradoxical effects of K^+ and D-600 on parathyroid hormone secretion and cytoplasmic Ca^{2+} in normal bovine and pathological human parathyroid cells. Biochim Biophys Acta **847**:263–269.
434. Hove K, Sand O 1981 Evidence for a function of calcium influx in the stimulation of hormone release from the parathyroid gland in the goat. Acta Physiol Scand **113**:37–43.
435. Seely EW, LeBoff S, Brown EM, Chen C, Posillico JT, Hollenberg NK, Williams GH 1989 The calcium channel blocker diltiazem lowers serum parathyroid hormone levels *in vivo* and *in vitro*. J Clin Endocrinol Metab **68**:1007–1012.
436. Cooper CW, Borosky SA, Farrell PE, Steinsland S 1986 Effects of the calcium channel activator BAY-K-8644 on *in vitro* secretion of calcitonin and parathyroid hormone. Endocrinology **118**:545–549.
437. Ross III AJ, Cooper CW, Ramp WK, Wells Jr SA 1979 Concurrent secretion of calcitonin and parathyroid hormone *in vitro*: Effects of drugs that alter cellular calcium transport. Surg Forum **30**:102–104.
438. Fox J, Della-Santina CP 1989 Oral verapamil and calcium and vitamin D metabolism in rats: Effect of dietary calcium. Am J Physiol **257**:E632–E638.
439. Bogin E, Chagnac A, Juppner H, Levi J 1987 Effect of verapamil on plasma parathyroid hormone. J Clin Chem Clin Biochem **25**:83–85.
440. Frishman WH, Klein NA, Charlap S, Klein P, Norsratian F, Strom JA, Sherwood LM 1984 Comparative effects of verapamil and propanolol on parathyroid hormone and serum calcium concentration. In: Packer M, Frishman WH (eds) Calcium Channel Antagonists in Cardiovascular Disease. Appleton-Century-Crofts: Norwalk, Connecticut, pp. 1445–1449.
441. Gozzelino G, Fubini A, Isaia GG, Soagliotti G, Gamna G 1981 Evaluation of calcium metabolism and pituitary gonadotropic secretion during treatment with nifedipine. G Ital Cardiol **11**:1445–1449.
442. Townsend R, Dipette DJ, Evans RR, Davis WR, Green A, Graham GA, Wallace JM, Holland OB 1990 Effects of calcium channel blockade on calcium homeostasis in mild to moderate essential hypertension. Am J Med Sci **300**: 133–137.
433. Albers MM, Johnson W, Vivian V, Jackson RD 1991 Chronic use of the calcium channel blocker nifedipine has no significant effect on bone metabolism in men. Bone **12**:39–42.
434. Renton KW 1985 Inhibition of hepatic microsomal drug metabolism by the calcium channel blockers diltiazem and verapamil. Biochem Pharmacol **34**:2549–2553.
435. Avioli LV 1975 Heparin-induced osteopenia: An appraisal. Adv Exp Med Biol **52**:375–387.
436. Sackler JP, Liu L 1973 Heparin-induced osteoporosis. Br J Radiol **46**:548–550.
437. Wise PH, Hall AJ 1980 Heparin-induced osteopenia in pregnancy. Br Med J **2**:110–111.
438. Griffiths HT, Liu DTY 1984 Severe heparin osteoporosis in pregnancy. Postgrad Med J **60**:424–425.
439. Mutoh S, Takeshita N, Yoshino T, Yamaguchi I 1993 Characterization of heparin-induced osteopenia in rats. Endocrinology **133**:2743–2748.
440. Matzsch T, Bergqvist D, Hedner U, Nilsson B, Ostergaard P 1986 Heparin-induced osteoporosis in rats. Thromb Haemost **56**:293–294.
441. Monreal M, Vinas L, Monreal L, Lavin S, Lafoz E, Angles AM 1990 Heparin-related osteoporosis in rats. Haemostasis **20**:204–207.
442. Aarskog D, Aksnes L, Lehmann V 1980 Low 1,25-dihydroxyvitamin D in heparin-induced osteopenia. Lancet **2**:650–651.
443. Haram K, Hervig T, Thordarson H, Aksnes L 1993 Osteopenia caused by heparin treatment in pregnancy. Acta Obstet Gynecol Scand **72**:674–675.
444. Hurley MM, Gronowicz G, Kream BE, Raisz LG 1990 Effect of heparin on bone formation in cultured fetal rat calvaria. Calcif Tissue Int **46**:183–188.
445. Hurley MM, Kream BE, Raisz LG 1990 Structural determinants of the capacity of heparin to inhibit collagen synthesis in 21-day fetal rat calvariae. J Bone Miner Res **5**:1127–1133.
446. Chowdhury MH, Hamada C, Dempster DW 1992 Effects of heparin on osteoclast activity. J Bone Miner Res **7**:771–777.

447. Hahnemann S 1965 Heparin and osteoporosis. Lancet **2**:855–856.
448. Ellis HA 1965 Effects of long-term administration to animals of dextran sulfate. J Pathol Bacteriol **89**:437–460.
449. Kock HJ, Handschin AE 2002 Clin Appl Thromb Hemost **8**:251–255.
450. Street J, McGrath M, O'Regan K, Wakai A, McGuinness A, Redmond P 2000 Clin Orthopaed and Related Res **381**:278–289.
451. Casele HL, Laifer SA 2000 J Matern Fetal Med **9**:122–125.
452. Williams GA, Longley RS, Bowser EN, Hargis GK, Kukreja SC, Vora NM, Johnson PA, Jackson BL, Kawahara WJ, Henderson WJ 1981 Parathyroid hormone secretion in normal man and in primary hyperparathyroidism: Role of histamine H_2 receptors. J Clin Endocrinol Metab **52**:122–127.
453. Abboud HE, Zimmerman D, Edis AJ, Dousa TP 1980 Histamine and parathyroid adenoma: Effect on cyclic AMP accumulation. Clin Res **28**:515A.
454. Sherwood JK, Ackroyd FW, Garcia M 1980 Effect of cimetidine on circulating parathyroid hormone in primary hyperparathyroidism. Lancet **1**:616–620.
455. Wiske PS, Epstein S, Norton JA Jr, Bell NH, Johnston CC Jr 1983 The effects of intravenous and oral cimetidine in primary hyperparathyroidism. Horm Metab Res **15**:245–248.
456. Jacob AI, Lanier D Jr, Canterbury J, Bourgoignie JJ 1980 Reduction by cimetidine of serum parathyroid hormone levels in uremic patients. N Engl J Med **302**:671–674.
457. Beehler CJ, Beckner JR, Rosenquist RC, Shankel SW 1980 Parathyroid hormone suppression by cimetidine in uremic patients. Ann Intern Med **93**:840–841.
458. Palmer FJ, Sawyers TM, Wierzbinski SJ 1980 Cimetidine and hyperparathyroidism. N Engl J Med **302**:692.
459. Ghishan FK, Walker F, Meneely R, Patwardhan R, Speeg KV Jr 1981 Intestinal calcium transport: Effect of cimetidine. J Nutr **111**:2157–2161.
460. Speeg KV Jr, Patwardhan RV, Avant GR, Mitchell MC, Schenker S 1982 Inhibition of microsomal drug metabolism by histamine H_2-receptor antagonists studied *in vivo* and *in vitro* in rodents. Gastroenterology **82**:89–96.
461. Henry DA, Macdonald IA, Kitchingman G, Bell GD, Langman MJS 1980 Cimetidine and ranitidine: Comparison of effects on hepatic drug metabolism. Br Med J **281**:775–777.
462. Bengoa JM, Bolt MJ, Rosenberg IH 1984 Hepatic vitamin D 25-hydroxylase inhibition by cimetidine and isoniazid. J Clin Lab Med **104**:546–552.
463. Wyatt C, Jensen LS, Rowland III GN 1990 Effect of cimetidine on eggshell quality and plasma 25-hydroxycholecalciferol in laying hens. Poultry Sci **69**:1892–1899.
464. Odes HS, Fraser GM, Krugliak P, Lamprecht SA, Shany S 1980 Effect of cimetidine on hepatic vitamin D metabolism in humans. Digestion **46**:61–64.
466. Klein GL, Horst RL, Norman AW, Ament ME, Slatopolsky E, Coburn JW 1981 Reduced serum levels of la,25-dihydroxyvitamin D during long-term total parenteral nutrition. Ann Intern Med **94**:638–643.
467. Klein GL, Alfrey AC, Miller NL, Sherrard DJ, Hazlet TK, Ament ME, Coburn JW 1982 Aluminum loading during parenteral nutrition. Am J Clin Nutr **35**:1425–1429.
468. Ott SM, Maloney NA, Klein GL, Alfrey AC, Ament ME, Coburn JW, Sherrard DJ 1983 Aluminum is associated with low bone formation in patients receiving chronic parenteral nutrition. Ann Intern Med **98**:910–914.
469. Parkinson IS, Feest TG, Ward MK, Fawcett RWP, Kerr DNS 1979 Fracturing dialysis osteodystrophy and dialysis encephalopathy. Lancet **1**:406–409.
470. Pierides AM, Edwards WG Jr, Cullum UX Jr, McCall JT, Ellis HA 1980 Hemodialysis encephalopathy with osteomalacic fractures and muscle weakness. Kidney Int **18**:115–124.
471. Ott SM, Maloney NA, Coburn JW, Alfrey AC, Sherrard DJ 1982 The prevalence of bone aluminum deposition in renal osteodystrophy and its relation to the response to calcitriol therapy. N Engl J Med **307**:709–713.
472. Maloney NA, Ott SM, Alfrey AC, Miller NL, Coburn JW, Sherrard DJ 1982 Histological quantitation of aluminum in iliac bone from patients with renal failure. J Clin Lab Med **99**:206–216.
473. Cournot-Witmer G, Zingraff J, Plachot JJ, Escaig F, Lefevre R, Boumati P, Bourdeau A, Garabedian M, Galle P, Bourdon R, Drueke T, Balsan S 1981 Aluminum localization in bone from hemodialyzed patients: Relationship to matrix mineralization. Kidney Int **20**:375–385.
474. Ellis HA, McCarthy JH, Herrington J 1979 Bone aluminum in haemodialysed patients and in rats injected with aluminium chloride: Relationship to impaired bone mineralization. J Clin Pathol **32**:832–844.
475. Hodsman AB, Sherrard DJ, Alfrey AC, Ott S, Brickman AS, Miller NS, Maloney NA, Coburn JW 1982 Bone aluminum and histomorphometric features of renal osteodystrophy. J Clin Endocrinol Metab **54**:539–546.
476. Alfrey AC, Hegg A, Craswell P 1980 Metabolism and toxicity of aluminum in renal failure. Am J Clin Nutr **33**:1509–1516.
477. Cann CE, Prussin SG, Gordan GS 1979 Aluminum uptake by the parathyroid glands. J Clin Endocrinol Metab **49**:543–545.
478. Morrissey J, Rothstein M, Mayor G, Slatopolsky E 1983 Suppression of parathyroid hormone secretion by aluminum. Kidney Int **23**:699–704.
479. Robertson JA, Felsenfeld AJ, Haygood CC, Wilson P, Clarke C, Llach F 1983 Animal model of aluminum-induced osteomalacia: Role of chronic renal failure. Kidney Int **23**:327–335.
480. Chan YL, Alfrey AC, Posen S, Lissner D, Hills E, Dunstan CR, Evans RA 1983 Effect of aluminum on normal and uremic rats: Tissue distribution, vitamin D metabolites, and quantitative bone histology. Calcif Tissue Int **35**:344–351.
481. Henry DA, Goodman WG, Nudelman RK, DiDomenico NC, Alfrey AC, Slatopolsky E, Stanley TM, Coburn JW 1984 Parenteral aluminum administration in the dog: I. Plasma kinetics, tissue levels, calcium metabolism, and parathyroid hormone. Kidney Int **25**:362–369.
482. Goodman WG, Henry DA, Horst R, Nudelman RK, Alfrey AC, Coburn JW 1984 Parenteral aluminum administration in the dog: II. Induction of osteomalacia and effect on vitamin D metabolism. Kidney Int **25**:370–375.
483. DeLuca HF 1994 Third F Raymond Keating Jr Memorial symposium parathyroid hormone, calcitonin, and vitamin D. Clinical consideration. II. Vitamin D—1973. Am J Med **57**:1–12.
484. Berlyne GM, Pest D, Ben-Ari J, Weinberger J, Stern M, Gilmore GR, Levine R 1970 Hyperaluminaemia from aluminum resins in renal failure. Lancet **2**:494–496.
485. Clarkson EM, Luck VA, Hynson WV, Bailey RR, Eastwood JB, Woodhead JS, Clements VR, O'Riordan JLH, De Wardener HE 1972 The effect of aluminum hydroxide on calcium, phosphorus, and aluminum balances, the serum parathyroid hormone concentration and the aluminum content of bone in patients with chronic renal failure. Clin Sci **43**:519–531.
486. Recker RR, Blotcky AJ, Leffler JA, Rack EP 1977 Evidence for aluminum absorption from the gastrointestinal tract and bone deposition by aluminum carbonate ingestion with normal renal function. J Clin Lab Med **90**:810–815.

487. Villa ML, Packer E, Cheema M, Holloway L, Marcus R 1991 Effects of aluminum hydroxide on the parathyroid vitamin D axis of postmenopausal women. J Clin Endocrinol Metab **73**:1256–1261.
488. Bloom WL, Flinchum D 1960 Osteomalacia and pseudofractures caused by ingestion of aluminum hydroxide. JAMA **174**:1227–1230.
489. Chines A, Pacifi R 1990 Antacid and sulcrafate-induced hypophosphatemic osteomalacia. A case report and review of the literature. Calcif Tissue Int **47**:291–295.
490. Woodson GC 1998 An interesting case of osteomalacia due to antacid use associated with stainable bone aluminum in a patient with normal renal function. Bone **22**:695–698.
491. Pattaragarn A, Alon US 2001 Antacid-induced rickets in infancy. Clin Pediatr **40**:389–393.
492. Shah SC, Sharma RK, Chitle H, Chitle AR 1981 Rifampicin-induced osteomalacia. Tubercle **62**:207–209.
493. Brodie MJ, Boobis AR, Dollery CT, Hillyard CJ, Brown DJ, Maclntyre I, Park BK 1980 Rifampicin and vitamin D metabolism. Clin Pharmacol Ther **27**:810–814.
494. Onhaus E, Park BK 1979 Measurement of urinary 6-/3-hydroxycortisol excretion as an *in vivo* parameter in the clinical assessment of the microsomal enzyme-inducing capacity of antipyrine, phenobarbitone, and rifampicin. Eur J Clin Pharmacol **15**:139–145.
495. Madhok TC, Schnoes HK, DeLuca HF 1978 Incorporation of oxygen-18 into the 25-position of cholecalciferol by hepatic cholecalciferol 25-hydroxylase. Biochem J **175**:479–482.
496. Kutt H, Verebely K, McDowell F 1968 Inhibition of diphenylhydantoin metabolism in rats and in rat liver microsomes by antitubercular drugs. Neurology **18**:706–710.
497. Ghazarian JG, DeLuca HF 1977 Kidney microsomal metabolism of 25-hydroxyvitamin D_3. Biochem Biophys Res Commun **75**:550–555.
498. Brodie MJ, Boobis AR, Hillyard CJ, Abeyasekera G, Stevenson JC, Maclntyre I, Park BK 1982 Effect of rifampicin and isoniazid on vitamin D metabolism. Clin Pharmacol Ther **32**:525–530.
499. Toppet M, Vainsel M, Vertongen F, Fuss M, Cantraine F 1988 Evolution sequentielle des metabolites de la vitamine D sous isoniazide et rifampicine. Arch Francaises Pediatr **45**:145–148.
500. Saggese G, Cesaretti G, Bertelloni S, Morganti E, Bottone E 1985 Isoniazid and vitamin D metabolism. In: Norman AW, Schaefer K, Grigoleit H-G, Herrath DV (eds) Vitamin D: Chemical, Biochemical, and Clinical Update. de Gruyter: Berlin, pp. 1123–1124.
501. Perry W, Brown J, Erooga MA, Stamp TCB 1982 Calcium metabolism during rifampicin and isoniazid therapy for tuberculosis. J R Soc Med **75**:533–536.
502. Williams SE, Wardman AG, Taylor GA, Peacock M, Cooke NJ 1985 Long-term study of the effect of rifampicin and isoniazid on vitamin D metabolism. Tubercle **66**:49–54.
503. Martinez ME, Gonzalez J, Sanchez MJ, Pena JM, Vasquez JJ 1996 Remission of hypercalcuria in patients with tuberculosis after treatment. Calcif Tissue Int **59**:17–20.
504. Daniell HW 1976 Osteoporosis of the slender smoker. Arch Intern Med **136**:298–304.
505. Heany RP, Recker RR 1982 Effects of nitrogen, phosphorus, and caffeine on calcium balance in women. J Clin Lab Med **99**:46–55.
506. Heaney RP 2002 Effects of caffeine on bone and calcium economy. Food and Chem Toxicol **40**:1263–1270.
507. Barger-Lux MJ, Heaney RP 1995 Caffeine and the calcium economy revisited. Osteopor Internat **5**:97–102.
508. Hannan MT, Felson DT, Dawson-Hughes B, Tucker KL, Cupples LA, Wilson PW, Kiel DP 2000 Risk factors of longitudinal bone loss in elderly men and women. The Framingham Osteoporosis Study. J Bone Miner Res **14**:710–720.
509. Rico H, Canal ML, Manas P, Lavado JM, Costa C, Pedrera JD 2002 Nutrition **18**:189–193.
510. Lloyd T, Rollings N, Eggli DF, Kieselhorst K, Chinchilli VM 1997 Dietary caffeine intake and bone status of postmenopausal women. Am J Clin Nutr **65**:1826–1830.
511. Rapuri PR, Gallagher JC, Kinyamu HK, Ryschon KL 2001 Caffeine intake increases the rate of bone loss in elderly women and interacts with the vitamin D receptor genotypes. Am J Clin Nutr **74**:694–700.
512. Mitoma C, Lombrozo L, LeValley SE, Dehn F 1969 Nature of the effect of caffeine on the drug metabolizing enzymes. Arch Biochem Biophys **134**:434–441.
513. Thithapandha A, Chaturapit S, Limlomwongse L, Sobhon P 1974 The effects of xanthines on mouse liver cell. Arch Biochem Biophys **161**:178–186.
514. Taft JL, French M, Danks JA, Larkins RG 1984 Opposing actions of methylxanthines and dibutyryl cAMP on 1,25 dihydroxyvitamin D_3 production and calcium fluxes in isolated chick renal tubules. Biochem Biophys Res Commun **121**:355–363.
515. Yeh JK, Aloia JF, Semla HM, Chen SY 1986 Influence of injected caffeine on the metabolism of calcium and the retention and excretion of sodium, potassium, phosphorus, magnesium, zinc, and copper in rats. J Nutr **116**:273–280.
516. Yeh JK, Aloia JF 1986 Differential effects of caffeine administration on calcium and vitamin D metabolism in young and adult rats. J Bone Miner Res **1**:251–258.
517. Glajchen N, Ismail F, Epstein S, Jowell PS, Fallen M 1988 The effect of chronic caffeine administration on serum markers of bone mineral metabolism and bone histomorphometry in the rat. Calcif Tissue Int **43**:277–280.
518. Kulkowski JA, Chan T, Martinez J, Ghazarian JG 1979 Modulation of 25-hydroxyvitamin D_3-24-hydroxylase by aminophylline: A cytochrome P-450 monooxygenase system. Biochem Biophys Res Commun **90**:50–57.
519. Pedersen JI, Shobaki HH, Holmberg I, Bergseth S, Bjorkhem I 1983 25-Hydroxyvitamin D_3-24-hydroxylase in rat kidney mitochondria. J Biol Chem **258**:742–746.
520. Fortenbery EJ, McDermott MT, Duncan WE 1999 Effect of theophylline on calcium metabolism and circulating vitamin D metabolites. J Bone Miner Res **5**:321–324.
521. Lohmann SM, Miech RP 1976 Theophylline metabolism by the rat liver microsomal system. J Pharmacol Exp Ther **196**:213–225.
522. Colin A, Kraiem Z, Kahana KL, Hochberg Z 1984 Effects of theophylline on urinary excretion of cyclic AMP, calcium, and phosphorus. Miner Electrolyte Metab **10**:359–361.
523. McPherson ML, Prince SR, Atamer ER, Maxwell DB, Ross-Clunis H, Estep HL 1986 Theophylline-induced hypercalcemia. Ann Intern Med **105**:52–54.
524. Movsowitz C, Epstein S, Fallen M, Ismail F, Thomas S 1988 Cyclosporin A *in vivo* produces severe osteopenia in the rat: Effect of dose and duration of administration. Endocrinology **123**:2571–2577.
525. Movsowitz C, Epstein S, Ismail F, Fallon M, Thomas S 1989 Cyclosporin A in the oophorectomized rat: Unexpected severe bone resorption. J Bone Miner Res **4**:393–398.
526. Schlosberg M, Movsowitz C, Epstein S, Fallon MD, Thomas S 1989 The effect of cyclosporin A administration and its

withdrawal on bone mineral metabolism in the rat. Endocrinology **124**:2179–2184.
527. Stein B, Halloran BP, Reinhardt G, Engstrom GW, Bales CW, Drezner MK, Currie KL, Takizawa M, Adams JS, Epstein S 1991 Cyclosporin-A increases synthesis of 1,25-dihydroxyvitamin D_3 in the rat and mouse. Endocrinology **128**: 1369–1373.
528. Katz I, Li M, Joffe I, Stein B, Jacobs T, Liang X, Ke H, Jee W, Epstein S 1994 The influence of age on cyclosporin A-induced alterations in bone mineral metabolism in the rat *in vivo*. J Bone Miner Res **9**:59–67.
529. Grenet O, Bobadilla M, Salah-Dine C, Steiner S 2000 Evidence for the impairment of the vitamin D activation pathway by cyclosporine A. Biochemical Pharmacology **59**:267–272.
530. Buchinsky FJ, Ma YF, Mann G, Rucinski B, Bryer HP, Romero DF, Jee WSS, Epstein S 1996 T-lymphocytes play a critical role in the development of cyclosporine-induced osteopenia. Endocrinology **137**:2278–2285.
531. Rooth P, Dawidson I, Diller K, Clothier N 1988 *In vivo* fluorescence microscopy reveals cyclosporine G to be less nephrotoxic than cyclosporine A. Trans Proc **20**:707–709.
532. Tejani A, Lancman I, Pomarantz A, Khawar M, Chen C 1988 Nephrotoxicity of cyclosporine A and cyclosporine G in a rat model. Transplantation **45**:184–187.
533. Jacobs TW, Katz IA, Joffe II, Stein B, Takizawa M, Epstein S 1991 The effect of FK 506, cyclosporine A, and cyclosporine G on serum 1,25-dihydroxyvitamin D_3 levels. Trans Proc **23**: 3188–3189.
534. Stein B, Takizawa M, Schlosberg M, Movsowitz C, Fallon M, Berlin JA, Epstein S 1992 Evidence that cyclosporine G is less deleterious to rat bone *in vivo* than cyclosporine A. Transplantation **53**:628–632.
535. Cvetkovic M, Mann GN, Romero DF, Liang X, Ma YF, Jee WSS, Epstein S 1994 The deleterious effects of long-term cyclosporin A, cyclosporin G, and FK506 on bone mineral metabolism *in vivo*. Transplantation **57**:1231–1237.
536. Morris R 1994 Modes of action of FK506, cyclosporin A, and rapamycin. Trans Proc **26**:3272–3275.
537. Bryer HP, Isserow JA, Armstrong EC, Mann GN, Rucinski B, Buchinsky FJ, Romero DF, Epstein S 1995 Azathioprine alone in bone sparing and does not alter cyclosporin A-induced osteopenia in the rat. J Bone Miner Res **10**:132–138.
538. Romero DF, Buchinsky FJ, Rucinski B, Cvetkovic M, Bryer HP, Liang XG, Ma YF, Jee SS, Epstein S 1995 Rapamycin: A bone sparing immunosuppressant? J Bone Miner Res **10**:760–768.
539. Joffe I, Katz I, Sehgal S, Bex F, Kharode Y, Tamasi J, Epstein S 1993 Lack of change of cancellous bone volume with short-term use of the new immunosuppressant rapamycin in rats. Calcif Tissue Int **53**:45–52.
540. Monier-Faugere MC, Mawad H, QI Q, Friedler RM, Malluche HH 2000 High prevalence of low bone turnover and occurrence of osteomalacia after kidney transplantation. J Am Soc Nephrol **11**:1093–1099.
541. Saha HHT, Salmela KT, Ahonen PJ, Pietila KO, Morsky PJ, Mustonen JT, Lalla MLT, Pasternak AI 1994 Sequential changes in vitamin D and calcium metabolism after successful renal transplantation. Scand J Urol Nephrol **28**:21–27.
542. Riancho JA, de Francisco ALM, del Arco C, Amado JA, Cotorruelo JG, Arias M, Gonzalez-Marcias J 1988 Serum levels of 1,25-dihydroxyvitamin D after renal transplantation. Miner Electrolyte Metab **14**:332–337.
543. Valero MA, Loinaz C, Larrodera L, Leon M, Moreno E, Hawkins F 1995 Calcitonin and bisphosphonates treatment in bone loss after liver transplantation. Calcif Tissue Int **57**:15–19.
544. McDonald JA, Dunstan CR, Dilworth P, Sherbon K, Ross Sheil AG, Evans RA, McCaughan GW 1991 Bone loss after liver transplantation. Hepatology **14**:613–619.
545. Shane E, Rivas M, Staron RB, Silverberg SJ, Seibel MJ, Kuiper J, Mancini D, Addresso V, Michler RE, Factor-Litvak P 1996 Fracture after cardiac transplantation: A prospective longitudinal study. J Clin Endocrinol Metab **81**:1740–1746.
546. Cundy T, Kanis JA, Heynen G, Morris PJ, Oliver DO 1983 Calcium metabolism and hyperparathyroidism after renal transplantation. Q J Med **205**:67–78.
547. Felsenfeld AJ, Gutman RA, Drezner M, Llach F 1986 Hypophosphatemia in long-term renal transplant recipients: Effects on bone histology and 1,25-dihydroxycholecalciferol. Miner Electrolyte Metab **12**:333–341.
548. Sakhee K, Brinker K, Helderman JH, Bengfort JL, Nicar MJ, Hull AR, Pak CYC 1985 Disturbances in mineral metabolism after successful renal transplantation. Miner Electrolyte Metab **11**:167–172.
549. Reichel H, Griibinger A, Knehans A, Kiihn K, Schmidt-Gayk H, Ritz E 1992 Long-term therapy with cyclosporin A does not influence serum concentrations of vitamin D metabolites in patients with multiple sclerosis. Clin Invest **70**:595–599.
550. Hanley DA, Ayer LM, Gundberg CM, Minuk GY 1991 Parameters of calcium metabolism during a pilot study of cyclosporin A in patients with symptomatic primary biliary cirrhosis. Clin Invets Med **14**:282–287.
551. Stempfle H, Werner C, Siebert U, Assum T, *et al*. 2002 The role of tacrolimus-based immunosuppression on bone density and bone turnover after cardiac transplantation: a prospective, longitudinal, randomized, double-blind trial with calcitriol. Transplantation **73**:547–552.
552. Epstein S, Shane E 1996 Transplantation osteoporosis. In: R Marcus, D Feldman, J Kelsey (eds) Osteoporosis Academic Press: New York, pp. 947–957.
553. Shane E, Epstein S 1994 Immunosuppressive therapy and the skeleton. Trends Endocrinol Metab **4**:169–175.
554. Turner RT, Francis R, Brown D, Garand J, Hannon KS, Bell NH 1989 The effects of fluoride on bone and implant histomorphometry in growing rats. J Bone Miner Res **4**: 477–484.
555. Manzke E, Rawley R, Vose G, Roginsky M, Rader JI, Baylink DJ 1977 Effect of fluoride therapy on nondialyzable urinary hydroxyproline, serum alkaline phosphatase, parathyroid hormone, and 25-hydroxyvitamin D. Metabolism **26**:1005–1010.
556. Dure-Smith BA, Parley SM, Linkhart SG, Parley JR, Baylink DJ 1996 Calcium deficiency in fluoride-treated osteoporotic patients despite calcium supplementation. J Clin Endocrinol Metab **81**:269–275.
557. Fallat RW, Glueck CJ, Lutmer R, Mattson FH 1976 Short-term study of sucrose polyester a nonabsorbable fa-like material as a dietary agent for lowering plasma cholesterol. Am J Clin Nutr **29**:1204–1215.
558. Grouse JR, Grundy SM 1979 Effects of sucrose polyester on cholesterol metabolism in man. Metabolism **28**:994–1000.
559. Mattson FH, Volpenhein RA 1972 Rate and extent of absorption of the fatty acids of fully esterified glycerol, erythritol, xylitol, and sucrose as measured in thoracic duct cannulated rats. J Nutr **102**:1177–1180.
560. Mattson FH, Volpenhein RA 1987 Hydrolysis of fully esterified alcohols containing from one to eight hydroxyl groups by the lipolytic enzymes of rat pancreatic juice. J Lipid Res **13**:325–328.

561. Kuksis A 1987 Absorption of fat-soluble vitamins In: Kuksis A (ed) Fat Absorption, Vol 2. CRC Press: Boca Raton, Florida, pp. 65–86.
562. Lueck CJ, Hastings MM, Alien C, Hogg E, Baehler E, Gartside PS, Phillips D, Jones M, Hollenboch EJ, Braun B, Anastasia JV 1982 Sucrose polyester and covert caloric dilution. Am J Clin Nutr **35**:1352–1359.
563. Mellies MJ, Vitale C, Jandacek RJ, Lamkin GE, Glueck CJ 1985 The substitution of sucrose polyester for dietary fat in obese, hypercholestrolemic outpatients. Am J Clin Nutr **41**:1–12.
564. Jones DY, Miller KW, Koonsvitsky BP, Ebert ML, Lin PYT, Jones MB, DeLuca HF 1991 Serum 25-hydroxyvitamin D concentrations of free-living subjects consuming olestra. Am J Clin Nutr **53**:1281–1287.
565. Mellies MJ, Jandacek RJ, Taulbee JD, Tweksbury MB, Lamkin G, Baehler L, King P, Boggs D, Goldman S, Gouge A, Tsang R, Glueck CJ 1983 A double-blind, placebo-controlled study of sucrose polyester in hypercholesterolemic outpatients. Am J Clin Nutr **37**:339–346.
566. Thornquist MD, Kristal AR, Patterson RE, Neuhouser ML, Rock CL, Neumark-Sztainer D, Cheskin LJ 2000 J Nutr. **130**:1711–1718.
567. Miller KW, Wood FE, Stuard SB, Alden CL 1991 A 20-month olestra feeding study in dogs. Food Chem Toxic **29**:427–435.
568. Lawson DEM, Paul AA, Black AE, Cole TJ, Mandal AR, Davie M 1979 Relative contributions of diet and sunlight to vitamin D state in the elderly. Br Med J **2**:303–305.
569. Poskitt EME, Cole TJ, Lawson DEM 1979 Diet, sunlight, and 25-hydroxyvitamin D in healthy children and adults. Br Med J **1**:221–223.
570. Haddad JG, Hahn TJ 1973 Natural and synthetic sources of circulating 25-hydroxyvitamin D in man. Nature **244**: 515–516.
571. McDuffie JR, Calis KA, Booth SL, Waifo GI, Yanovski JA 2002 Effects of orlistat on fat soluble vitamins in obese adolescents. Pharmacotherapy 814–822.
572. Gotfredsen A, Westergren Hendel H, Anderson T 2001 Influence of orlistat on bone turnover and body composition. Int J Obes Relat Metab Disord **25**:1154–1160.
573. Mellerup ET, Lauritsen B, Dam H, Rafaelson OJ 1976 Lithium effects on diurnal rhythms of calcium, magnesium, and phosphate metabolism in manic-melancholic disorder. Acta Psychiat Scand **53**:360–370.
574. Davis BM, Pfefferbaum A, Krutzik S, Davis KL 1981 Lithium's effect on parathyroid hormone. Am J Psychiatry **138**:489–492.
575. Plenge P, Rafaelsen OJ 1982 Lithium effects on calcium, magnesium, and phosphate in man: Effects on balance, bone mineral content, faecal, and urinary excretion. Acta Psychiat Scand **66**:361–373.
576. Mallette LE, Khouri K, Zengotita H, Hollis BW, Malini S 1989 Lithium treatment increases intact and midregion parathyroid hormone and parathyroid volume. J Clin Endocrinol Metab **68**:654–660.
577. Nielsen JL, Christiansen MS, Pedersen EB, Darling S, Amdisen A 1977 Parathyroid hormone in serum during lithium therapy. Scand J Clin Lab Invest **37**:369–372.
578. Miller PD, Dubovsky SL, McDonald KM, Arnaud C, Schrier RW 1978 Hypocalciuric effect of lithium on man. Miner Electrolyte Metab **1**:3–11.
579. Bjorum N, Hornum I, Mellerup ET, Plenge PK, Rafaelsen OJ 1975 Lithium, calcium, and phosphate. Lancet **1**:1243.
580. Birnbaum J, Klandorf H, Giuliano A, Van Herle A 1988 Lithium stimulates the release of human parathyroid hormone *in vitro*. J Clin Endocrinol Metab **66**:1187–1191.
581. Christiansen C, Baastrup PC, Transbol I 1976 Lithium, hypercalcemia, hypermagnesaemia, and hyperparathyroidism. Lancet **1**:969.
582. Shen F-H, Sherrard DJ 1982 Lithium-induced hyperparathyroidism: An alteration of the "set point." Ann Intern Med **96**:63–65.
583. Christiansen C, Baastrup PC, Lindgreen P, Transol I 1978 Endocrine effects of lithium: II. Primary hyperparathyroidism. Acta Endocrinol **88**:528–534.
584. Christiansen C, Baastrup P, Transol I 1980 Development of primary hyperparathyroidism during lithium therapy. Neuropsychobiology **6**:280–283.
585. Nordenstrom J, Elvius M, Bagedahl-Strindlund M, Zhao B, Torring O 1994 Biochemical hyperparathyroidism and bone mineral status in patients treated long-term with lithium. Metabolism **43**:1562–1567.
586. Perez GO, Oster JR, Vaavioucle CA 1975 Incomplete syndrome of renal tubular acidosis induced by lithium carbonate. J Clin Lab Med **86**:386–394.
587. Nordenstrom J, Strigard K, Perbeck L, Willems J, Bagedahl-Strindlund M, Linder J 1992 Hyperparathyroidism associated with treatment of manic-depressive disorders by lithium. Eur J Surg **158**:207–211.
588. Mallette LE, Eichhorn E 1986 Effects of lithium carbonate on human calcium metabolism. Arch Intern Med **146**:770–776.
589. Hestbech J, Hansen HE, Amdisen A, Olsen S 1977 Chronic renal lesions following long-term treatment with lithium. Kidney Int **12**:205–213
590. Hansen HE, Hestbech J, Sorensen JL, Norgaard K, Heiskov J, Amidisen A 1979 Chronic interstitial nephropathy in patients on long-term lithium treatment. Q J Med **192**:577–591.
591. Rosenblatt S, Chanley JD, Segal RL 1989 The effect of lithium on vitamin D metabolism. Biol Psychiatry **26**:206–208.

CHAPTER 75

Bone Disorders Associated with Gastrointestinal and Hepatobiliary Disease

MICHAEL DAVIES, JACQUELINE L. BERRY AND ANDREW P. MEE
University of Manchester, Vitamin D Research Group, Department of Medicine,
Manchester Royal Infirmary, Manchester M13 9WL, UK

I. INTRODUCTION

The major bone diseases associated with gastrointestinal and liver disease are osteomalacia, caused by severe and prolonged deficiency of vitamin D, which may more correctly be described as vitamin D depletion, and, more commonly, osteoporosis in which decreased bone density may be partially attributable to low calcium intake or absorption and excessive bone resorption, which may be mediated by raised PTH. This latter state may arise as a result of an insufficiency (as opposed to depletion) of vitamin D or of calcium.

The gastrointestinal tract plays a primary role in the biological activity of vitamin D, so it is not surprising that diseases of the liver and intestine have consequences for vitamin D metabolism and function that may result in bone disease. The liver is the site of the first stage of the metabolic activation of vitamin D, namely the introduction of a hydroxyl group at C25, and is also important in the elimination of vitamin D metabolites in the bile (see Chapter 4). Furthermore, the liver is also the site of synthesis of the plasma vitamin D–binding protein (DBP, Chapters 8 and 9). Most importantly, the mucosa of the small intestine is a major target organ for the active metabolite of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which promotes the absorption of calcium from the diet. This is effected by genomic reactions in which $1,25(OH)_2D$, binding to its specific intranuclear receptor mediates the transcription of genes for proteins involved in calcium transport; recent evidence also supports the concept of a rapid nongenomic action on calcium flux involving a membrane receptor (see Chapters 11, 23, 24, 42).

II. METABOLIC DISTURBANCES IN GASTROINTESTINAL DISEASE

A. Development of Vitamin D Deficiency and Depletion

1. Controlling Factors in the Supply of Vitamin D

The physiological source of vitamin D is solar irradiation of the precursor 7-dehydrocholesterol in skin to give vitamin D_3, but both vitamin D_2 and D_3 can also be supplied in the diet. There is presently no agreed-upon definition of a sufficient level of vitamin D, in terms of serum 25-hydroxyvitamin D (25OHD) concentration (see discussion in Chapters 46, 61, and 62). However, the idea that an adequate vitamin D status can be defined as the absence of osteomalacia (see Section IIA1b) has been largely rejected. It is now evident that there is an intermediate state in which vitamin D levels are insufficient, but not frankly depleted. Such a state is associated with increased levels of parathyroid hormone (PTH), and is characterized by increased synthesis of $1,25(OH)_2D$, presumably driven by PTH, when a dose of vitamin D is given [1,2]. Because the suppression of PTH is seen as beneficial for bone, many now regard serum 25OHD concentrations of 50–75 nmol/l [3,4] or even higher as desirable [5], since at these concentrations PTH approaches a minimum in relation with 25OHD. A recent paper by Veith *et al.* [6], however, found no such plateau in PTH with increasing 25OHD, but demonstrated that, irrespective of age, PTH concentration decreased as 25OHD increased, although the decline in PTH was more gradual in the older

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patients for the same level of 25OHD. They suggested that to ensure that all adults have 25OHD levels of at least 40 nmol/l they should consume, at least, 20–25 μg (800–1000IU) of vitamin D daily. This is consistent with the findings of Glerup *et al.* [7] in their study of Danish women, who also proposed that adults with limited sunlight exposure should receive 800–1000 IU/day.

a. The Importance of the Intestine Gastrointestinal and hepatobiliary diseases are characterized by general problems with absorption of lipids, whether because of poorly functioning enterocytes, a reduction in their number, or lack of biliary secretion; as vitamin D is a lipid soluble molecule, malabsorption of dietary vitamin D may then contribute to vitamin D deficiency [8–10]. However, for people in those parts of the world where there is little fortification of foodstuffs with the vitamin, endogenous synthesis of vitamin D in the skin, as a result of solar exposure, is the major source of the vitamin [11,12] (also see Chapters 3 & 47). In this case, the diet supplies only a fraction, often less than 25–30%, of the daily requirement (see Fig. 1). Thus, in normal circumstances, partial impairment of dietary absorption would not be expected to have a major effect on vitamin D status. However, ill health, such as may be encountered in patients with serious gastrointestinal or liver disease, may lead to a reluctance or inability to spend time outdoors, thus decreasing the endogenous synthesis of vitamin D. When the opportunity to make vitamin D in the skin is minimal, provision of the vitamin in the diet, however little, becomes crucial [13], and any impairment of absorption is more likely to have a significant effect. Factors affecting the elimination of vitamin D by the liver may also play a critical role in the face of gastrointestinal and hepatobiliary disease and may contribute to a state of "acquired vitamin D deficiency," see Section IIA2c.

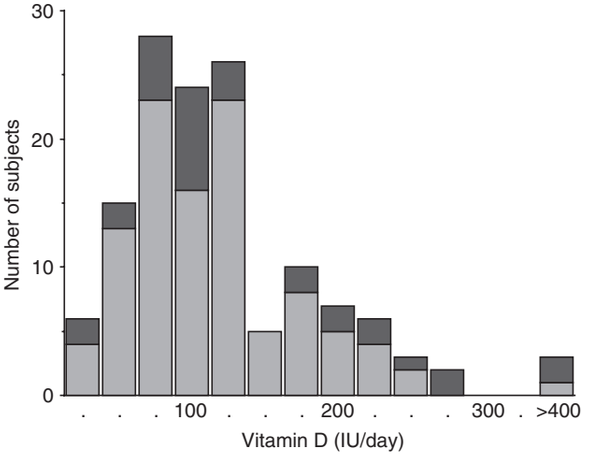

FIGURE 1 Daily dietary intake of vitamin D in a sample of Caucasians (light bars) and Asian immigrants (dark bars) living in northwest England.

b. The Significance of Changes in 1,25-dihydroxyvitamin D Concentration Vitamin D deficiency has been defined in various ways, the most extreme of which is the development of osteomalacia or rickets. An earlier indication is the measurement of serum 25OHD levels; concentrations associated with the clinical signs of deficiency are usually associated with levels below 12–20 nmol/l [14,15]. Also, biochemical indices such as the plasma concentrations of calcium, inorganic phosphate, parathyroid hormone, alkaline phosphatase, or osteocalcin are sometimes used. Ultimately, vitamin D deficiency must be characterized by the inability to synthesize sufficient $1,25(OH)_2D$ to enable adequate absorption of calcium from the intestine or to stimulate osteoblastic activity, and this state can then be defined as vitamin D depletion. During the development of vitamin D deficiency, whether arising from a lack of solar exposure, an inadequate dietary supply, or intestinal malabsorption, the concept of a normal range for the serum $1,25(OH)_2D$ concentration becomes invalid. The process can be envisaged as a progressive descending spiral in which low 25OHD results in a reduction of $1,25(OH)_2D$ synthesis, thus impairing calcium absorption (Fig. 2). The tendency for calcium to fall stimulates PTH secretion by the parathyroid glands, and the trophic action of PTH on the renal 1α-hydroxylase increases $1,25(OH)_2D$ synthesis, thus temporarily increasing the efficiency of intestinal calcium absorption. However, increased $1,25(OH)_2D$ synthesis depletes still further a diminishing supply of 25OHD, and the process will continue, (in the absence of a new supply of vitamin D) until there is insufficient substrate to provide the level of $1,25(OH)_2D$ required for adequate absorption of calcium, despite an increasing degree of secondary hyperparathyroidism. There is also evidence (Section IIA2c) that a raised concentration of $1,25(OH)_2D$ may itself influence the process, by shortening the serum half-life of 25OHD, thus accelerating the onset of vitamin D deficiency.

2. THE ROLE OF THE LIVER

The liver plays two major roles in vitamin D metabolism; 25-hydroxylation of vitamin D occurs in the endoplasmic reticulum of hepatocytes, and the liver is a major excretory organ, both of vitamin D and 25OHD (when these are present in excessive amounts), but mainly of water soluble compounds, principally glucuronide conjugates, all of which are present in bile [16–19]. There is a minimal effect on 25-hydroxylation in the liver if hepatocellular function is maintained, (Section V), and impairment of 25-hydroxylation is not

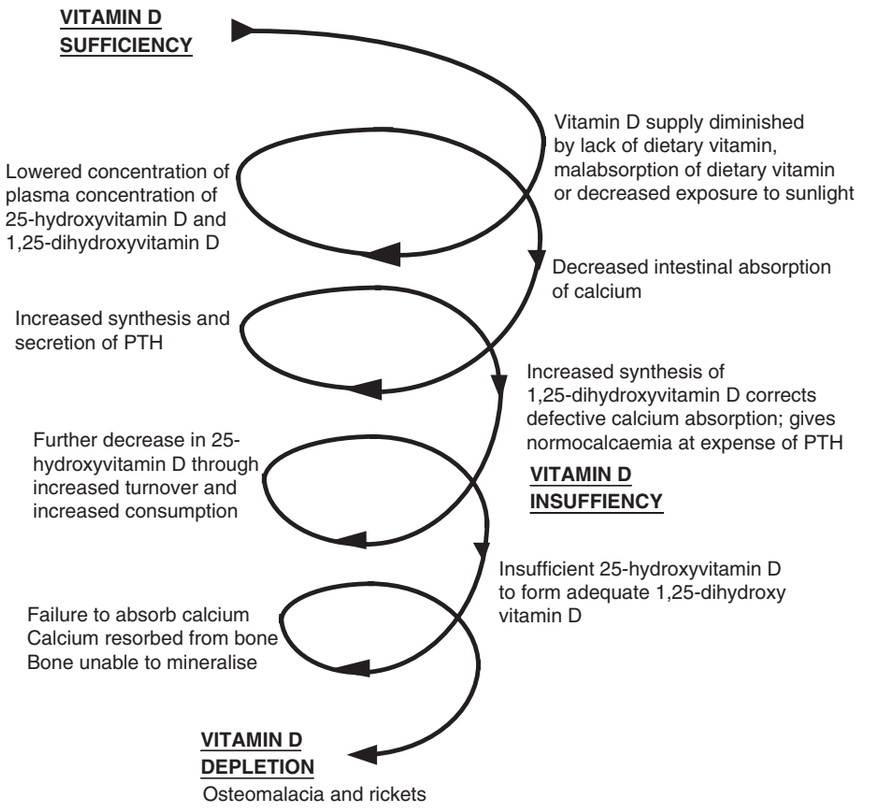

FIGURE 2 Spiral of developing vitamin D deficiency showing progression through a state of vitamin D insufficiency, characterized by secondary hyperparathyroidism and increased synthesis of 1,25-dihydroxyvitamin D.

believed to make a major contribution to the loss of vitamin D activity until the later stages of disease.

a. Biliary Secretion of Vitamin D Metabolites In normal subjects, the biliary-fecal route appears to be the major excretory pathway for vitamin D, but precise chemical identification of many of the excretory products is still awaited. The recognition of the importance of the renal 24-hydroxylase in producing metabolites such as 24,25-dihydroxyvitamin D ($24,25(OH)_2D$), which can then undergo sidechain cleavage and form carboxylic acids similar to bile acids [20,21], raises the question as to whether urine may be a major excretory route for water soluble vitamin D metabolites. However, the results of tracer experiments in humans show in general, preferential excretion via the intestinal tract, and hence do not support this argument [16–19]. Some experiments in rats seem to indicate considerable urinary excretion in this species [22], but in humans the balance of evidence is that the fecal excretion is much more important, except in the particular case of biliary obstruction in which metabolites normally excreted by the biliary-fecal route are instead passed through the kidneys, see Section V,B.

b. The Enterohepatic Circulation of Vitamin D and Its Metabolites The polar vitamin D derivatives in bile probably have no significant biological activity and only insignificant amounts of unchanged vitamin D or 25OHD are usually present [16–19]. Nevertheless, the idea that biologically active metabolites of vitamin D might enter a conservative enterohepatic circulation has proved an attractive one to attempt to explain the acquired vitamin D deficiency associated with various gastrointestinal diseases in which lipid absorption is impaired. It has been suggested that interruption of such a circulation would explain the wastage of vitamin D that occurs in conditions such as celiac disease and gastric or intestinal resection. However, most studies that appear to support such a hypothesis have used an intravenous bolus dose of labeled vitamin D or 25OHD [23]. In these circumstances, especially if a nonphysiological vehicle has been used, the labeled compound may be seen as a foreign compound and

cleared rapidly by the liver, mainly as hydrophilic metabolites, but also, immediately after injection, in the form of unchanged sterol. It has been argued that vitamin D, absorbed from the diet and entering the circulation rapidly in the form of chylomicrons, constitutes a nonphysiological presentation to the liver. The relative inefficiency of oral vitamin D, as opposed to that synthesized in the skin and transported to the liver on DBP, has been attributed to such a mechanism [24].

In a study often quoted to support the enterohepatic circulation hypothesis, a high proportion of injected radioactivity given as $25OHD_3$ was recovered in the bile, and was assumed still to be present in that form [23]. In fact, by comparison with other studies, it is most likely that, after the initial equilibration period when the 25OHD would have become bound to DBP, the label would be largely in the form of polar derivatives. Experiments in rats have demonstrated that if biliary excretion products of isotopically labeled vitamin D are collected and given to other animals, they can be absorbed and re-excreted in the bile [25]. There is little evidence, however, that these metabolites have biological activity; any conjugates would need to be cleaved enzymatically to release the vitamin D moiety, which is itself likely to be a catabolic product with low activity. It is, therefore, unlikely that any enterohepatic circulation of vitamin D metabolites that does occur is of a conservative nature or of physiological significance. Indeed, biliary excretion of vitamin D metabolites appears to be dose-dependent, increasing both in humans and rats when vitamin D is plentiful, and being suppressed in vitamin D deficiency [18,25]. An alternative hypothesis for the significance of the biliary secretion of vitamin D compounds is that this mechanism enables the body to dispose of a highly potent biological compound if present in excess amounts, and may be seen as a form of detoxification, rather than an attempt to conserve a scarce resource [24–26]. The potential importance of bile acids as ligands for the VDR is discussed in Chapter 53.

c. The Development of Acquired Vitamin D Deficiency

The concept of interruption of an enterohepatic circulation of vitamin D metabolites is also inadequate to explain the development of vitamin D deficiency in patients with gastrointestinal and hepatobiliary disease, see above [19,24]. In addition to patients with fat malabsorption, various other conditions are characterized by wasting of vitamin D. These include primary hyperparathyroidism, anticonvulsant therapy, and high cereal and high fiber diets. A theory has been proposed by Clements *et al.* [27] in an attempt to explain this phenomenon. In the rat, these authors showed that withdrawal of calcium from the diet increased the plasma clearance of 25OHD; a similar effect was observed when phenobarbitone was administered to animals on a normal calcium diet, and also when fiber was added to the diet. In addition, the plasma half-life of 25OHD was decreased by secondary hyperparathyroidism. Parallel studies on human subjects have shown that both primary and secondary hyperparathyroidism can increase the catabolism of $25OHD_3$ [28,29]. In all these studies, there was a strong inverse relationship between the plasma half-life of 25OHD and the prevailing concentration of $1,25(OH)_2D$, (see Fig. 3). In those conditions in which $1,25(OH)_2D$ was raised, ^{3}H-25OHD was removed from the circulation more rapidly, and there was a corresponding increase in fecal radioactivity [28]. The reasons for these phenomena are not fully understood, although it has been postulated that $1,25(OH)_2D$ may stimulate, perhaps by a receptor-mediated mechanism, the hepatic conjugation reactions that render vitamin D and 25OHD water soluble [29]. In addition, $1,25(OH)_2D$ is known to stimulate the renal 24-hydroxylase, which initiates the sidechain cleavage cascade, though the subsequent metabolic fate of these compounds in humans is unclear. The relationship between $1,25(OH)_2D$ level and the turnover of 25OHD is obtained in a variety of gastrointestinal conditions in which, as a result of problems with calcium absorption leading to secondary hyperparathyroidism, serum $1,25(OH)_2D$ is raised (see Sections IIB and III). The phenomenon of increased loss of plasma 25OHD in patients, in whom either dietary intake of the vitamin or solar exposure is inadequate, could help to explain the prevalence of vitamin D deficiency in gastrointestinal disease.

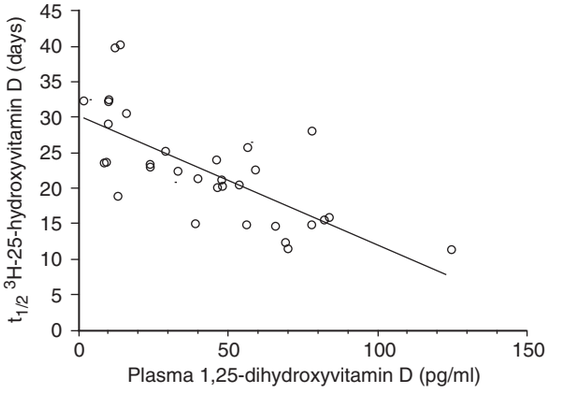

FIGURE 3 Strong inverse relationship between the plasma half-life of 25-hydroxyvitamin D and the prevailing concentration of 1,25-dihydroxyvitamin D, $r_s = -0.706$, $P < 0.001$. Data from studies reported by Clements *et al*. [28,29].

B. Malabsorption of Calcium

The development of osteomalacia in patients with steatorrhea is usually attributed to malabsorption of dietary vitamin D, but the degree of malabsorption is rarely severe enough to account for the development of vitamin D deficiency in populations with a high oral intake of the vitamin. Since the small intestine is a target organ for vitamin D, it is possible that the functional activity of $1,25(OH)_2D$ may be compromised (Chapter 24). Colston *et al*. [30] concluded that, although VDR were abundant in crypt cells from patients with celiac disease, malabsorption of calcium resulted from the loss of vitamin D–regulated proteins and enzymes located in the most mature enterocytes of the mid and tip villous regions. Absorption of dietary calcium has been found to be deficient in various types of gastrointestinal disease, including celiac disease, and Crohn's disease, in which fat malabsorption may lead to the formation of calcium soaps. The phenomenon may also be observed following gastrectomy and intestinal bypass surgery, where fat malabsorption is not a problem, see Section III. The resulting tendency to hypocalcemia with the induction of secondary hyperparathyroidism has been shown in many of these conditions to lead to raised levels of $1,25(OH)_2D$, the condition which is known to lead to wastage of 25OHD and the development of vitamin D deficiency, as discussed previously in Section IIA2c. Even with an adequate supply of vitamin D, malabsorption of calcium can lead to secondary hyperparathyroidism; vitamin D deficiency will not occur, but the skeleton may be subject to the effects of high levels of PTH for many years. It is recognized that PTH excess is detrimental to the skeleton, especially at cortical sites. Any increase in bone turnover will also exacerbate the remodeling imbalances that accompany the bone loss associated with aging. Raised levels of $1,25(OH)_2D$ also stimulate bone resorption and may be deleterious to the skeleton. These phenomena may explain why the principal bone disease in patients with gastrointestinal disease is osteoporosis.

III. ACQUIRED BONE DISEASE IN GASTROINTESTINAL DISORDERS

Osteomalacia has been associated with diseases of the gastrointestinal tract for many years, but the pathophysiological mechanisms responsible for this bone disease have only recently been recognized. Furthermore, the mechanisms that lead to osteomalacia may also lead to loss of skeletal tissue, osteoporosis, and easy fracture. Varying degrees of perturbation of calcium and vitamin D metabolism occur in patients with gastrointestinal disease, and the extent, duration, and severity of these derangements are important in determining the nature of any underlying bone disease. Since the major source of vitamin D is cutaneous synthesis rather than the diet (Section IIA1), adequate solar exposure will protect against vitamin D deficiency.

A. The Role of Secondary Hyperparathyroidism

It is now well recognized that certain diseases of the gastrointestinal tract are accompanied by secondary hyperparathyroidism with an increase in the circulating concentration of $1,25(OH)_2D$. It is assumed that these changes are an adaptation to the calcium malabsorption that has been documented in many diseases affecting the gut. Secondary hyperparathyroidism may be successful in correcting calcium malabsorption provided there is a sufficient supply of vitamin D. However, if there is an increase in the catabolism of vitamin D (Section IIA2c), the body may become depleted of vitamin D, the amount of $1,25(OH)_2D$ will be insufficient for normal calcium absorption, and so secondary hyperparathyroidism will be intensified (Fig. 2). This leads to an increase in bone resorption and an increase in remodeling imbalance. In addition, the effects of excess PTH on the kidney will produce phosphate wastage and a reduction in the serum phosphate concentration. These changes, if present for a prolonged period of time, result in the development of osteomalacia due to insufficient $1,25(OH)_2D$ and mineral for normal bone formation. If the supply of vitamin D from diet or solar exposure is adequate for the increased demands resulting from calcium malabsorption and secondary hyperparathyroidism, then osteomalacia will not occur, but the skeleton will be subjected to the effects of excess PTH perhaps over many years. This can lead to loss of bony tissue, particularly at cortical sites with the development of osteoporosis and increased tendency to fracture.

B. The Nature of the Bone Disease

While osteomalacia may occur in patients with gastrointestinal disease, osteoporosis is far more common; this may be explained in part by the state of vitamin D nutrition in patients with gut disease. Where the amount of solar exposure is limited by geographical

latitude, then vitamin D deficiency and osteomalacia are more likely to be seen.

Following intestinal bypass surgery for obesity, it has been estimated that 12% of patients in Europe and 4% in the United States of America develop osteomalacia [31]. This reinforces the importance in Northern Europe, including the United Kingdom, of extrinsic (privational) vitamin D deficiency in determining the development of osteomalacia in intestinal [32] and hepatic [33] disorders.

The frequency of osteomalacia in gastrointestinal and hepatobiliary disease has previously been greatly overestimated. This has resulted from misinterpretation of both biochemical and histological findings. Although a raised serum alkaline phosphatase, often accompanied by serum calcium values in the lower part of the normal range, may be an early sign of underlying osteomalacia, the same biochemical changes are to be found in other clinical situations and may not be due to disturbances in calcium and vitamin D metabolism. Both hepatic and intestinal isoforms of alkaline phosphatase may be increased in certain diseases, and to be certain that a raised level of alkaline phosphatase arises from bone, it is necessary to use a bone-specific assay. Hyperosteoidosis due to secondary hyperparathyroidism has been misinterpreted as osteomalacia, as have been measurements of increased osteoid volume. Clinically, skeletal pain in a patient with primary biliary cirrhosis is more likely to arise from multiple osteoporotic related fractures than from osteomalacia.

Osteoporosis is therefore by far the most common metabolic bone disease complicating disorders of the liver and gastrointestinal tract. There is a high incidence of fractures (especially vertebral) in patients with chronic liver disease or a past history of partial gastrectomy. Bone densitometry has demonstrated a significant reduction in bone mineral content in both the axial and appendicular skeleton, even in asymptomatic patients.

C. The Role of Factors Other Than Vitamin D and Calcium

Gastrointestinal diseases may result in bone disease by mechanisms other than disturbances in calcium and vitamin D metabolism. Protein deficiency is common in some diseases of the liver and small bowel and may adversely affect bone [34]. Hypoalbuminemia can depress osteoblast function, and other nutrients not immediately considered relevant to the skeleton may be important in bone cell function [35]. Deficiencies of other vitamins, including vitamins A, C, and K may affect bone, although their significance in intestinal disease is not fully established. Vitamin C is a co-factor for prolyl hydroxylase, which is necessary for the formation of stable collagen polymers. And two proteins, bone Gla protein (osteocalcin) and matrix Gla protein are generated by vitamin K–dependent enzymes. Deficiencies in vitamin A levels have been demonstrated in one third of patients with primary biliary cirrhosis [10].

D. The Development of Metabolic Bone Disease in Gastrointestinal Disease

In many situations, however, an absolute or relative deficiency of calcium or vitamin D will be pivotal to the development of metabolic bone disease. Rao *et al*. [36] studied the histological evolution of vitamin D deficiency in patients with intestinal malabsorption and a plasma 25OHD of less than 25 nmol/l. In early vitamin D depletion, there is a lack of clinical symptoms, although osteopenia may be present with histological evidence of hyperparathyroidism; there may also be an increased risk of fracture. As the severity increases, mineralization becomes more defective, bone formation rate declines, osteoid tissue increases in both thickness and surface extent, and changes of hyperparathyroidism become more severe [36].

Bone disease resulting from intestinal disease will display features of osteopenia, hyperosteoidosis, osteitis fibrosa, and osteomalacia. The extent and degree of these changes will be determined by the duration and severity of vitamin D deficiency, calcium malabsorption, and accompanying secondary hyperparathyroidism. Additional factors such as the use of corticosteroids or parenteral nutrition (see Section IV,F) may also affect bone. Since the mucosal barrier is often compromised in small bowel disease, it may also be possible for unspecified nutritional or perhaps toxic substances to affect bone.

Histomorphometric analysis of transiliac bone biopsy material has revealed several differing abnormalities, some of which represent the evolution of osteomalacia [31] (see Chapter 63). The first stage in the development of osteomalacia is secondary hyperparathyroidism where the osteoid surface and volume are increased, but osteoid thickness and mineralization lag times are normal. Some patients with vitamin D deficiency show reduced adjusted appositional rate and prolongation of the mineralization lag time. This latter condition is described as hypovitaminosis D osteopathy type I (HVO-I) by Parfitt [31], who divides osteomalacia into HVO-II and HVO-III when osteoid thickness exceeds 15 μm, and mineralization lag time exceeds 100 days. In HVO-III there is no mineralization occurring (using double tetracycline labels), while in HVO-II, mineralization still occurs. Atypical and focal

osteomalacias are also seen occasionally. Florid hyperparathyroidism can result in high turnover osteoporosis, but low turnover osteoporosis is perhaps the most common lesion seen. Osteoid thickness is normal or reduced, bone formation and appositional rates are reduced, and there is little or no evidence of hyperparathyroidism. Low turnover osteoporosis is often associated with general undernutrition and protein malnutrition.

IV. GASTROINTESTINAL CONDITIONS ASSOCIATED WITH BONE DISORDERS

The various pathophysiological mechanisms resulting in metabolic bone disease have been discussed earlier. Whether any gastroenterological disturbance produces bone disease will depend upon the extent to which calcium and vitamin D metabolism are disturbed. Thus, disturbances of colonic function are unlikely to affect calcium metabolism, but since inflammatory bowel disease of the colon is commonly treated by systemic steroids, treatment of colonic disease may impact upon the skeleton (see Chapters 73 and 74).

A. Post Gastrectomy Bone Disease

1. The Pattern of Bone Disease

a. The Incidence of Osteomalacia The changing patterns in both incidence and medical management of peptic ulcer disease has meant that the problem of post gastrectomy bone disease is in decline, and this trend is likely to continue as the need for stomach resection is reduced. However, surgery to alleviate problems with obesity can also lead to problems with bone disease [37]. Osteopenia or osteoporosis is far more common than frank osteomalacia. The overall reported incidence of osteomalacia varies in different centers, and this is in part explained by the differing criteria used for diagnosing osteomalacia. In a survey of 1228 patients following partial gastrectomy, Paterson *et al*. [38] found only 6 cases of osteomalacia using clinical, biochemical, and histological criteria. Tovey *et al*. [39] found osteomalacia in 10 of 227 postgastrectomy subjects, but bone biopsies were performed in only 15 of the patients. When more extensive use is made of bone histology, the incidence of diagnosed osteomalacia tends to increase, and in some series is over 20% [40,41]. Despite the numerous explanations for postgastrectomy bone disease, one unanswered question is why the problem of osteomalacia is greater in women when 80% of gastrectomy patients are men. However, bone disease related to gastrectomy does not develop for several years after surgery, and since many of those affected are middle-aged or elderly, it may not be easy to uncouple the effects of the menopause and the aging process upon the skeleton from those of gastric surgery.

b. Vitamin D Metabolism Osteomalacia responds to small doses of vitamin D [42], and vitamin D absorption is normal in the absence of steatorrhea and only reduced by 40% of intake in the most severe states of malabsorption [43]. The etiology of vitamin D deficiency has therefore never been clear. However, it has been shown that some subjects with gastrectomy show a reduced half-life of 25OHD. These patients had evidence of secondary hyperparathyroidism with increased serum $1,25(OH)_2D$ levels. Lowering of the hormone levels by large calcium supplements was accompanied by a prolongation of the half-life of 25OHD [44]. Other workers have also shown evidence of hyperparathyroidism in gastrectomized patients [45,46]. Nilas *et al*. [47] showed reduced serum 25OHD levels, increased $1,25(OH)_2D$ levels, reduced calcium absorption and osteopenia in subjects several years after gastrectomy. Rümenapf *et al*. [48,49], using dynamic bone histomorphometry, found high turnover osteopenia using a rat model of total gastrectomy. Animals had high $1,25(OH)_2D$ levels, but normal serum calcium and PTH. Using minipigs, Maier *et al*. [50] showed the animals to have low 25OHD, a reduced serum calcium, and secondary hyperparathyroidism. Other potential factors in producing abnormalities in calcium metabolism in this group are the reduced food intake resulting from loss of stomach area, reduced acid secretion, which is important for calcium absorption, poor admixture of food with digestive juices, and intestinal hurry. However, long term treatment with H2-receptor antagonists does not appear to affect the skeleton adversely [51].

c. Bone Turnover Vertebral deformity and fracture are more common in patients with a past history of gastrectomy [52–54], when compared with controls. Rao *et al.* [45] found a past history of gastrectomy in 5% of patients seen with vertebral fracture compared with 1% of controls. Histologically, the bone shows thin but extensive osteoid seams, a low appositional rate resulting from decreased collagen synthesis, and a low bone formation rate. Evidence of secondary hyperparathyroidism may be present and Parfitt *et al*. argue that these changes are the result of accelerated bone turnover with net loss of bone. Impaired recruitment and activity of osteoblasts then results in defective bone repair, predisposing to fracture [35,55].

2. Clinical Features of Postgastrectomy Bone Disease

Osteoporosis is usually without symptoms until a fracture occurs. In postgastrectomy patients, there are

often symptoms from disturbed bowel function, for example loose motions and steatorrhea, and these should be taken as indications for assessing the patient for bone disease. Multiple vertebral fractures lead to loss of height, thoracic kyphosis, and chronic back pain, which is difficult to control. Not surprisingly, accompanying depression is common, and may exacerbate the pain.

Osteomalacia presents insidiously with vague bone pain and muscle weakness, particularly affecting the proximal muscles. As the bone disease progresses, the patient becomes increasingly incapacitated by bone pains and muscle weakness. Walking becomes labored, the patient developing a waddling gait and often having to climb stairs "crab-like," holding the stair rail with both hands. In the most extreme cases, pain around joints from an enthesopathy, due to the effects of hyperparathyroidism, may simulate active arthritis. Fractures may occur, particularly in chronic cases, and especially where pseudofractures have been present; a frequent site is the femoral neck. Insufficiency fractures from osteoporosis can mimic Loosers zones radiologically [56], but the clinical picture is one of acute pain in relation to the fracture, rather than the history of chronic vague bone pain that characterizes osteomalacia.

3. BIOCHEMISTRY

Serum biochemistry in cases of osteoporosis is normal, although the alkaline phosphatase may be slightly increased, especially if there has been a recent fracture. Evidence for secondary hyperparathyroidism may be present together with raised $1,25(OH)_2D$. Bone densitometry will show a reduced bone mass that may be more marked at cortical sites in the forearm and hip than in the spine.

In osteomalacia there is usually a reduction in serum phosphate with a low normal or frankly reduced serum calcium and elevated alkaline phosphatase. If measured, PTH will be found to be increased, 25OHD reduced, and $1,25(OH)_2$ low, normal, or increased, depending upon the amount of vitamin D recently provided from the diet or by solar exposure.

4. MANAGEMENT

The management aim for patients at risk should be to ensure normal plasma biochemistry by providing sufficient calcium and vitamin D as a dietary supplement, and thus suppress any increase in PTH and ensure normal 25OHD levels. When osteomalacia is present, oral vitamin D supplements can be given as a large single bolus using doses of several hundred thousand units, or much smaller doses of 1000–4000 IU (25–100 μg) daily. Patients with steatorrhea will require larger oral doses, or parenteral vitamin D, and should be monitored by measuring serum 25OHD regularly, until it can be shown that normal 25OHD values are being achieved. In the light of difficulties with calcium absorption, a large calcium supplement (1–2 g of elemental calcium) should also be given long term. Patients without overt bone disease should have blood assayed for PTH and 25OHD. If there is any evidence of secondary hyperparathyroidism, sufficient oral calcium should be given to suppress the elevated PTH. Vitamin D should also be given if the serum 25OHD is low. Bone densitometry should be performed and if evidence of osteoporosis is found, then appropriate antiresorptive therapy, such as bisphosphonate treatment, should be considered.

B. Celiac Disease

1. CLINICAL FEATURES

Celiac disease is an inflammatory condition of the small intestine triggered by ingesting gluten, present in wheat, rye, or barley. The development of sensitive and specific serological screening tests such as antiendomysial and tissue transglutaminase antibodies [57] has resulted in an increase in the estimated prevalence of celiac disease from an often quoted 1 in 2000–4000 of the population to a more recent study where 0.4% of healthy blood donors had positive serology results for celiac disease [58]. The prevalence varies in different ethnic groups, and is particularly common in those of Irish descent. While reduced bone mass is found in a significant proportion of patients compared with age- and sex-matched controls [59–63], symptomatic bone disease is uncommon in celiac disease. Using antiendomysial antibodies in asymptomatic osteoporotic subjects, Lindh *et al.* [64] showed that the incidence of celiac disease was tenfold higher than in the normal population. In adults on treatment for celiac disease, osteopenia is twice that expected for a normal population with a reduction in bone density of between 7 and 13% [65,66]. Until recently, no data existed to show an increase of fracture in celiac patients, but in a cross-sectional case control study by Vazquez *et al.* [67], a quarter of 165 patients with celiac disease had a history of 1 to 5 peripheral fractures compared with 8% of an age- and sex-matched control group. The fractures occurred before the diagnosis of celiac disease, or in those subjects who did not comply with a gluten-free diet. The findings of Selby *et al.* [68], that cortical bone is more affected than trabecular bone almost certainly because of the effects of excess parathyroid hormone upon the skeleton, helps to explain the findings of Vazquez *et al.* [67]. More recently, Valdimarsson *et al.* have confirmed these findings [69].

In children, institution of treatment with a gluten-free diet results in a normal bone mass when they become teenagers [70,71]. In a small group of prepubertal

children with celiac disease, the addition of calcium and vitamin D to gluten-free diet showed increments in BMD greater than in an age- and sex-matched control population, but the Z scores of the celiac group remained lower than the control group after two years of treatment [72].

These observations in children, adolescents, and adults indicate that there may be an irreversible element to the bone loss (or reduced accrual of bone), which may be a function of the duration of untreated celiac disease, a condition that is much more common than previously considered. An adult diagnosed with celiac disease may have had the problem since childhood and failed to achieve adequate peak bone mass, whereas the developing child can fully develop the skeleton if the gut is returned to normality.

Occult celiac disease with no overt signs or symptoms of intestinal malabsorption may occasionally present as an osteomalacic syndrome [73,74], and small bowel biopsy should be considered in the assessment of all patients with osteomalacia in whom the cause is unclear. Alternatively, gastrointestinal symptoms may be so florid as to obscure the symptoms of an underlying osteomalacia. As celiac disease affects the duodenum and jejunum more severely than the ileum, malabsorption of calcium and vitamin D are common [8,75]. However, not all patients with celiac disease malabsorb vitamin D; in a small study, the celiac disease patient with the most severe malabsorption of vitamin D had the highest serum level of 25OHD, whereas absorption of vitamin D was normal in two patients who showed some degree of vitamin D deficiency [43]. These findings re-emphasize the importance of cutaneous synthesis of vitamin D.

2. Development of Vitamin D Deficiency

Evidence of poor vitamin D nutrition can be found in untreated celiac disease. Dibble *et al.* [76] found two untreated patients with low serum 25OHD levels (<12 nmol/l) but normal values in 12 subjects successfully treated with a gluten-free diet. Ferretti *et al*. [57] and Arnaud *et al*. [77] also found low 25OHD values in celiac patients. In the small series reported by Melvin *et al*. [75], 5 of 9 patients had histological osteomalacia (this study was performed before measurements of vitamin D metabolites were possible).

The mechanisms leading to vitamin D deficiency have previously been attributed to interruption of a conservative enterohepatic circulation of 25OHD [23], but this concept has been largely discarded, see Section IIA2b. A patient with celiac disease, secondary hyperparathyroidism, and elevated serum $1,25(OH)_2D$ was described in a study by Clements *et al*. [29]. The half-life of radio-labeled $25OHD_3$ was shortened in the hyperparathyroid state compared with the euparathyroid state, which was achieved by using large supplements of calcium. In a recent study by Selby *et al*. [68] and Valdimarsson *et al*. [69], a high proportion of patients with treated celiac disease showed evidence of secondary hyperparathyroidism, suggesting that there may be a continuing problem with calcium absorption despite a gluten-free diet. Support for these last two observations is also forthcoming from the recent papers of Corazza *et al*. [78] and Ferretti *et al*. [63]. These workers found evidence of low bone mass in celiac patients, which was improved but not completely corrected by a gluten-free diet. Many untreated patients have secondary hyperparathyroidism with significantly raised serum $1,25(OH)_2D$ levels and significantly lower 25OHD levels in the untreated state, compared with the treated condition.

3. Management

The treatment of choice is a gluten-free diet, but not all patients will be able to adhere to such a strict regime. Some individuals either overtly or covertly eat a diet containing gluten, which may explain the findings of Selby *et al.* above [68]. Additional vitamin D will cure any osteomalacia, and there is probably a good case for giving long-term calcium supplements of 1–2 g per day to guard against the effects of occult secondary hyperparathyroidism on the skeleton.

C. Pancreatic Disease

Pancreatic insufficiency is not normally associated with metabolic bone disease, unless complicated by other conditions such as cystic fibrosis or alcoholism. This paradox implies that the disturbances to mineral and bone metabolism seen in other forms of bowel and liver disease have little to do with steatorrhea. Low 25OHD levels have been reported in cystic fibrosis [79,80], and both osteoporosis and osteomalacia have been described in this condition [81,82]. Meredith and Rosenberg [9] have questioned the rarity of bone disease associated with pancreatic steatorrhea, and they consider that osteomalacia may be more common than previously believed.

Patients should receive pancreatic supplements and, if serum 25OHD is shown to be low, oral vitamin D at 800 IU/day. If, however, steatorrhoea remains a problem, larger oral doses will be necessary. Serum 25OHD levels should be measured every 1–2 months if daily doses in excess of 10,000 IU/day (0.25 mg) are used. Occasionally, parenteral vitamin D 100,000 IU per month may prove necessary.

D. Inflammatory Bowel Disease

1. Clinical and Biochemical Features

Of the two main inflammatory bowel diseases, bone disease is more often seen in Crohn's disease than in ulcerative colitis. Since the colon is principally an organ for conservation of salt and water, disturbance of its function does not normally affect the skeleton. However, loss of bone and osteoporosis may result from the use of corticosteroids to control disease (see Chapter 73).

Malabsorption of vitamin D and 25OHD have been documented in Crohn's disease [9,43], and calcium is also malabsorbed but perhaps less often than previously considered. In one study [83], only 4 of 31 patients with active Crohn's disease were found to have reduced net absorption of calcium and negative calcium balance and 2 of the 4 had undergone ileal resection. Driscoll *et al.* [84] studied 82 patients with Crohn's disease from 9 of whom transiliac crest bone biopsies were taken. Six of these patients were found to have osteomalacia; 3 had a repeat biopsy, which showed improvement following treatment with vitamin D. There was a high incidence of vitamin D deficiency as assessed by serum 25OHD, 65% having a low 25OHD; in 25% of cases, serum 25OHD was below 25 nmol/l. It was in this lowest group where the cases of osteomalacia occurred. The lowest 25OHD values were found in those patients who had had a previous ileal resection. Nine of 25 patients with Crohn's disease, assessed by Compston *et al.* [85] had increased osteoid on bone biopsy. More recently, vitamin D status was assessed in 112 patients ranging from 5–22 years of age [86]. The overall prevalence of hypovitaminosis in this group of patients was only 16%, however hypovitaminosis was much more common in winter (31%), in African Americans (56%), in patients with disease confined to the upper GI tract (44%), and in patients with a greater lifelong exposure to corticosteroids [86].

Osteoporosis is more common than osteomalacia. Compston *et al.* [87] found a reduced bone mass in 30% of an unselected group of patients with Crohn's disease. The pathogenesis of osteomalacia may be similar to that in celiac disease, but no reports have shown good evidence of secondary hyperparathyroidism with high serum concentrations of $1,25(OH)_2D$ as has been seen in celiac disease. The fact that calcium absorption is often normal [83] does not exclude excess parathyroid activity because normal calcium absorption may only be maintained at the expense of increased parathyroid drive. However, $1,25(OH)_2D$ induced hepatic wastage of 25OHD seems less common in patients with Crohn's disease.

Glucocorticoids, which are used frequently to control inflammatory bowel disease, can both impair calcium absorption and reduce osteoblast function thus exacerbating those processes known to produce bone loss. Protein and calorie malnutrition, which are seen in severe cases of inflammatory bowel disease, will also adversely affect the skeleton [88].

There is increasing interest in factors of the inflammatory process as possible mediators of bone resorption and osteoblast function. While the nature of these factors is unknown, a candidate cytokine is interleukin 6 (IL-6). Pollak *et al.* [89] showed an increase in serum IL-6 in osteoporotic patients with inflammatory bowel disease, with normal BMD in patients with normal IL-6 levels.

Finally, Hewison *et al*. [90] have produced data suggesting that ectopic production of $1,25(OH)_2D$ by activated macrophages in Crohn's granuloma may be detrimental to the skeleton. These authors showed a negative correlation in serum $1,25(OH)_2D$ levels and lumbar spine BMD, which was independent of corticosteroid use. Serum $1,25(OH)_2D$ values were elevated (>60 pg/ml) in 42% of 138 patients with Crohn's disease without evidence of secondary hyperparathyroidism. Despite the known beneficial effects of corticosteroids on suppressing extra-renal 1α-hydroxylase (Chapter 79), the Crohn's patients receiving high doses of steroid had similar $1,25(OH)_2D$ levels compared with Crohn's patients receiving low doses of corticosteroid.

2. Management

Patients with inflammatory bowel disease should be managed in a similar way to patients with partial gastrectomy. Osteoporosis may pose a problem for management using oral bisphosphonates because of their poor absorption and tendency to cause bowel upset. Intravenous bisphosphonates or nasal calcitonin can be used as an alternative to oral antiresorptive therapy. There is also a possible role for $1,25(OH)_2D$ or analog in the treatment of inflammatory bowel disease itself. It has been shown that isolated T lymphocytes from patients with ulcerative colitis are particularly sensitive to cyclosporin A, and that there is a synergistic effect on proliferation when the cyclosporin was given in combination with $1,25(OH)_2D$ and several analogs [91], suggesting that this combination therapy may be more useful in patients than cyclosporin A alone. (Cyclosporin is further discussed in Chapter 74.)

E. Jejuno-ileal bypass

1. Clinical Features

Intestinal bypass surgery for obesity became popular during the 1970s but was followed by a considerable mortality and morbidity, including a high incidence of

skeletal complications. Metabolic bone disease developed after a variable number of years in a significant proportion of patients following bypass surgery for obesity, however, obesity *per se* is known to be sometimes associated with an increase in PTH [92]. Dano and Christiansen [93] found the bone mineral content to be reduced compared with controls in a group of 37 obese patients before undergoing bypass surgery. Surgery, resulting in severe intestinal malabsorption, may exacerbate pre-existing, and perhaps subtle, abnormalities of calcium metabolism. In about half the patients, there is a fall in serum calcium and magnesium after surgery, and although serum 25OHD levels fall, $1,25(OH)_2D$ levels are maintained [94–96]. Osteomalacia is seen on bone biopsy in up to 60% of patients and in some, $1,25(OH)_2D$ levels are low and PTH values are raised [35,37,94,97]. Profound hypocalcemia may occur secondary to the hypoparathyroidism of magnesium deficiency when osteomalacia is believed to be less severe [35]. Osteomalacia resulting from bypass surgery is seen more frequently in Europe than in America.

The etiology of osteomalacia in bypass patients has been attributed to malabsorption of vitamin D, but the functional abnormalities produced by intestinal bypass surgery should favor hepatic wastage of 25OHD, although this hypothesis has not been tested. Osteopenia is also found following surgery with loss of bone in both the appendicular and axial skeleton [95].

2. MANAGEMENT

Vitamin D and 1α-hydroxyvitamin D have been used with success to treat osteomalacia, but the native vitamin should be adequate to correct any deficiency. The parenteral route may be necessary in view of loss of functional bowel. Liver disease may follow this type of operation, but impaired hepatic hydroxylation of vitamin D is rarely a problem (see Section V). Treatment failures have responded to antibiotics used to eradicate bacterial overgrowth in the bypass segment [98].

F. Total Parenteral Nutrition (TPN)

1. CLINICAL FEATURES

TPN is used for those individuals in whom disease of the bowel is so severe that oral feeding cannot achieve adequate nutrition. Most patients commencing TPN do so after years of chronic bowel disease, and therefore are likely to have pre-existing bone disease. This section is primarily concerned with the effects of TPN *per se* on the bone.

TPN may not provide all the nutrients or micronutrients required and historically calcium and phosphate were omitted from solutions, resulting in rickets in some children [99]. However, the major iatrogenic bone lesion resulted from the contamination of casein hydrolysate by aluminum at concentrations of up to 1 mg/l [100]. Serum samples from these patients contained large amounts, and bone contained more than 30 times the normal level of aluminum [99]. It is not surprising, therefore, that bone biopsies in most of these patients showed signs of osteomalacia. Clinically, the problem presented with peri-articular bone pains, especially in the legs, back, and ribs, and was only improved by cessation of TPN [101]. The disease has largely disappeared following the substitution of casein by amino acids [102]. Several biochemical abnormalities were found, but these may have been related to the amount of protein present in the feed. Hypercalciuria, low PTH, and low $1,25(OH)_2D$ values returned to normal when purified amino acids were introduced. However, hypercalciuria persists if the amino acid content is kept too high [103]. The bone disease seen in these patients is mainly osteopenia [104,105], probably related at least in part to bone changes before TPN was commenced.

2. MANAGEMENT

Bone density, biochemistry, and bone histology should be evaluated at the onset of TPN and any hyperparathyroidism, vitamin D deficiency, or osteomalacia corrected by the appropriate use of calcium and vitamin D. If only osteoporosis is present, parenteral bisphosphonate treatment should be considered, and the response should be monitored by bone densitometry.

Whenever possible, a return to enteral feeding should be encouraged. In cases of severe aluminum poisoning, chelation with desferoxamine, as used in dialysis patients, may be effective.

V. LIVER DISEASE

A. Hepatic Osteodystrophy

While both osteomalacia and osteopenia contribute to the syndrome of "hepatic osteodystrophy," the condition is characterized by reduced bone mass leading to severe osteoporosis and fractures. Osteomalacia occurs more commonly in the presence of severe long-term cholestasis, as in primary biliary cirrhosis (PBC), but even in PBC, it is rare and its incidence appears largely restricted to Northern Europe [106–108]. Earlier studies in which a high incidence of osteomalacia in bone biopsies was reported (e.g. 22 out of 32 patients, mainly with cholestatic disease) used diagnostic methods, which have now been superseded [109,110]. Compston *et al.* [111], using more stringent criteria including the presence of calcification fronts,

reported a much lower incidence; only 4 out of 32 patients had evidence of osteomalacia. A similarly low incidence was recently reported by Crosbie *et al.* [112]; only 2 of 27 patients with chronic liver disease showed biochemical evidence of osteomalacia. A rather higher incidence was reported by Dibble *et al.* [33], who found osteomalacia in 9 out of 29 patients; of these 9, 5 had PBC, 2 had chronic active hepatitis, and one each had sclerosing cholangitis and alcoholic cirrhosis. Indeed, using his strict criteria for diagnosing osteomalacia, Parfitt [31] has argued that only eight histologically proven cases of hepatic osteomalacia have been published [33,111,113–116]. Many other reported cases have used invalid methods involving decalcified bone [110,117] or not excluding HVOI (Section III). By such strict criteria, osteomalacia is most uncommon, but when present is associated with severe vitamin D deficiency and secondary hyperparathyroidism [32,105].

Conflicting reports have appeared on the levels of PTH in severe liver disease; an earlier study [118], claiming a defect in hepatic cleavage of PTH, was challenged by Klein *et al*. [119] who failed to find hyperparathyroidism using a range of different assays for the hormone. The latter authors concluded that raised PTH was unlikely to play a part in hepatic osteodystrophy. Dibble *et al*. [33] found evidence of slightly increased PTH in advanced cirrhosis, but osteomalacia was only found together with chronic cholestasis and vitamin D deficiency.

The central role of the liver in maintaining a balance between the synthesis of 25OHD, eliminating excess vitamin and its metabolites, and thus regulating plasma levels of 25OHD is discussed elsewhere (Chapter 4). It had been supposed that osteomalacia in liver disease could be explained by impairment of hepatic 25-hydroxylation, and low plasma levels of 25OHD have been reported in many types of liver disease, including alcoholic hepatitis and cirrhosis, lupoid and cryptogenic cirrhosis, and primary biliary cirrhosis [117]. It is now recognized that where hepatocellular function is maintained, 25-hydroxylation of vitamin D remains normal until a very late stage of disease, and that malabsorption of vitamin D as a result of steatorrhea is a more likely cause of deficiency. Where steatorrhea is present, absorption of calcium may well be impaired, leading to osteoporosis. Malabsorption of both vitamin D and calcium may be compounded by the use of bile sequestration agents [120] (see Chapter 74).

Osteoporosis is the most common bone problem seen in chronic liver disease [121] and it may be exacerbated by drugs used to control the disease, such as steroids in chronic active hepatitis. The most common histomorphometric parameter is not that of increased bone turnover, as is often associated with intestinal disease, but a low turnover state with low values for osteoid volume and surface extent. Appositional rate is also low when tetracycline labeling is used [108,121]. Although eroded surfaces without osteoclasts may be increased, this may reflect delayed formation rather than increased resorption [31]. This low turnover state reflects depressed osteoblastic function, which may be part of the chronic debilitating nature of liver disease, or may be due to the persistence of toxins normally removed by the liver. The histological features are, however, similar to those seen in patients after jejunoileal bypass.

B. Primary Biliary Cirrhosis

1. Clinical Features

Vitamin D deficiency has been frequently reported in PBC, at times associated with osteomalacia, and characterized by low plasma levels of 25OHD [117,122]. The explanation is probably poor intestinal absorption [123] from a diet containing little vitamin D; in addition, there may be problems with endogenous synthesis of the vitamin. Sunshine may be deliberately avoided for cosmetic reasons or because of itching and discomfort, and it is possible that UV irradiation is less effective because of the pigmented skin. Claims that the osteomalacia of PBC was refractory to treatment with vitamin D were based upon early studies, the results of which may have been misinterpreted. Reports claiming that parenteral vitamin D therapy was ineffective in PBC may be explained by the use of intramuscular oily preparations of the vitamin that have been shown to have low bioavailability [124]. Similarly, the availability of vitamin D from oral preparations may depend on the vehicle. Absorption from tablets tends to be poor, especially in the presence of fat malabsorption, whereas absorption from an ethanol solution given in milk is relatively efficient [112]. The deficiency can be corrected by a modest increase in the oral supply of vitamin D or by giving UV irradiation [106,113]. Successful treatment has also been given using 25OHD, which being less lipophilic, is absorbed more efficiently than vitamin D [43,125]. Osteomalacia, as noted above (Section VA), is rare, and may be easily missed because of the severity of symptoms of the liver disease. Changes in alkaline phosphatase, phosphate, and calcium may reflect more the liver disease than changes in the skeleton. The clinician needs to be alert to the possibility of vitamin D deficiency and to identify patients at risk by measuring serum 25OHD. Bone pain from rib and vertebral fractures is more likely to result from osteoporosis, and the prevention and treatment of this disease is far more difficult than the prevention or treatment of osteomalacia.

2. VITAMIN D METABOLISM

Given an adequate supply of vitamin D, the patient with PBC can metabolize the vitamin normally [126]. Reported low levels of 25OHD and $1,25(OH)_2D$ in PBC [116,119,126] seem to be related to malabsorption of the vitamin, rather than to any intrinsic defect in metabolism. Subnormal concentrations of vitamin D metabolites are associated with established osteomalacia, and provide an adequate explanation for the development of the disease. There is little evidence for raised levels of either $1,25(OH)_2D$ or PTH in PBC, and so the conditions favoring the $1,25(OH)_2D$–induced increased turnover of 25OHD are not present.

Where there is considerable biliary obstruction, water-soluble vitamin D metabolites appear in unusually large amounts in urine [106, 123, 126], see Fig. 4. Jung *et al.* [106] found urinary excretion of metabolites after administering vitamin D but not after 25OHD, and concluded that the water soluble metabolites had arisen from vitamin D but not via the 25-hydroxylation pathway. It is doubtful whether this urinary excretion contributes quantitatively to vitamin D deficiency in PBC, since these metabolites would otherwise be excreted via the fecal route and do not appear to constitute an increased net loss.

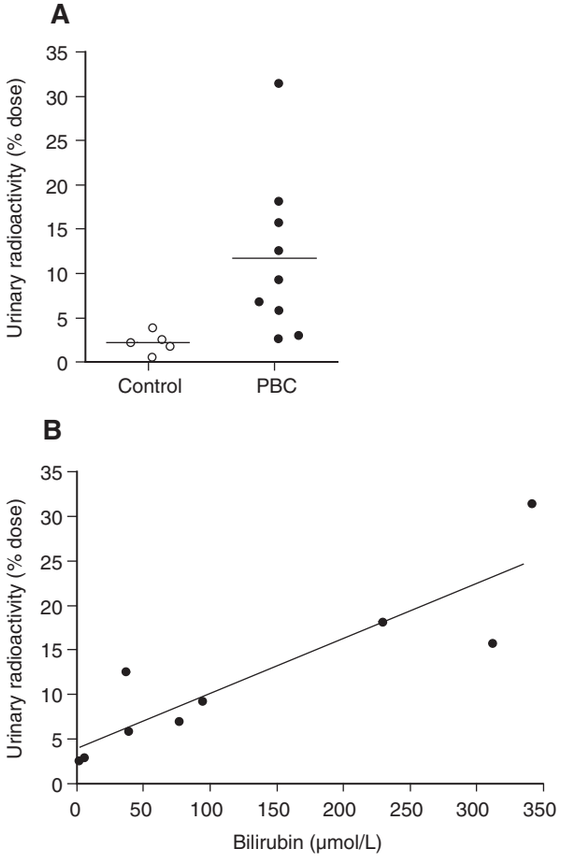

FIGURE 4 Urinary excretion of vitamin D metabolites in patients with primary biliary cirrhosis (PBC) showing (**A**) percentage dose excreted compared to control subjects and (**B**) the strong relationship to the degree of biliary obstruction as indicated by plasma bilirubin, Kendall's coefficient of concordance $\tau = 0.788$, $P = 0.001$. Data taken from Mawer *et al.* [126].

3. MANAGEMENT

Oral doses of vitamin D have been found to be effective in treating the osteomalacia in PBC patients. Biochemistry and calcium balance were normalized, and bone histology improved after treatment [113]. The precise dose of vitamin D was selected in this study by first measuring the absorption of a ^3H-labeled test dose and calculating the administered dose to fit the patient's needs. The greatest degree of malabsorption was 26% of the administered dose. Doses of 400–4000 IU in solution per day were used, but, in the absence of information on absorption, a dose of 2000 IU per day should be adequate for most patients. Treatment with UV irradiation is also effective [106,113]. Tablets of vitamin D are less well absorbed than solutions, and treatment with high dose tablets needs to be monitored. If, after a trial of 10,000 IU per day serum 25OHD does not rise towards 50 nmol/l, then regular monthly or three-monthly injections of 300,000 IU (7.5 mg) should be tried and the response assessed by measuring serum 25OHD. If a response greater than 125 nmol/l is produced, then the dose should be reduced. If the extent of steatorrhea or cholestasis increase, then the oral dose may need to be increased. At no time should a patient receive more than 10,000 IU per day by mouth without checking 25OHD levels, as eventually intoxication may result. There is no need to use 25OHD or 1α–hydroxylated derivatives, although these are effective in curing bone disease.

Osteoporosis is a difficult problem to address, and any treatment is experimental. Currently, treatment with bisphosphonates or hormone replacement therapy may be considered for those with a low, or falling bone mineral density, but given that the state is one of low turnover, it is difficult to envisage much benefit accruing from antiresorptive treatment. It is possible that fluoride, or parathyroid hormone, which stimulate bone formation, might be beneficial, but this awaits a detailed clinical trial.

Despite beneficial effects on the underlying liver disease, treatment with ursodeoxycholic acid conferred no benefits on the skeleton in a study of 88 patients with PBC [127]. Supplementation with calcium was superior to treatment with calcitonin in maintaining

bone mass, albeit transiently, in a study of 25 women with PBC-associated osteoporosis [128]. The anabolic effects of 1–34 parathyroid hormone recently licensed for postmenopausal osteoporosis would seem to the authors to be the most promising clinical trial for the low turnover osteoporosis of PBC.

C. Alcoholic Liver Disease

1. Clinical Features

Osteoporosis is the more frequent bony abnormality in patients with alcoholic liver disease, as in PBC, but osteomalacia has been reported occasionally [129–131]. The osteoporosis is characterized by a high incidence of fractures [129–131], but these do not follow a classic pattern. In a recent series, 27 of 76 chronic alcoholic male patients had vertebral fractures, but in only 5 of these was bone mineral density below the fracture threshold [132]. In evaluating fractures, it is important to distinguish between bone disease, which arises directly from the cirrhosis, ethanol associated osteopenia [133,134], and increased trauma (including seizures), resulting from the lifestyle of some alcoholic patients [131,132]. A study of the histomorphometry of alcohol-induced bone disease established major changes, even though chronic liver damage was not severe [135]. A significant decrease in bone volume was recorded together with increased resorption surfaces and increased osteoclast number.

Low assayed levels of plasma 25OHD have been reported frequently in patients with alcoholic cirrhosis. The development of vitamin D deficiency in alcoholic liver disease probably has causes in common with the same problem in PBC, namely malabsorption of vitamin D (in patients with cholestasis) and lack of exposure to sunlight. Poor nutrition in alcoholic patients may also reduce the intake of vitamin D.

2. Vitamin D Metabolism

In contrast to PBC, abnormal hepatocellular function in alcoholic liver disease may be associated with disturbances of vitamin D metabolism. Plasma levels of 25OHD were shown to correlate with the degree of hepatic dysfunction, as measured by the antipyrine breath test [136], and defective synthesis of 25OHD was demonstrated by Jung *et al*. [137]. In a subsequent study, the impairment was shown to be correlated to plasma levels of bilirubin and prothrombin [118] (see Fig. 5). In this study, synthesis of $1,25(OH)_2D$ was also shown to be defective in three of the patients studied, and it was attributed to the low level of precursor 25OHD and to poor renal function.

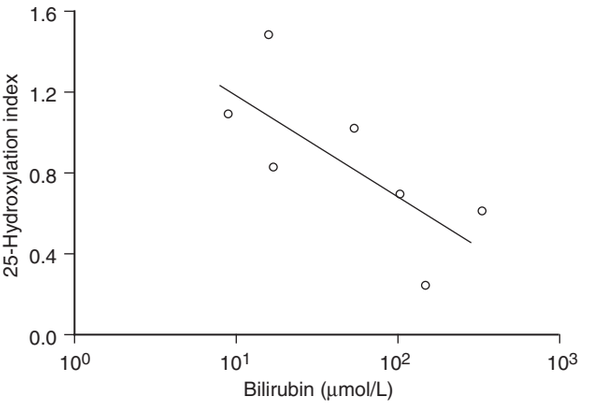

FIGURE 5 Impaired 25-hydroxylation of vitamin D in patients with alcoholic liver disease related to the plasma level of bilirubin, Kendall's coefficient of concordance $\tau = -0.788$, $P < 0.02$. Data taken from Mawer *et al*. [126].

Assayed concentrations of $1,25(OH)_2D$ were low in a minority of alcoholic patients reported by Lalor *et al*. [130], but despite this evidence for defective vitamin D metabolism, osteomalacia is a comparatively rare component of alcoholic liver disease.

3. Management

Alcohol intake should cease, though this is a difficult goal to achieve. Vitamin D and calcium supplements should be given to those with evidence of low serum 25OHD and a poor (< 1g) intake of calcium. This simple maneuver will protect the skeleton from osteomalacia and reduce the tendency toward increased bone resorption. If alcohol continues to be consumed to the extent where amnesia is a problem, then compliance with the rigors of bisphosphonate therapy is unlikely. However, reformed alcoholics with osteoporosis seem as likely to respond favorably to antiresorptive therapy as any other group. Monitoring of bone mineral density at the start of therapy with follow-up after 12–18 months will give an indication as to whether treatment should continue long-term.

VI. SUMMARY

It should not be surprising that metabolic bone disease arises when there is chronic disease affecting the gastrointestinal tract. The mineral phase of the skeleton is maintained by the absorption of calcium and other elements from the diet. It has sometimes been difficult to disentangle the effects of the disease itself on the skeleton from any effects, which might be

exerted by the treatment given for that disease, but understanding of these factors is improving. Interpretation of the pathophysiological mechanisms involved has sometimes been clouded by misinterpretation of data, and there are still many problem areas. The mechanisms that result in low turnover osteoporosis are not fully understood, nor why states of secondary hyperparathyroidism should result in vitamin D wastage. It seems clear, however, that osteomalacia results primarily from vitamin D deficiency although calcium deficiency may also cause rickets and osteomalacia (see Chapters 64 and 65). The predisposition to osteomalacia may be present in some individuals before development of the gastrointestinal disease, but it can be reversed by appropriate, sometimes parenteral, treatment with the native vitamin. Likewise, HVOI may be managed by large calcium supplements, and perhaps vitamin D, to suppress secondary hyperparathyroidism. It seems that individuals with chronic debilitating bowel or liver disease are programmed to develop osteoporosis through poorly understood disturbances of osteoblast function, which may include effects from interleukins and other cytokines, produced by the inflammatory process itself. Research into this aspect of bone disease needs to be actively pursued, as the lives of many patients with advanced disease can now be prolonged by artificial feeding or by organ transplantation; these procedures themselves may introduce compounding factors into the genesis of metabolic bone disease.

References

1. Peacock M, Selby PL, Francis RM, Brown WB, Hordon L 1985 Vitamin D deficiency, insufficiency, and intoxication. What do they mean? In: Norman AW, Schaefer K, Grigoleit H-G, Herrath D (eds) Vitamin D, Chemical, Biochemical, and Clinical Update. de Gruyter: Berlin, 569–570.
2. Krall EA, Sahyoun N., Tannenbaum S, Dallal GE, Dawson-Hughes B 1989 Effect of vitamin D intake on seasonal variations in parathyroid hormone secretion in postmenopausal women. New Engl J Med **321**:1777–1783.
3. Dawson-Hughes B, Harris SS, Krall EA, Dallal GE 1997 Effect of calcium and vitamin D supplementation on bone density in men and women 65 years of age or older. N Engl J Med **327**:670–676.
4. Malabanan A, Veronikis IE Holick MF 1998 Redefining vitamin D insufficiency. Lancet **351**:805–806.
5. Chapuy MC, Preziosi P, Maamer M, Arnaud S, Galan P, Hercberg S, Meunier PJ 1997 Prevalence of vitamin D insufficiency in an adult normal population. Osteoporosis Int **7**:439–443.
6. Veith R, Ladak Y, Walfish PG 2003 Age-related changes in the 25-hydroxyvitamin D versus parathyroid hormone relationship suggests a different reason why older adults require more vitamin D. J Clin Endocrinol Metab **88**:185–191.
7. Glerup H, Mikkelsen K, Poulsen L, Hass E, Overbeck O, Thomsen J, Charles P, Eriksen EF 2000 Commonly recommended daily intake of vitamin D is not sufficient if sunlight exposure is limited. J Int Med **247**:260–268.
8. Thompson GR, Lewis B, Booth CC 1966 Absorption of vitamin D_3-^3H in control subjects and patients with intestinal malabsorption. J Clin Invest **45**:94–102.
9. Meredith SC, Rosenberg IH 1980 Gastrointestinal-hepatic disorders and osteomalacia. In: Avioli LV, Raisz LG (eds) Clinics in Endocrinology and Metabolism 9. WB Saunders and Co Ltd: London, pp. 131–150.
10. Phillips JR, Angulo P, Petterson T, Lindor KD 2001 Fat-soluble vitamin levels in patients with primary biliary cirrhosis. Am J Gastroenterol **96**:2745–2750.
11. Poskitt EME, Cole TJ, Lawson DEM 1979 Diet, sunlight, and 25(OH)D in healthy children and adults. Br Med J **i**:221–223.
12. Stamp TCB, Haddad JG, Twigg CA 1977 Comparison of oral 25-hydroxycholecalciferol, vitamin D, and ultraviolet light as determinants of circulating 25-hydroxyvitamin D. Lancet **i**:1341–1343.
13. Davies M, Mawer EB, Hann JT, Taylor JL 1986 Seasonal changes in the biochemical indices of vitamin D-deficiency in the elderly. A comparison of people in residential homes, long-stay wards, and attending a day hospital. Age and Aging **15**:77–83.
14. Mawer EB 1980 Clinical implications of measurements of circulating vitamin D metabolites. In: Avioli LV, Raisz LG, (eds) Clinics in Endocrinology and Metabolism. WB Saunders & Co Ltd: London **9**:131–150.
15. Berry JL, Davies M, Mee AP 2002 Vitamin D metabolism, rickets, and osteomalacia. Sem Musculoskel Radiol **6**:173–181.
16. Avioli LV, Lee SW, McDonald JE, Lund J, De Luca HF 1967 Metabolism of vitamin D_3-^3H in human subjects distribution in blood, bile, feces, and urine. J Clin Invest **46**: 983–992.
17. De Luca HF, Schnoes HK 1983 Vitamin D: recent advances. Ann Rev Biochem **52**:411–439.
18. Mawer EB, Backhouse J, Holman CA, Lumb GA, Stanbury SW 1972 The distribution and storage of vitamin D and its metabolites in human tissues. Clin Sci **43**:413–431.
19. Clements MR, Chalmers TM, Fraser DR 1984 Enterohepatic circulation of vitamin D: a reappraisal of the hypothesis. Lancet **i**:1376–1379.
20. Makin G, Lotues D, Buford V, Ray R, Jones G 1989 Target cell metabolism of 1,25-dihydroxyvitamin D_3 to calcitroic acid. Biochem J **262**:173–180.
21. Jones G, Kung M, Kano K 1983 The isolation and identification of the new metabolites of 25-hydroxyvitamin D_3 produced in the kidney. J Biol Chem **258**:12920–12928.
22. Bolt MJG, Jensen WE, Sitrin MD 1992 Metabolism of 25-hydroxyvitamin D_3 in rats: low calcium diet vs. calcitriol infusion. Am J Physiol (Endocrinol Metab) **262**(25): E359–E367.
23. Arnaud SB, Goldsmith RS, Lambert PW, Go VLW 1975 25-hydroxyvitamin D_3: Evidence of an enterohepatic circulation in man. Proc Soc Exp Biol **149**:570–572.
24. Fraser DR 1983 The physiological economy of vitamin D. Lancet **i**:969–972.
25. Gascon-Barre M 1986 Is there any physiological significance to the enterohepatic circulation of vitamin D sterols. J Am Coll Nutr **5**:317–324.

26. Mawer EB 1979 The role of the liver in the control of vitamin D metabolism. In: Norman AW, (ed) Vitamin D, Basic Research and its Clinical Application. De Gruyter: Berlin, 533–561.
27. Clements MR, Johnson L, Fraser DR 1987 A new mechanism for induced vitamin D deficiency in calcium deprivation. Nature **325**:62–65.
28. Clements MR, Davies M, Fraser DR, Lumb GA, Mawer EB, Adams PH 1987 Metabolic inactivation of vitamin D is enhanced in primary hyperparathyroidism. Clin Sci **73**:659–664.
29. Clements MR, Davies M, Hayes ME, Hickey CD, Lumb GA, Mawer EB, Adams PH 1992 The role of 1,25-dihydroxyvitamin D in the mechanism of acquired vitamin D deficiency. Clin Endocrinol 3 **7**:17–27.
30. Colston KW, Mackay AG, Finlayson C, Wu JCY, Maxwell JD 1994 Localization of vitamin D receptor in normal human duodenum and in patients with celiac disease. Gut **35**:1219–1225.
31. Parfitt AM 1990 Osteomalacia and related disorders. In: Avioli LV, Krane SM (eds) Metabolic Bone Disease and Clinically Related Disorders. WB Saunders and Co: Philadelphia, London, Toronto, Montreal, Sydney, Tokyo, 329–396.
32. Pittet PG, Davies M, Lawson DEM 1979 Role of nutrition in the development of osteomalacia in the elderly. Nutr Metab **23**:109–116.
33. Dibble JB, Sheridan P, Hampshire R, Hardy GJ, Losowsky MS 1982 Osteomalacia, vitamin D deficiency, and cholestasis in chronic liver disease. Quart J Med **51**:89–103.
34. Parfitt AM 1983 Dietary risk factors for age-related bone loss and fractures. Lancet **ii**:1181–1184.
35. Parfitt AM, Podenphant J, Villanueva AR, Frame B 1985 Metabolic bone disease with and without osteomalacia after intestinal bypass surgery. A bone histomorphometric study. Bone **6**:211–220.
36. Rao DS, Villanueva AR, Mathews M, Pumo B, Kleerekoper M, Parfitt AM 1983 Histologic evolution of vitamin D–depletion in patients with intestinal malabsorption or dietary deficiency. In: Frame B, Potts Jnr JT (eds) Clinical Disorders of Bone and Mineral Metabolism. Excerpta Medica: Amsterdam, 224–226.
37. Goldner WS, O'Dorisio TM, Dillon JS, Mason EE 2002 Severe metabolic bone disease as a long-term complication of obesity surgery. Obesity Surgery **12**:685–692.
38. Paterson CR, Woods CG, Pulvertaft CN, Fourman P 1965 Search for osteomalacia in 1228 patients after gastrectomy and other operations on the stomach. Lancet **ii**:1085–1088.
39. Tovey FI, Karamanolis DG, Godfrey J, Clark CG 1985 Postgastrectomy nutrition: methods of outpatient screening for early osteomalacia. Hum Nutr Clin Nutr **39**C:439–446.
40. Eddy RL 1971 Metabolic bone disease after gastrectomy. Am J Med **50**:442–449.
41. Deller DJ, Begley MD, Edwards RG, Addison M 1964 Metabolic effects of partial gastrectomy with special reference to calcium and folic acid. Gut **5**:218–225.
42. Morgan DB, Hunt G, Paterson CR 1970 The osteomalacia syndrome after stomach operations. Quart J Med **39**:395–410.
43. Davies M, Mawer EB, Krawitt EL 1980 Comparative absorption of vitamin D_3 and 25 hydroxyvitamin D_3 in intestinal disease. Gut **21**:287–292.
44. Davies M, Heys SE, Selby PS, Berry JL, Mawer EB 1997 Increased catabolism of 25-hydroxyvitamin D in patients with partial gastrectomy and elevated 1,25-dihydroxyvitamin D levels. Implications for metabolic bone disease. J Clin Endocrinol Metab **82**:209–212.
45. Rao RS, Kleerekoper M, Rogers M, Frame B, Parfitt AM 1984 Is gastrectomy a risk factor for osteoporosis? In: Christiansen C, Arnaud CD, Nordin BEC, Parfitt AM, Peck WA, Riggs BL (eds) Osteoporosis: Proceedings of the Copenhagen International Symposium on Osteoporosis. Aalborg Stiftsborgtrykkeri: Copenhagen, pp. 775–777.
46. Bisballe S, Eriksen FF, Melsen F, Mosekilde L, Sorensen O, Hessov I 1991 Osteopenia and osteomalacia after gastrectomy: interrelations between biochemical markers of bone remodeling, vitamin D metabolites, and bone histomorphometry. Gut **32**:1303–1307.
47. Nilas L, Christiansen C, Christiansen J 1985 Regulation of vitamin D and calcium metabolism after gastrectomy. Gut **26**:252–257.
48. Rümenapf G, Schwille PO, Erben RG, Schreiber M, Fries W, Schmiedl A, Hohenberger W 1997 Osteopenia following total gastrectomy in the rat: state of mineral metabolism and bone histomorphometry. Eur J Surg Res **29**:209–221.
49. Rümenapf G, Schwille PO, Erben RG, Schreiber M, Berge B, Fries W, Schmiedl A, Koroma S, Hohenberger W 1998 Gastric fundectomy in the rat: effects on mineral and bone metabolism, with emphasis on the gastrin-calcitonin-parathyroid hormone-vitamin D axis. Calcif Tissue Int **62**:433–441.
50. Maier GW, Kreis ME, Zittel TT, Becker HD 1997 Calcium regulation and bone mass loss after total gastrectomy in pigs. Ann Surg **225**:181–192.
51. Adachi Y, Shiota E, Matsumata T, Iso Y, Yoh R, Kitano S 1998 Bone mineral density in patients taking H_2-receptor antagonist. Calcif Tissue Int **62**:283–285.
52. Deller DJ, Begley MD 1983 Calcium metabolism and the bones after partial gastrectomy. Clinical features and radiology of bones. Aust Ann Med **12**:282–294.
53. Nilsson BE, Westlin NE 1971 The fracture incidence after gastrectomy. Acta Chir Scand **137**:533–534.
54. Mellstrom D, Johansson C, Johnell O, Lindstedt G, Lundberg PA, Obrant K, Schoon I, Toss G, Ytterberg B 1993 Osteoporosis, metabolic aberrations, and increased risk of vertebral fractures after partial gastrectomy. Calcif Tissue Int **53**:370–377.
55. Parfitt AM, Mathews CHE, Villanueva AR, Rao DS, Rogers M, Kleerekoper M, Frame B 1983 Microstructural and cellular basis of age-related bone loss and osteoporosis. In: Frame B, Potts Jnr JT (eds) Clinical Disorders of Bone and Mineral Metabolism. Excerpta Medica: Amsterdam, 328–332.
56. McKenna MJ, Kleerekoper M, Ellis BI, Rao DS, Parfitt AM, Frame B 1987 Atypical insufficiency fractures confused with Looser zones of osteomalacia. Bone **8**:71–78.
57. Troncone R, Maurano F, Rossi M, Micillo M, Greco L, Auricchio R, Salerno G, Salvatore F, Sacchetti L 1999 IgA antibodies to tissue transglutaminase: an effective diagnostic test for celiac disease. J Pediatr **134**:166–171.
58. Trier JS 1998 Diagnosis of celiac sprue. Gastroenterology **115**:211–216.
59. Bode S, Hassager C, Gudmand-Hoyer E, Christiansen C 1991 Body composition and calcium metabolism in adult-treated celiac disease. Gut **32**:1342–1345.
60. Caraceni MP, Molteni N, Bardella MT, Ortolani S, Gandolini GG, Bianchi P 1988 Bone and mineral metabolism in adult celiac disease. Am J Gastroenterol **83**:274–277.
61. Pistorius LR, Sweidan WH, Purdie DW, Stee SA, Howey S, Bennett JR, Sutton DR 1995 Celiac disease and bone mineral density in adult female patients. Gut **37**:639–642.

62. Walters JRF 1994 Bone mineral density in celiac disease. Gut **35**:150–151.
63. Ferretti J, Mazure R, Tanoue P, Marino A, Cointry G, Vazquez H, Niveloni S, Pedreira S, Maurino E, Zanchetta J, Bai JC 2003 Analysis of the structure and strength of bones in celiac disease patients. Am J Gastroenterol **98**: 382–390.
64. Lindh E, Ljunghall S, Larsson K, Lavo B 1992 Screening for antibodies against gliadin in patients with osteoporosis. J Intern Med **231**:403–406.
65. McFarlane J, Bhalla A, Morgan L, Reeves D, Robertson DAF 1992 Osteoporosis: a frequent finding in treated adult celiac disease. Gut **33**:S48.
66. Butcher GP, Banks LM, Walters JRF 1992 Reduced bone mineral density in celiac disease—the need for bone densitometry estimations. Gut **33**:S54.
67. Vazquez H, Mazure R, Gonzalez D, Flores D, Pedreira S, Niveloni S, Smecuol E, Maurino E, Bai JC 2000 Risk of fractures in celiac disease patients: a cross-sectional, case-control study. Am J Gastroenterol **95**:183–189.
68. Selby PL, Davies M, Adams JE, Mawer EB 1999 Bone loss in celiac disease is related to secondary hyperparathyroidism. J Bone Min Res **14**:652–657.
69. Valdimarsson T, Toss G, Löfman O, Ström M 2000 Three-years follow-up of bone density in adult celiac disease: significance of secondary hyperparathyroidism. Scand J Gastroenterol **35**:274–280.
70. Molteni N, Caraceni MP, Bardella MT, Ortolani S, Gandolini GG, Bianchi P 1990 Bone mineral density in adult celiac patients and the effect of gluten-free diet from childhood. Am J Gastroenterol **85**:51–53.
71. Bianchi ML, Bardella MT 2002 Bone and celiac disease. Calcif Tissue Int **71**:465–471.
72. Muzzo S, Burrows R, Burgueno M, Rios G, Bergenfreid C, Chavez E, Leiva L 2000 Effect of calcium and vitamin D supplementation on bone mineral density of celiac children. Nutr Res **20**:1241–1247.
73. Moss AJ, Waterhouse C, Terry R 1965 Gluten-sensitive enteropathy with osteomalacia but without steatorrhoea. N Engl J Med **272**:825–830.
74. Hajjar ET, Vincenti F, Salti IS 1974 Gluten-induced enteropathy. Arch Intern Med **134**:565–566.
75. Melvin KEW, Hepner GW, Bordier P, Neale G, Joplin GF 1970 Calcium metabolism and bone pathology in adult celiac disease. Quart J Med **39**:83–113.
76. Dibble JB, Sheridan P, Losowsky MS 1984 A survey of vitamin D deficiency in gastrointestinal and liver disorders. Quart J Med **209**:119–134.
77. Arnaud SB, Newcomer AD, Dickson ER, Arnaud CD, Go VLW 1976 Altered mineral metabolism in patients with gastrointestinal (GI) diseases. Gastroenterology **70**:860.
78. Corazza GR, Di Sario A, Cecchetti L, Tarozzi C, Corrao G, Bernardi M, Gasbarrini G 1995 Bone mass and metabolism in patients with celiac disease. Gastroenterology **109**: 122–128.
79. Hubbard VS, Farrell PM, de Sant'Agnese PA 1979 25-hydroxycholecalciferol levels in patients with cystic fibrosis. J Pediatr **94**:84–86.
80. Donovan DS Jr, Papadopulos A, Staron RB, Addesso V, Schulman L, McGregor C, Cosman F, Lindsay RL, Shane E 1998 Bone mass and vitamin D deficiency in adults with advanced cystic fibrosis lung disease. Am J Respir Crit Care Med **158**:1892–1899.
81. Hahn TJ, Squires AE, Halstead LR, Strominger DB 1979 Reduced serum 25-hydroxyvitamin D concentration and disordered mineral metabolism in patients with cystic fibrosis. J Pediatr **94**:38–42.
82. Haworth CS, Selby PL, Webb AK, Dodd ME, Musson H, Niven RMcL, Economou G, Horrocks AW, Freemont AJ, Mawer EB, Adams JE 1999 Low bone mineral density in adults with cystic fibrosis. Thorax **54**:961–967.
83. Krawitt EL, Beeken WL, Janney CD 1976 Calcium absorption in Crohn's disease. Gastroenterology **71**:251–254.
84. Driscoll RH, Meredith SC, Sitrin M, Rosenberg IH 1982 Vitamin D deficiency and bone disease in patients with Crohn's. Gastroenterology **83**:1252–1258.
85. Compston JE, Ayers AB, Horton LW, Tighe JR, Creamer B 1978 Osteomalacia after small intestinal resection. Lancet **i**:9–12.
86. Sentongo TA, Semaeo EJ, Stettler N, Piccoli DA, Stallings VA, Zemel BS 2002 Vitamin D status in children, adolescents, and young adults with Crohn's disease. Am J Clin Nutr **76**: 1077–1081.
87. Compston JE, Judd D, Crawley EO, Evans WD, Evans C, Church HA, Reid EM, Rhodes J 1987 Osteoporosis in patients with inflammatory bowel disease. Gut **28**:410–415.
88. Geinoz G, Rapin CH, Rizzoli R, Kraemer R, Buchs B, Slosman D, Michel JP, Bonjour JP 1993 Relationship between bone mineral density and dietary intakes in the elderly. Osteoporosis Int **3**:242–248.
89. Pollak RD, Karmeli F, Eliakim R, Ackerman Z, Tabb K, Rachmilevitz D 1998 Femoral neck osteopenia in patients with inflammatory bowel disease. Am J Gastroenterol **93**:1483–1490.
90. Hewison M, Abreu MT, Kantorovitch V, Vasiliauskas EA, Gruntmanis U, Matuk R, Daigle K, Chen S, Zehnder D, Lin Y-C, Yang H, Adams JS 2003 Elevated 1,25-dihydroxyvitamin D in patients with Crohn's disease: A novel risk factor for low bone mineral density. J Bone Min Res **18**(Suppl 2):417.
91. Stio M, Treves C, Celli A, Tarantion O, d'Albasio G, Bonanomi AG 2002 Synergistic inhibitory effect of ciclosporine A and vitamin D derivatives on T-lymphocyte proliferation in active ulcerative colitis. Am J Gastroenterol **97**:679–689.
92. Atkinson RL, Dahms WT, Bray GA, Schwartz AA 1978 Parathyroid hormone in obesity: effects of intestinal bypass. Miner Electrolyte Metab **1**:315–320.
93. Dano P, Christiansen C 1978 Calcium malabsorption and absence of bone decalcification following intestinal shunt operation for obesity. Scand J Gastroenterol **13**:81–85.
94. Teitelbaum SL, Halverson JD, Bates M, Wise L, Haddad JG 1977 Abnormalities of circulating 25-OH vitamin D after jejuno-ileal bypass for obesity and evidence of an adaptive response. Ann Int Med **856**:288–293.
95. Rickers H, Christiansen C, Balsev I, Rodbro P 1984 Impairment of vitamin D metabolism and bone mineral content after intestinal bypass for obesity. Scand J Gastroenterol **19**:184–189.
96. Hey H, Stokholm KH, Lund BJ, Lund BI, Sorensen OH 1982 Vitamin D deficiency in obese patients and changes in circulating vitamin D metabolites following jejunoileal bypass. Int J Obes **6**:473–479.
97. Mosekilde L, Melsen F, Hessov I, Christensen MS, Lund BJ, Lund BI, Sorensen OH 1980 Low serum levels of 1,25 dihydroxyvitamin D and histomorphometric evidence of osteomalacia after intestinal bypass for obesity. Gut **21**:624–631.
98. Compston JE, Horton LWL, Laker MF, Merrett AL, Woodhead JS, Gazet J-C, Pilkington TRE 1980 Treatment of bone disease after jejunoileal bypass for obesity with oral 1-hydroxyvitamin D_3. Gut **21**:669–674.

99. Klein GL, Chesney RW 1986 Metabolic bone disease associated with total parenteral nutrition. In: Lebenthal E (ed). Total parenteral nutrition: Indication, utilization, complications and pathophysiological considerations. Raven Press: New York, 431–442.
100. Klein GL, Alfrey AC, Miller ML, Sherrard DJ, Hazlet TK, Ament ME, Coburn JW 1982 Aluminum loading during total parenteral nutrition. Am J Clin Nutr **35**:1425–1429.
101. Klein GL, Targoff CM, Ament ME, Sherrard DJ, Bluestone R, Young JH, Norman AW, Coburn JW 1980 Bone disease associated with total parenteral nutrition. Lancet **ii**: 1041–1044.
102. Vargas JH, Klein GL, Ament ME, Ott SM, Sherrard DJ, Horst RL, Berquist WE, Alfrey AC, Slatopolsky E, Coburn JW 1988 Metabolic bone disease of total parenteral nutrition: course after changing from casein to amino acids in parenteral solutions with reduced aluminum content. Am J Clin Nutr **48**:1070–1078.
103. Bengoa JM, Sitrin MD, Wood RJ, Rosenberg IH 1983 Amino acid-induced hypercalciuria in patients on total parenteral nutrition. Am J Clin Nutr **38**:264–269.
104. Lipkin EW, Ott SM, Klein GL 1987 Heterogeneity of bone histology in parenteral nutrition patients. Am J Clin Nutr **46**:673–680.
105. Shike M, Shils ME, Heller A, Alcock N, Vigorita V, Brockman R, Holick MF, Lane J, Flombaum C 1986 Bone disease in prolonged parenteral nutrition: osteopenia without mineralization defect. Am J Clin Nutr **44**:89–98.
106. Jung RT, Davie M, Siklos P, Chalmers TM, Lawson DEM 1979 Vitamin D metabolism in acute and chronic cholestasis. Gut **20**:840–847.
107. Recker RR, Maddrey W, Herlong F, Sorrell M, Russell R 1983 Primary biliary cirrhosis and alcoholic cirrhosis as examples of chronic liver disease associated with bone disease. In: Frame B, Potts JT Jr (eds) Clinical Disorders of Bone and Mineral Metabolism, Excerpta Medica: International Congress Series, **617**:227–230.
108. Stellon AJ, Webb A, Compston J, Williams R 1987 Low bone turnover state in primary biliary cirrhosis. Hepatology **7**:137–142.
109. Atkinson M, Nordin BEC, Sherlock S 1956 Malabsorption and bone disease in prolonged obstructive jaundice. Quart J Med **25**:299–312.
110. Long RG, Varghese Z, Meinhard EA, Skinner RK, Wills MR, Sherlock S 1978 Parenteral 1,25 dihydroxycholecalciferol in hepatic osteomalacia. Br Med J **i**:75–77.
111. Compston JE, Crowe JP, Wells IP, Horton LWL, Hirst D, Merrett AL, Woodhead JS, Williams R 1980 Vitamin D prophylaxis and osteomalacia in chronic cholestatic liver disease. Dig Dis Sci **25**:28–32.
112. Crosbie OM, Freaney R, McKenna MJ, Hegarty JE 1999 Bone density, vitamin D status, and disordered bone remodeling in end-stage chronic liver disease. Calcif Tissue Int **64**:295–300.
113. Davies M, Mawer EB, Klass HJ, Lumb GA, Berry JL, Warnes TW 1983 Vitamin D deficiency osteomalacia and primary biliary cirrhosis, the response to orally administered vitamin D_3. Dig Dis Sci **28**:145–153.
114. Compston JE, Horton LWL, Thompson RPH 1979 Treatment of osteomalacia associated with PBC with parenteral vitamin D_2 or oral 25 hydroxyvitamin D_3. Gut **20**:133–136.
115. Compston JE, Crowe JP, Horton LWL 1979 Treatment of osteomalacia associated with primary biliary cirrhosis with oral 1 alpha hydroxyvitamin D_3. Br Med J **2**:309.
116. Danielson A, Lorentzon R, Larsson S-E 1982 Normal hepatic vitamin D metabolism in icteric primary biliary cirrhosis associated with pronounced vitamin D deficiency symptoms. Hepatogastroenterology **29**:6–8.
117. Long RG, Skinner RK, Wills MR, Sherlock S 1976 Serum 25-Hydroxy-Vitamin-D in untreated parenchymal and cholestatic liver disease. Lancet **ii**:650–652.
118. Atkinson MJ, Vido I, Keck E, Hesch RD 1983 Hepatic osteodystrophy in primary biliary cirrhosis: a possible defect in Kupffer cell mediated cleavage of parathyroid hormone. Clin Endocrinol **118**:21–28.
119. Klein GL, Endres DB, Colonna II JD, Berquist WE, Goldstein LI, Busuttil RW, Deftos LJ 1989 Absence of hyperparathyroidism in severe liver disease. Calcif Tissue Int **44**:330–334.
120. Thompson WG, Thompson GR 1969 Effect of cholestyramine on the absorption of vitamin D_3 and calcium. Gut **10**:717–722.
121. Hodgson SF, Dickson ER, Eastell R, Eriksen EF, Bryant SC, Riggs BL 1993 Rates of cancellous bone remodeling and turnover in osteopenia associated with primary biliary cirrhosis. Bone **14**:819–827.
122. Wagonfield JB, Nemchausky BA, Bolt M, Horst JV, Boyer JH, Rosenberg IH 1976 Comparison of vitamin D and 25-hydroxyvitamin D in the therapy of primary biliary cirrhosis. Lancet **ii**:391–394.
123. Krawitt EL, Grundman MJ, Mawer EB 1977 Absorption hydroxylation and excretion of vitamin D_3 in primary biliary cirrhosis. Lancet **ii**:1246–1249.
124. Davies M, Mawer EB 1979 The absorption and metabolism of vitamin D_3 from subcutaneous and intramuscular injection sites. In: Norman AW (ed) Vitamin D: Basic Research and Its Clinical Application. De Gruyter: Berlin, pp. 609–612.
125. Reed JS, Meredith SC, Nemohausky BA, Rosenberg IH, Boyer JL 1980 Bone disease in primary biliary cirrhosis: reversal of osteomalacia by 25-hydroxyvitamin D. Gastroenterology **78**:512–517.
126. Mawer EB, Klass HJ, Warnes TW, Berry JL 1985 Metabolism of vitamin D in patients with primary biliary cirrhosis and alcoholic liver disease. Clin Sci **69**: 561–570.
127. Lindor KD, James CH, Crippin JS, Jorgensen RA, Dickson ER 1995 Bone disease in primary biliary cirrhosis: does urodeoxycholic acid make a difference? Hepatology **21**:389–392.
128. Camisasca M, Crosignani A, Battezzati PM, Albisetti W, Grandinetti G, Pietrogrande L, Biffi A, Zuin M, Podda M 1994 Parenteral calcitonin for metabolic bone disease associated with primary biliary cirrhosis. Hepatology **20**: 633–637.
129. Nilsson BE 1970 Conditions contributing to fracture of the femoral neck. Acta Chir Scand **136**:383–384.
130. Lalor BC, France MW, Powell D, Adams PH, Counihan TB 1986 Bone and mineral metabolism and chronic alcohol abuse. Quart J Med **229**:497–511.
131. Israel Y, Orrego H, Holt S, Macdonald DW, Meema HE 1980 Identification of alcohol abuse: thoracic fractures on routine X-rays as indicators of alcoholism. Alcohol Clin Exp Res 420–422.
132. Peris P, Guanabens N, Pares A, Pons F, de Rio L, Monegal A, Suris X, Caballeria J, Rodes J, Munoz-Gomez J 1995 Vertebral fractures and osteopenia in chronic alcoholic patients. Calcif Tiss Int **57**:111–114.

133. Diamond T, Stiel D, Lunzer M, Wilkinson M, Posen S 1989 Ethanol reduces bone formation and may cause osteoporosis. Am J Med **86**:282–288.
134. Crilly RG, Anderson C, Hogan D, Delaquerriere-Richardson L 1988 Bone histomorphometry, bone mass, and related parameters in alcoholic males. Calcif Tissue Int **43**:269–276.
135. Diez A, Puig J, Serrano S, Marinoso M-L, Bosch J, Marrugat J, Mellibovsky L, Nogues X, Knobel H, Aubia J 1994 Alcohol-induced bone disease in the absence of severe chronic liver damage. J Bone Min Res **9**:825.
136. Hepner GW, Roginsky M, Moo HF 1986 Abnormal vitamin D metabolism in patients with cirrhosis. Dig Dis **21**: 527–532.
137. Jung RT, Davie M, Hunter JO, Chalmers TM, Lawson DEM 1978 Abnormal vitamin D metabolism in cirrhosis. Gut **19**:290–293.

CHAPTER 76

Vitamin D and Renal Failure

ADRIANA S. DUSSO, ALEX J. BROWN, AND EDUARDO A. SLATOPOLSKY
Renal Division, Washington University School of Medicine, St. Louis, Missouri

I. INTRODUCTION

The kidney is a central component of the powerful endocrine system evolved to maintain extracellular calcium within narrow limits, a process vital for normal cellular physiology as well as skeletal integrity. Fig. 1A summarizes the main secretory control and hormonal interactions between the kidney, the parathyroid glands, intestine, and bone responsible for the maintenance of normal calcium homeostasis, and the alterations in the functions of this system induced by kidney disease Fig. 1B shows the changes found in kidney disease and will be discussed further below in Section IV.

Kidney disease not only impairs the renal handling of calcium and phosphate ions, but also the endocrine capacity of the kidney to secrete the potent calcitropic hormone 1,25-dihydroxyvitamin D [$1,25(OH)_2D$ or calcitriol] [1]. The immediate consequence of calcitriol deficiency is decreased intestinal calcium absorption. The resulting hypocalcemia, along with the low circulating calcitriol levels, enhances the synthesis and secretion of parathyroid hormone (PTH) and eventually induces hyperplastic parathyroid-cell growth in an effort to re-establish calcium balance [2]. High circulating levels of PTH cause bone loss, osteitis fibrosa, and various degrees of skeletal abnormalities known as *renal osteodystrophy*. High serum levels of PTH also cause systemic toxicities, including cardiovascular, endocrine, nervous, immunologic, and cutaneous dysfunctions that markedly increase morbidity and mortality rates in renal failure patients [2].

Calcitriol not only has a critical role in the maintenance of calcium homeostasis and serum PTH levels, but also has potent effects in the differentiation and function in many cell types including skin, pancreas, and muscle, as well as hematopoietic, immune, and nervous systems [1]. In fact, epidemiological studies suggest an association between calcitriol deficiency and hypertension, diabetes, as well as immunological and neuromuscular abnormalities, all of which are common disorders in patients with advanced kidney disease.

This chapter presents the current understanding of the molecular mechanisms leading to calcitriol deficiency and resistance to calcitriol action in chronic renal failure. The pathophysiological implications of these defects is examined in classical vitamin D target organs (intestine, parathyroid glands, bone, and the kidney), as well as in tissues unrelated to calcium homeostasis, which, as mentioned, compromise the well being of patients with kidney disease.

The last section, on vitamin D therapy in predialysis and patients undergoing hemodialysis, includes an overview of the treatment of renal osteodystrophy with special emphasis on recent clinical trials with calcitriol analogs, as well as the 2003 recommendations from the United States National Kidney Foundation.

II. ALTERATIONS IN VITAMIN D BIOACTIVATION TO $1,25(OH)_2D$

A. Decreased Renal Mass

The kidney proximal convoluted tubule is the principal site for the final and most critical step in vitamin D biological activation: 1α-hydroxylation of 25-hydroxyvitamin D (25OHD) to the potent calcitropic-steroid hormone 1,25-dihydroxyvitamin D ($1,25(OH)_2D$ or calcitriol) [1]. To maintain extracellular calcium within narrow limits, renal 1α-hydroxylase, a cytochrome-P450 mixed-function oxidase, is tightly regulated. PTH, hypocalcemia, and hypophosphatemia are the major inducers, whereas hyperphosphatemia, hypercalcemia, and calcitriol, the enzyme product, repress its activity (see Chapters 5 and 7).

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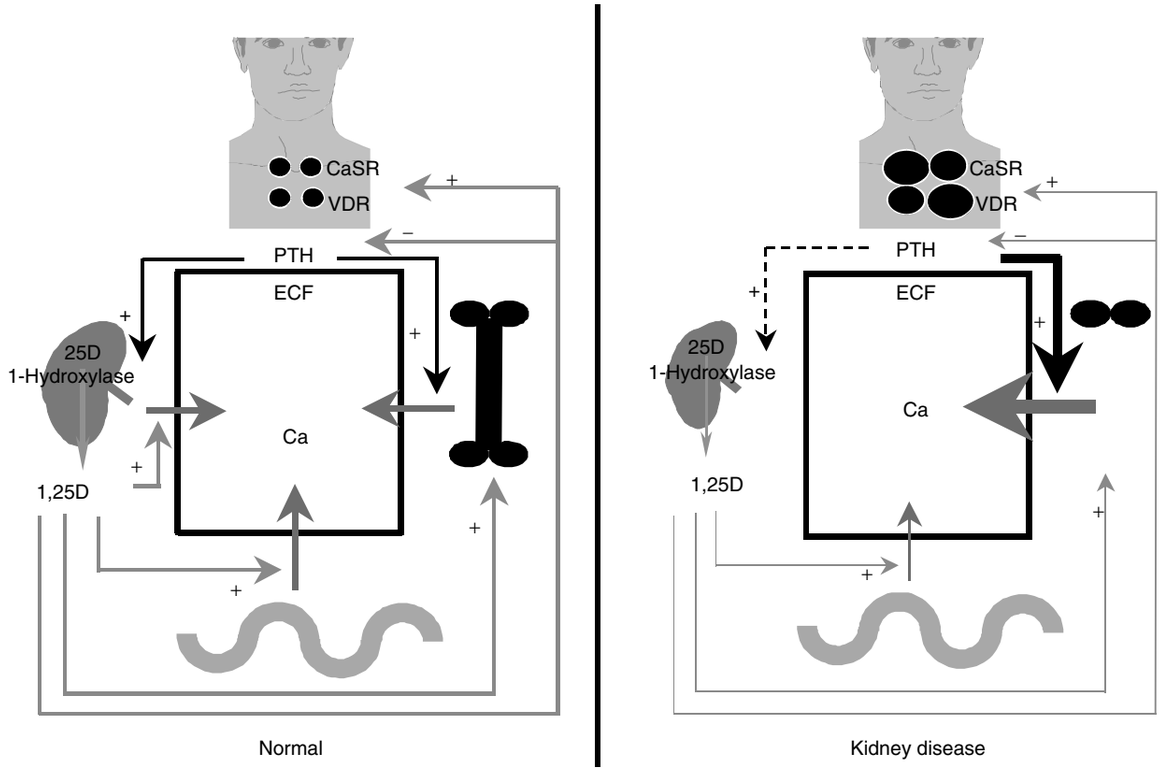

FIGURE 1 **Left panel.** Calcium fluxes and hormonal interactions between the kidney, bone, intestine, and parathyroid glands responsible for the maintenance of extracellular calcium homeostasis. **Right panel.** Alterations to this powerful endocrine system induced by chronic kidney disease. (See text for details.)

Martinez *et al*. (see Figure 2) [3], as well as other investigators, showed that, in the course of chronic kidney disease (CKD), the levels of calcitriol in blood remain in the normal range until the glomerular filtration rate (GFR) falls below 50% of normal [4–7]. Some investigators, however, have described patients whose plasma levels of calcitriol were below normal with creatinine clearances between 50 and 80 ml per minute [8]. Importantly, even normal levels of calcitriol should be considered abnormally low for the elevated circulating concentrations of PTH at these early stages of kidney disease. From these findings, it can be inferred that, in addition to the progressive reduction in functional renal mass, other abnormalities impair calcitriol production by the remnant 1α-hydroxylase. These abnormalities include impaired delivery of its substrate, 25OHD, to mitochondrial-renal 1α-hydroxylase, as well as a blunted induction of enzymatic activity in response to PTH. Direct inhibition of the activity of remnant renal 1α-hydroxylase by hyperphosphatemia, acidosis, and/or the accumulation of uremic toxins is an additional contributor to reduced calcitriol production in CKD. This section presents the mechanisms underlying the abnormalities in renal calcitriol synthesis induced by CKD.

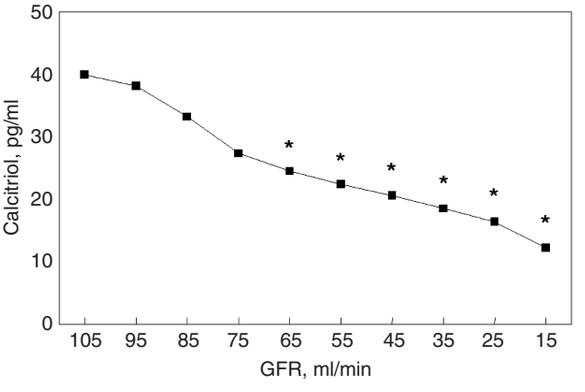

FIGURE 2 Correlation between serum calcitriol and glomerular filtration rates (GFR) in 165 patients with different degrees of CKD. * indicates GFR levels at which the decreases in calcitriol reached statistical significance. Adapted from Martinez *et al*. [3].

B. Reduced Substrate Availability to Renal 1α-hydroyxlase

In patients with CKD with a GFR below 25 ml/min and therefore very limiting renal-1α-hydroxylase activity, serum calcitriol levels are low only in the presence of normal serum 25OHD concentrations [9]. However, serum levels of calcitriol were normalized in these severely uremic patients by increasing the serum concentrations of the substrate, 25OHD, to supraphysiological levels through oral supplementation [9]. Furthermore, a strong correlation was found between serum levels of substrate and product of renal 1α-hydroxylase. This association, which does not occur in individuals with normal kidney function, is believed to result from an impairment in 25OHD availability to renal 1α-hydroxylase in severe renal failure.

Studies by Nykjaer and collaborators in the megalin-null mice [10] provided new insights into the mechanisms responsible for impaired substrate availability and the strong correlation between serum levels of 25OHD and calcitriol in CKD (see Chapter 10). The megalin-null mice challenged the concept that renal 25OHD uptake by proximal tubular cells occurs through simple diffusion of the sterol through the basolateral membrane upon dissociation from its main carrier in circulation (the vitamin D binding protein, DBP). In fact, simple diffusion of 25OHD through the cell membrane cannot explain the impaired substrate availability to mitochondrial 1α-hydroxylase that is present in severe uremia. Instead, it was shown that the 25OHD/DBP complex in the circulation is filtered through the glomerulus and endocytosed into the proximal tubular cell via the apical-membrane receptor, megalin [10], a member of the LDL receptor superfamily. In CKD, the lower the GFR, the lower the amount of filtered 25OHD/DBP complex, which in turn, limits the amount of intracellular 25OHD available for conversion to calcitriol by renal 1α-hydroxylase. 25OHD supplementation increases the proportion of 25OHD/DBP-complex in the blood with the consequent enhancement of the amount of 25OHD filtered and the intracellular levels available for bioactivation to calcitriol by the renal 1α-hydroxylase. However, as discussed below, 25OHD supplementation in severe renal failure could also enhance circulating calcitriol by increasing substrate availability to nonrenal 1α-hydroxylase.

Importantly, in rats, renal megalin-mRNA levels progressively decrease by two weeks after the induction of renal failure [11]. In CKD, reduced renal-megalin levels would worsen the already abnormal uptake of 25OHD by the proximal convoluted tubules caused by the reduced glomerular filtration of the 25OHD/DBP-complex. The demonstration that calcitriol up-regulates megalin expression in renal cells in culture [12] raises the possibility that the low circulating calcitriol in renal failure may constitute an additional contributor to reduced megalin expression in cells of the proximal convoluted tubules.

Abnormal 25OHD delivery to extrarenal sources of calcitriol also occurs in kidney disease [13]. 1α-hydroxylase expression in numerous nonrenal cells has been well documented (see Chapter 79). The contribution of nonrenal 1α-hydroxylases to systemic calcitriol was conclusively demonstrated in bilaterally nephrectomized patients undergoing hemodialysis [14]. Importantly, in anephric humans, a strong correlation exists between serum levels of calcitriol and its precursor 25OHD, similar to that previously described in severely uremic patients (GFR below 25 ml/min). These findings led us to an evaluation of the impact of kidney disease on substrate availability to monocyte/macrophage 1α-hydroxylase, the same enzyme as renal 1α-hydroxylase, though more readily accessible. Peripheral blood monocytes from hemodialysis patients elicit a markedly impaired uptake of 25OHD compared to that in monocytes from normal individuals [13]. This defective uptake could be corrected by normalizing the low serum calcitriol levels of hemodialysis patients through intravenous calcitriol supplementation for 15 days. Neither the mechanisms mediating impaired-25OHD uptake nor those involved in its correction by calcitriol treatment are known. Calcitriol was shown to modulate LDL-receptor expression and function in the human monocytic cell line HL60 [15,16]. A similar mechanism could partially account for calcitriol-induced correction of the impaired uptake of 25OHD by peripheral blood monocytes from hemodialysis patients.

C. Blunted Induction of Renal-1α-hydroxylase by PTH- and Calcium

PTH is a potent stimulator of calcitriol synthesis. Patients with hypoparathyroidism have low calcitriol levels despite persistent hypocalcemia [17]. Furthermore, parathyroidectomy severely blunts the induction of renal 1α-hydroxylase by hypocalcemia [18]. As mentioned earlier in this section, with the elevations in serum PTH occurring at early stages of renal disease, normal calcitriol levels in patients with GFR between 50 and 80 ml/min reflect abnormalities in PTH induction of renal calcitriol synthesis. In fact, Ritz and collaborators demonstrated an impaired increase in serum calcitriol in response to pharmacological doses of PTH in patients with a GFR of 70 ml/min and normal calcitriol levels [19], compared to individuals with normal

kidney function. Mechanistically, PTH is known to activate 1α-hydroxylase-gene transcription through a cAMP-mediated mechanism [20–22].

Acidosis, a feature commonly present in renal failure, could contribute to impaired PTH induction of calcitriol production in these patients. In the dog, acidosis blunts the action of PTH on 1α-hydroyxlase of the proximal convoluted tubule. This effect can be overcome by cAMP, and it is unrelated to a loss of renal PTH receptors. Taken together, these findings suggest that acidosis induces an altered coupling of PTH receptor with adenylate cyclase [23] that blunts PTH enhancement of calcitriol production.

Phosphate retention, a common feature in renal failure and a potent inhibitor of renal 1α-hydroxylase [24], could also account for counteracting the stimulatory actions of PTH on calcitriol production. Since acidosis also blunts the response to PTH on phosphate reabsorption [25], it could affect PTH induction of renal 1α-hydroxylase indirectly through concurrent effects on phosphate homeostasis.

Similar to the impaired induction of 1α-hydroxylase activity by PTH in CKD, a blunted increase in serum calcitriol in response to calcium restriction was reported by Prince and collaborators [26].

D. Direct Inhibition of 1α-hydroxylase Expression and Activity by Hyperphosphatemia, Acidosis, and Accumulation of Uremic Toxins

In individuals with normal kidney function, phosphate restriction increases renal calcitriol production and serum calcitriol levels, despite a decrease in serum PTH [27]. See Chapter 26 for a discussion of phosphate. Interestingly, whereas hypophysectomy blocks the stimulating action of dietary phosphate restriction on 1α-hydroxylase activity, injection of growth hormone or IGF-I to hypophysectomized rats restores the induction of renal calcitriol production in response to a low dietary phosphate intake [28]. Recent studies in intact mice and rats demonstrated that phosphate restriction not only modulates the activity but also mRNA levels of renal 1α-hydroxylase [29,30]. Furthermore, they supported the involvement of a growth hormone mediated mechanism for low P induction of renal-1α-hydroxylase mRNA levels.

Kidney disease also appears to blunt growth hormone/low P-signaling pathway, mediating the induction of renal 1α-hydroxylase mRNA levels and activity. There are no increases in serum calcitriol in response to P restriction in patients with end-stage renal disease [31] or in severely uremic dogs [32]. The possibility also exists that the induction by phosphorus restriction of the very low amounts of remnant 1α-hydroxylase is insufficient to result in measurable increases in serum calcitriol levels.

Transepithelial inorganic phosphate transport by the renal tubule was suggested as the mechanism underlying low phosphate induction of renal-1α-hydroxylase. However, studies in the mice null for the phosphate-regulated renal Na/P cotransporter 2 (NPT2a) revealed that intact renal-Na/P co-transport is not required for the regulation of 1α-hydroxylase mRNA levels and activity by dietary phosphate restriction [33].

In contrast to the stimulatory effects of phosphate restriction, oral phosphate supplementation reduces serum calcitriol concentrations in patients with idiopathic hypercalciuria or in children with moderate renal insufficiency [34]. As mentioned in the prior section, hyperphosphatemia could partially account for the blunted response to PTH induction of renal calcitriol production. Also, metabolic acidosis, through alterations in phosphate homeostasis or by inducing increases in ionized calcium, could affect renal 1α-hydroxylase activity. However, the actual contribution of acidosis to modulate renal 1α-hydroxylase is controversial. Acidosis has been shown to decrease, increase, or not to change serum calcitriol levels [35–39].

Accumulation of uremic toxins in CKD could account for reduced renal-calcitriol synthesis. In normal rats, the infusion of uremic-plasma ultrafiltrate markedly decreases calcitriol production rates. Low molecular weight compounds in uremic plasma ultrafiltrate were identified *in vitro* as the inhibitors of 1α-hydroxylase activity [40]. The same authors have also shown that in partially nephrectomized rats, high dietary protein intake, which increases the production of uremic toxins, suppresses calcitriol synthesis.

Taken together these studies suggest that, in CKD, there is an active suppression of the reserve for synthetic calcitriol capacity in the remnant kidney. The actual contribution of impaired substrate availability and/or direct inhibition of transcriptional and/or post-transcriptional regulatory mechanisms on 1α-hydroxylase-expression and activity is unclear at present.

E. Abnormal Calcitriol Catabolism

Serum calcitriol levels are tightly regulated through simultaneous control of synthetic and catabolic rates [1]. The ubiquitously distributed enzyme 24-hydroxylase catalyzes most steps in the major pathway responsible for calcitriol metabolic inactivation. 24-Hydroxylase-gene expression is highly inducible by calcitriol (see Chapters 6 and 7). Calcitriol induction of its own

catabolism provides a fine-tuning for both calcitriol control of its own levels in circulation and the magnitude of the response to calcitriol in target organs [1]. In fact, in normal individuals, the metabolic clearance rate of calcitriol is accelerated when its production rate increases [41].

In contrast to calcitriol stimulation of its own degradation, PTH down-regulates 24-hydroxylase activity in normal individuals [42]. This reduction in calcitriol catabolism could partially account for PTH up-regulation of serum calcitriol levels and the subsequent increases in intestinal calcium absorption.

From the low circulating levels of calcitriol and the high PTH in CKD, reduced catabolic rates of calcitriol were expected. However, decreased, unaltered, and increased rates of calcitriol catabolism were reported in CKD.

Hsu and collaborators reported a decreased calcitriol metabolic clearance rate in patients with CKD [43] and in the rat model of experimental renal insufficiency [44]. This reduction was considered a compensatory mechanism to maintain normal serum calcitriol levels at early stages of kidney disease.

In contrast to these findings, studies of calcitriol metabolic clearance rate in dogs with mild to severe CKD demonstrated no changes in the rate of calcitriol catabolism with the progression of kidney disease. The changes in serum levels of calcitriol reflected the decrease in production rates with the progression of kidney disease [45].

In relation to enhanced calcitriol catabolism in CKD, it is possible that a mechanism similar to that blunting PTH induction of 1α-hydroxylase impairs PTH reduction of 24-hydroxylase. In fact, enhanced rather than reduced intestinal 24-hydroxylase was found in renal failure [46]. This increase in intestinal calcitriol degradation could partially account for the blunted response to calcitriol treatment to increase intestinal calcium absorption. Metabolic acidosis, commonly present in CKD, was also shown to increase 24-hydroxylase activity [47].

Independent of the controversial data on changes in the rate of calcitriol catabolism induced by kidney disease, it is obvious from the low serum calcitriol levels that reduced production rate is the main contributor to calcitriol deficiency.

III. ALTERATIONS IN $1,25(OH)_2D$/VDR ACTION

Most, if not all, of calcitriol biological actions are mediated by a high affinity receptor, the vitamin D receptor (VDR), which acts as a ligand-activated transcription factor (see Chapters 11, 13, 16, and 17). In kidney disease, in addition to the decreases in calcitriol synthesis described in the previous section, there is compelling evidence of resistance to calcitriol actions. Decreased VDR levels in target tissues and abnormalities in calcitriol/VDR regulation of the expression of vitamin D responsive genes have been observed in both patients and experimental animals with kidney disease. This section presents the current understanding of the mechanisms underlying both defects, as well as the potential contribution of VDR polymorphisms in the dialysis population to VDR expression and activity.

A. Defective Homologous Up-regulation of VDR

The best-known regulator of VDR expression in target tissues is calcitriol itself (see Chapters 11, 12, and 15). Calcitriol up-regulation of VDR expression involves dual mechanisms: It increases VDR mRNA levels and/or VDR protein stability [48,49]. The latter is the most common process in calcitriol up-regulation of VDR content. Calcitriol binding to the VDR prevents receptor degradation by the proteasome complex, thus enhancing the half-life of the ligand-bound VDR compared to that of the unliganded VDR [49]. In renal failure, because serum calcitriol levels are low, a decrease in VDR content is predictable. In fact, calcitriol-binding studies in the parathyroid glands of humans [50,51], rats [52], and dogs [53] support a reduction in VDR number in uremia without changes in the affinity of the receptor for calcitriol. Immunohistochemical studies in the parathyroid glands of uremic patients corroborated these results and also demonstrated that the lowest VDR content was found in areas of nodular growth, the most aggressive form of parathyroid hyperplasia in severe kidney disease [50]. The latter finding suggests that the mitogenic signals that trigger parathyroid hyperplasia could also down-regulate VDR expression.

A contribution of calcitriol deficiency to reduced VDR content was supported by studies in parathyroid glands from normal and uremic rats (see Fig. 3), which demonstrated a strong correlation between serum levels of calcitriol and parathyroid VDR content [54]. This association also suggested the potential of calcitriol therapy to correct the reduced parathyroid VDR content in renal failure patients. In fact, Fig. 4 shows that in uremic rats, the reduced parathyroid VDR content could be normalized by administration of either calcitriol or its less-calcemic analog 22-oxa-calcitriol [54].

Controversial reports exist on calcitriol binding to and regulation of intestinal VDR. Increased and reduced VDR binding was found in uremic rats

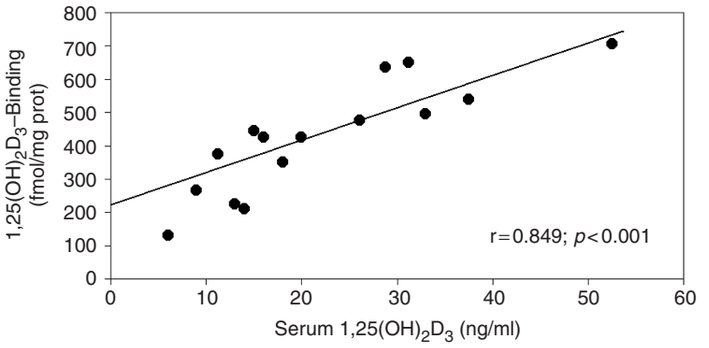

FIGURE 3 Correlation between parathyroid VDR content and serum calcitriol levels in uremic and control animals. Adapted from Denda *et al*. [54].

compared to normal animals. In normal rats, calcitriol has no effect on VDR mRNA levels but markedly increases VDR protein by 12 to 24 h [55]. In contrast, in uremic rats, there is a blunted response to calcitriol. Despite an increase in VDR mRNA levels by 5 h, there is only a mild increase in VDR protein after 12 to 24 h of calcitriol exposure [55]. High PTH levels or hypocalcemia in uremic rats could impair calcitriol up-regulation of intestinal VDR since induction of hyperparathyroidism by calcium restriction was shown to prevent calcitriol up-regulation of intestinal VDR [56]. In contrast to these reports, Patel and co-workers demonstrated similar increases in VDR binding after 18 h exposure to calcitriol that were preceded by transient increases in VDR mRNA [57].

In the kidney, vitamin D or calcitriol supplementation to rats with a normal calcium intake increases VDR content up to five fold [58–60]. While Brown and collaborators reported a mild induction of kidney VDR mRNA by calcitriol in rats fed a high calcium diet [61], other investigators found no induction of renal VDR mRNA by calcitriol [49,62].

Controversial reports also exist demonstrating either reduced or normal VDR levels in peripheral blood monocytes from hemodialysis patients compared to monocytes from normal individuals [13,63].

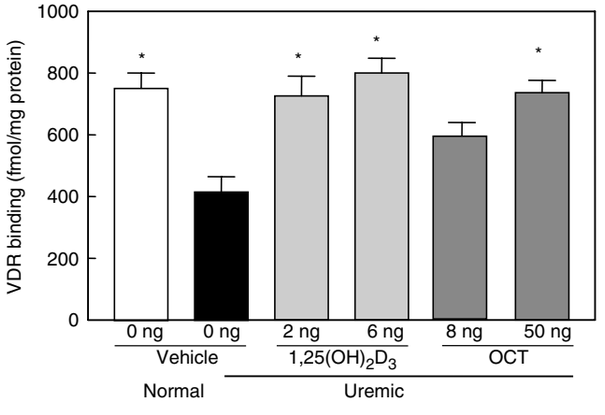

FIGURE 4 Calcitriol [1,25(OH)$_2$D$_3$] and 22-oxacalcitriol [OCT] supplementation corrected the reduced-VDR content in the parathyroid glands of uremic rats (0 ng) to the levels of rats with normal renal function (Normal). Adapted from Denda *et al*. [54].

B. Potential Contribution of VDR Polymorphisms to Reduced-VDR Expression and Function

The human VDR is encoded by a gene, which localizes in chromosome 12. The gene has 11 exons, and the first three are variably present in VDR mRNAs. There are normal genetic variants or polymorphisms of the human VDR [64,65]. This topic is the focus of Chapters 68 and 12. The most frequently studied of these polymorphisms are located at the 3′ untranslated region in the intron separating exon VIII and IX and were defined by the restriction enzymes BmsI, ApaI, and TaqI. None of these variants affects either the expression or the translated VDR protein. Linked to these polymorphisms is a microsatelite poly A repeat of variable length (long or short), which may affect mRNA stability. A most recent polymorphism to be studied, FokI, is not linked to the others and results in a three amino acid shorter VDR with higher biological activity. Controversial reports exist on the association between the frequency of certain alleles and changes in bone density [66], the propensity to hyperparathyroidism [67,68], and resistance to vitamin D therapy [69].

In patients with renal failure from Western populations, higher PTH levels were reported to associate

with the bb genotype [70]. However, a faster bone mineral loss is seen in the BB group [71]. Also patients in the BB group can remain longer in hemodialysis before they need parathyroidectomy [72].

In Japanese patients, high PTH associates with the bb genotype only in one group of patients [73], whereas no association with the bb, but with the aa genotype was also reported [74]. Bone density after renal transplantation was also lower in the bb group as a result of the impact of this genotype on the severity of secondary hyperparathyroidism [75]. Moreover, hemodialysis patients in the aa-group showed higher sensitivity to parathyroid responsiveness to changes in $[Ca^{++}]$ than those in the AA or Aa groups with repercussions on the onset and progression of secondary hyperparathyroidism [76]. The cause of these associations is still unclear because, as mentioned at the beginning of this section, the encoded VDR protein remains unchanged, and there are no changes in VDR mRNA stability to affect VDR expression. No association has been reported between these polymorphisms and accelerated VDR-targeting for proteosomal degradation.

As mentioned, important limitations of all of these epidemiological studies are that correlations were sought between a single specific polymorphism or between the Bsm-Apa-Taq linkage group and the physiological parameter of interest [77]. More importantly, most studies lack analysis of the direct influence of allelic variation on VDR protein expression or activity. These limitations leave open the possibility that the observed correlations might be due to another nearby site or even to a different gene.

In a recent study, the functional significance of two unlinked-human VDR-gene polymorphisms [at a FokI restriction site (F/f) in exon II and a singlet A repeat (L/S)] was examined simultaneously [77]. Higher activity of the F and L biallelic forms was found. Statistical significance emerged when genotypes at both sites were considered simultaneously [77]. This study suggests that simultaneous reassessment of F/f and L/S genotypes in the hemodialysis population could provide insights into the actual contribution of VDR polymorphisms to abnormal parathyroid function, important for prognosis and therapy. The same study raised the possibility that polymorphisms in the 5′ region of the VDR gene might affect the activity of the VDR promoters [78], leading to the expression of altered quantities of VDR protein. Recently, a polymorphism was described in a binding site for Cdx-2, a homeodomain protein involved in intestine-specific VDR expression [79]. In fact, in a large cohort of Japanese women, the A allele at the Cdx-2 locus, correlated with higher bone mineral density in the lumbar spine consistent with a slightly greater activity of a VDR promoter construct [80]. This polymorphism also should be considered in epidemiological studies relating VDR-mediated intestinal absorption of Ca and P, as they could impact both bone mineral density and parathyroid function in the hemodialysis patient population.

C. Additional Mechanisms for Calcitriol Resistance in CKD

The calcitriol synthesized in the kidney by mitochondrial 1α-hydroxylase is transported in the blood by carrier proteins. Vitamin D–binding protein (DBP) is the main carrier. However, calcitriol also binds albumin and lipoproteins [1]. Recently, reports showing that the free form of calcitriol triggers biological responses after entering target cells by simple diffusion have been challenged. The demonstration that 25OHD uptake by renal proximal tubular cells occurs through megalin-mediated endocytosis [10], raised the possibility that calcitriol entrance to target cells could also involve a megalin- or LDL receptor-mediated endocytosis. Megalin is expressed in several epithelial cell types [81], including parathyroid cells [82] that respond to calcitriol. Interestingly, the LDL receptor was shown to mediate calcitriol uptake by human fibroblasts [83]. Therefore, uremia-induced reduction in megalin expression could constitute an additional mechanism for calcitriol resistance independent of the abnormalities described in VDR content or function.

Once inside the target cell, calcitriol binding to the VDR activates the receptor to translocate from the cytosol to the nucleus where it heterodimerizes with its partner, the retinoid X receptor, RXR. The VDR/RXR complex binds specific sequences in the promoter region of target genes, called *vitamin D response elements* (VDRE), and recruits basal transcription factors and co-regulator molecules to either increase or suppress the rate of gene transcription by RNA-polymerase II [1]. Numerous genes, transcriptionally induced or suppressed by the calcitriol/VDR-complex, are relevant for the efficacy of calcitriol therapy in renal failure. The main biological action resulting from calcitriol regulation of the expression of these genes is a tight control of calcium homeostasis by the parathyroid glands, bone, intestine, and the kidney. Specifically, these genes affect calcitriol/VDR regulation of the rates of calcitriol synthesis and catabolism, suppression of PTH synthesis, and induction of the expression of the calcium-sensing receptor (CaSR) [1,84].

Calcitriol also controls the expression of genes unrelated to calcium homeostasis, such as those involved in the inhibition of the renin/angiotensin system [85], modulation of immune responses, and suppression of

cell proliferation [1]. Several mechanisms have been identified as responsible for the reduced efficacy of calcitriol to control the expression of these genes in renal failure. The remainder of this section addresses the pathophysiological implications of the abnormal calcitriol/VDR transcriptional activity in renal failure patients.

The magnitude of calcitriol/VDR induction or suppression of gene transcription is determined mainly by the intracellular levels of both calcitriol and the VDR, and both are reduced in renal failure. Thus, reduced VDR levels, which lead to decreased formation of the calcitriol/VDR complex, are major contributors to the resistance to calcitriol therapy in advanced kidney disease. However, abnormalities in steps downstream from ligand binding to the VDR were also demonstrated in studies comparing calcitriol action in peripheral blood monocytes from normal individuals and hemodialysis patients. Figure 5 shows that in the presence of a similar VDR content, the binding of endogenous VDR/RXR complex to DNA is markedy impaired in peripheral blood monocytes from hemodialysis patients compared to that in monocytes from normal individuals, thus leading to an 80% inhibition of the ability of exogenous calcitriol to induce 24-hydroxylase gene transcription. Figure 6 lists uremia-induced mechanisms underlying the reduced RXR/VDR heterodimerization, as well as the impaired binding of the VDR/RXR heterodimer to DNA with the consequent decrease in calcitriol/VDR regulation of gene expression.

Studies in unilaterally nephrectomized rats demonstrated a reduction in the content of a 50 kDa RXR isoform in cell extracts from the remnant kidney. This decrease in RXR results in a reduction of the binding of the endogenous VDR/RXR heterodimer to the VDRE of the mouse osteopontin promoter [86]. A similar reduction of RXR content in the parathyroid glands of these rats could explain their enhanced serum PTH levels in the absence of hypocalcemia or hypophosphatemia [86], since RXR mediates both VDR and retinoic acid suppression of PTH mRNA levels and protein expression [87].

The contribution of the accumulation of uremic toxins to calcitriol resistance has been well documented [88]. Ultrafiltrate from uremic plasma causes a dose-dependent inhibition of VDR/RXR binding to VDRE and calcitriol/VDR-transactivating function [89].

Increases in nuclear calreticulin also modulate calcitriol/VDR action. Calreticulin is a cytosolic protein that binds integrins in the plasma membrane

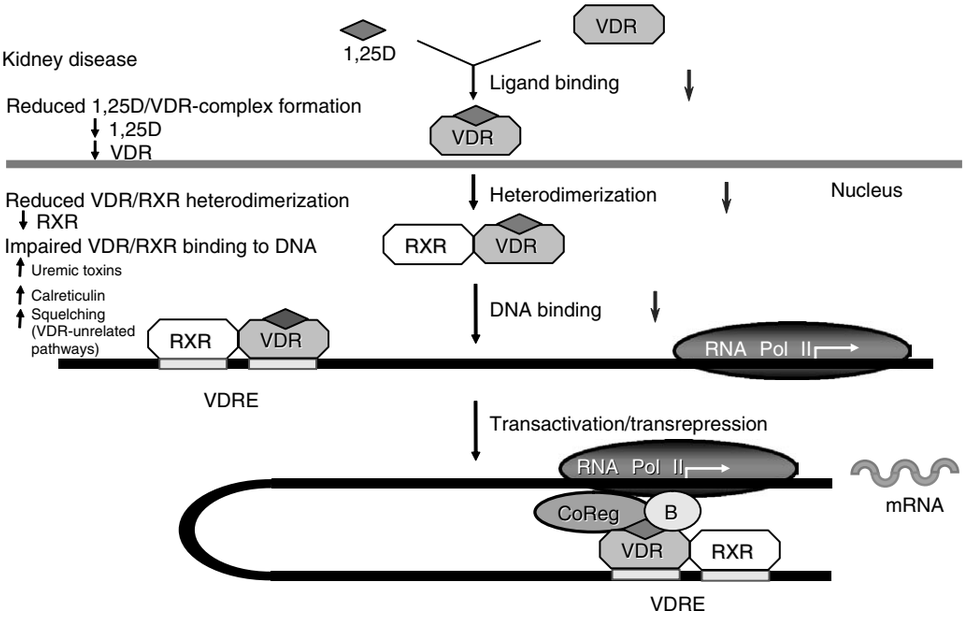

FIGURE 5 Calcitriol (1,25D)/VDR-transcriptional control of the expression of vitamin D responsive genes involves ligand binding to VDR; VDR heterodimerization with RXR; DNA binding of the VDR/RXR complex to the VDRE, and recruitment of basal transcription factors (B), co-regulator molecules (Co-reg), and RNA polymerase II (RNA Pol II) to activate or repress gene transcription. Kidney disease induces several mechanisms (listed on the left) responsible for impairing critical steps (indicated by blue arrows) in calcitriol/VDR transcriptional activity. See CD-ROM for color.

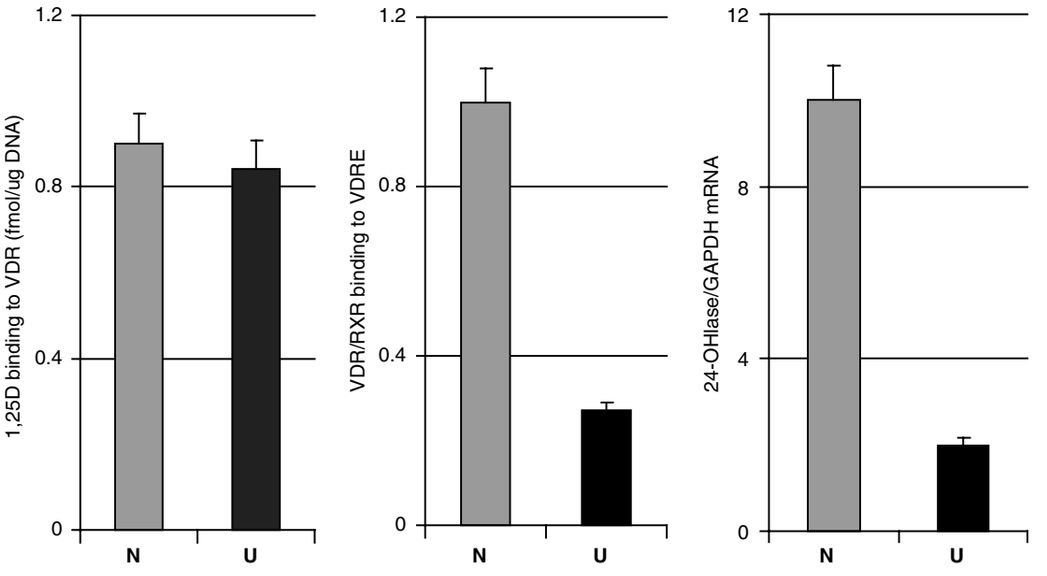

FIGURE 6 Uremia-induced impairment in calcitriol/VDR-mediated transcription unrelated to reduced-VDR content. Despite a similar VDR content [specific calcitriol (1,25D)-binding to VDR, left panel] between peripheral blood monocytes from hemodialysis patients (U; n=10) and normal individuals (N; n=10), the binding of the endogenous RXR/VDR complex to the VDRE of the human 24-hydroxylase promoter (middle panel) is markedly impaired in monocytes from hemodialysis patients. Also, a reduced induction of 24-hydroyxlase mRNA levels by 0.24 nM calcitriol (right panel) occurs in monocytes from hemodialysis patients.

and the DNA–binding domain of nuclear receptors, including the VDR, thus interfering with receptor-mediated transactivation [90]. Hypocalcemia, commonly present in renal failure and caused by either low serum calcitriol levels or hyperphosphatemia, enhances nuclear levels of parathyroid calreticulin. *In vitro* studies demonstrated that increases in nuclear calreticulin inhibit VDR/RXR binding to VDRE in a dose dependent manner and totally abolish calcitriol suppression of PTH gene transcription [90]. However, there is no report demonstrating actual increases in the nuclear levels of calreticulin induced by CKD in the parathyroid glands of experimental animals that could account for the resistance to calcitriol treatment.

Impaired binding of the VDR/RXR complex to DNA and a concomitant reduction in calcitriol/VDR-mediated transcription also occur as a result of activation of VDR-unrelated pathways. In human monocytes and macrophages, cytokine activation markedly inhibits calcitriol/VDR-mediated gene transcription. Specifically, activation by the cytokine gamma interferon of its signaling molecule, Stat1, induces physical interactions between Stat1 and the DNA-binding domain of the VDR that impair VDR/RXR binding to VDRE and consequently, calcitriol transactivation of the 24-hydroxylase and osteocalcin genes [91]. The higher levels of inflammatory cytokines after hemodialysis could contribute to vitamin D resistance.

Little is known at present on how renal failure affects the last and most critical step in calcitriol/VDR-mediated transactivation or transrepression of target genes. Binding of the VDR/RXR heterodimer to the VDRE of genes induced by vitamin D begins the recruitment of co-activator molecules that act synergistically with the VDR to markedly amplify calcitriol/VDR-mediated transcription [92–94]. The complex interactions of the VDR/RXR with VDRE and co-regulators of VDR-transactivation or transrepression of vitamin D responsive genes raises the possibility that, in uremia, vitamin D resistance could also result from decreased expression of essential coactivator or co-repressor molecules, or from defective recruitment of these molecules by the VDR. Megalin was shown to modulate VDR-mediated transactivation through sequestration of a component of the VDR-transcriptional complex [95]. Thus, the reduction in megalin expression induced by kidney disease could partially account for abnormalities in calcitriol/VDR transcriptional activity. Uremia-induced activation of VDR-unrelated signaling pathways may also interfere with the recruitment by the VDR of co-regulator molecules to the transcription pre-initiation complex.

The section below describes tissue specific consequences of calcitriol deficiency and vitamin D resistance that contribute to bone disease and systemic toxicity.

IV. TISSUE SPECIFIC EFFECTS OF LOW CALCITRIOL AND ABNORMAL VDR FUNCTION

A. Classical Calcitriol Responsive Organs

Renal osteodystrophy is the term used to describe the many different patterns of skeletal abnormalities that occur in the course of chronic kidney disease. Alterations in divalent ion, PTH, and vitamin D metabolism play an important part in the pathogenesis and maintenance of skeletal disorders, such as hyperparathyroid bone disease. This section addresses the specific contribution of low serum calcitriol and/or resistance to calcitriol/VDR action in intestine, parathyroid glands, bone, and kidney to alter the powerful calcium-control system causing renal osteodystrophy (see Fig. 1B).

1. INTESTINE

The most critical calcitriol action *in vivo* is the stimulation of the small intestine to absorb calcium and phosphate, as conclusively demonstrated by recent studies in the VDR null mice [96] (see Chapters 20 and 25). Calcitriol induces active cellular calcium uptake and transport mechanisms (see Chapters 24, 25, and 26). The active-calcium uptake requires the epithelial calcium channel ECaC2 or TRPV6 and, to a lesser extent, ECaC1 (TRPV5). Thereafter, calbindin 9K transports calcium across the cell, and the final delivery to the bloodstream involves the plasma membrane calcium pump. The initial active calcium uptake is highly dependent on vitamin D and the rate-limiting step in intestinal calcium absorption [97]. Studies in the VDR KO mice showed reduced expression of both channels [97,98]. Furthermore, the mRNA levels for both channels are up-regulated upon calcitriol supplementation [99]. Importantly, differential modulation of the expression of these channels, namely up-regulation by calcitriol but not by its analog, 19nor-1,25$(OH)_2D_2$, could partially explain the less calcemic properties of the latter [100].

In view of the low serum calcitriol concentrations, reduced intestinal calcium absorption is expected in patients with end-stage renal disease [101,102] and in animal models of experimental renal failure [103,104]. In fact, calcitriol treatment increases intestinal calcium transport in these patients [105]. However, low serum calcitriol levels can only partially account for the reduction in calcium absorption associated with CKD. A blunted calcitriol stimulation of intestinal calcium absorption occurs in patients with kidney disease and in experimental animals with renal failure compared to normal controls [106]. This finding suggests the existence of abnormalities in either intestinal VDR expression or function. There are no comparative studies on intestinal VDR content in normal individuals and patients with CKD. We also lack epidemiological studies addressing the distribution in the hemodialysis population of the 5′ polymorphism affecting Cdx-2 binding and, consequently, an intestine-specific reduction in VDR expression [79]. Although the contribution of reduced VDR to intestinal calcitriol resistance cannot be completely ruled out, the increases in calcium absorption following dialysis [107,108] suggests the involvement of steps in calcitriol action downstream of reduced intestinal calcitriol/VDR-complex formation. It is unclear whether the increase in calcium absorption after dialysis could be accounted for by the removal of uremic toxins that affect VDR function, or other changes such as a decrease in serum phosphate or volume depletion [108]. In CKD, there is a greater induction of intestinal 24-hydroxylase by calcitriol [46]. This suggests that induced degradation of calcitriol could contribute to the blunted response to the sterol to increase intestinal calcium absorption (see Chapter 24). As with intestinal calcium absorption, phosphate absorption is decreased in renal failure and could be enhanced by administration of calcitriol of 1alpha-hydroxy vitamin D_3 (see Chapter 26).

2. PARATHYROID GLANDS

Nearly all patients with end-stage renal disease develop secondary hyperparathyroidism. This condition is characterized by parathyroid hyperplasia and increased synthesis and secretion of parathyroid hormone. High circulating levels of PTH cause osteitis fibrosa, bone loss, and systemic toxicities, including cardiovascular complications, all of which markedly increase morbidity and mortality in hemodialysis patients. Hypocalcemia, hyperphosphatemia due to phosphate retention, and calcitriol deficiency are the three main causes of secondary hyperparathyroidism [109]. Hyperphosphatemia and calcitriol deficiency also enhance parathyroid function indirectly by lowering serum calcium [109].

The ability of calcitriol to inhibit PTH synthesis and arrest parathyroid cell growth *in vivo* and *in vitro* has been known for many years [1]. The mechanisms mediating calcitriol-transcriptional repression of the PTH gene are well characterized and described in Chapters 30 and 31. In contrast, direct characterization of the pathogenic mechanisms underlying both induction

of parathyroid cell proliferation by kidney disease and its suppression by calcitriol has been difficult because of a lack of an appropriate parathyroid cell line and the rapid dedifferentiation of primary cultures of hyperplastic parathyroid cells.

Studies in our laboratory showed that enhanced parathyroid co-expression of the growth promoter, transforming growth factor-α (TGFα) and its receptor, the epidermal growth factor receptor (EGFR), is a major mitogenic signal in experimental renal failure [110]. Similar to secondary hyperparathyroidism in humans [111], parathyroid levels of TGFα are higher in uremic rats than in normal controls. Furthermore, when uremia-induced hyperplasia is worsened by either a high phosphate [110] or a low calcium intake [112], the increases in parathyroid TGFα and EGFR expression correlate directly with high proliferating activity and gland enlargement (see Fig. 7). More importantly, highly

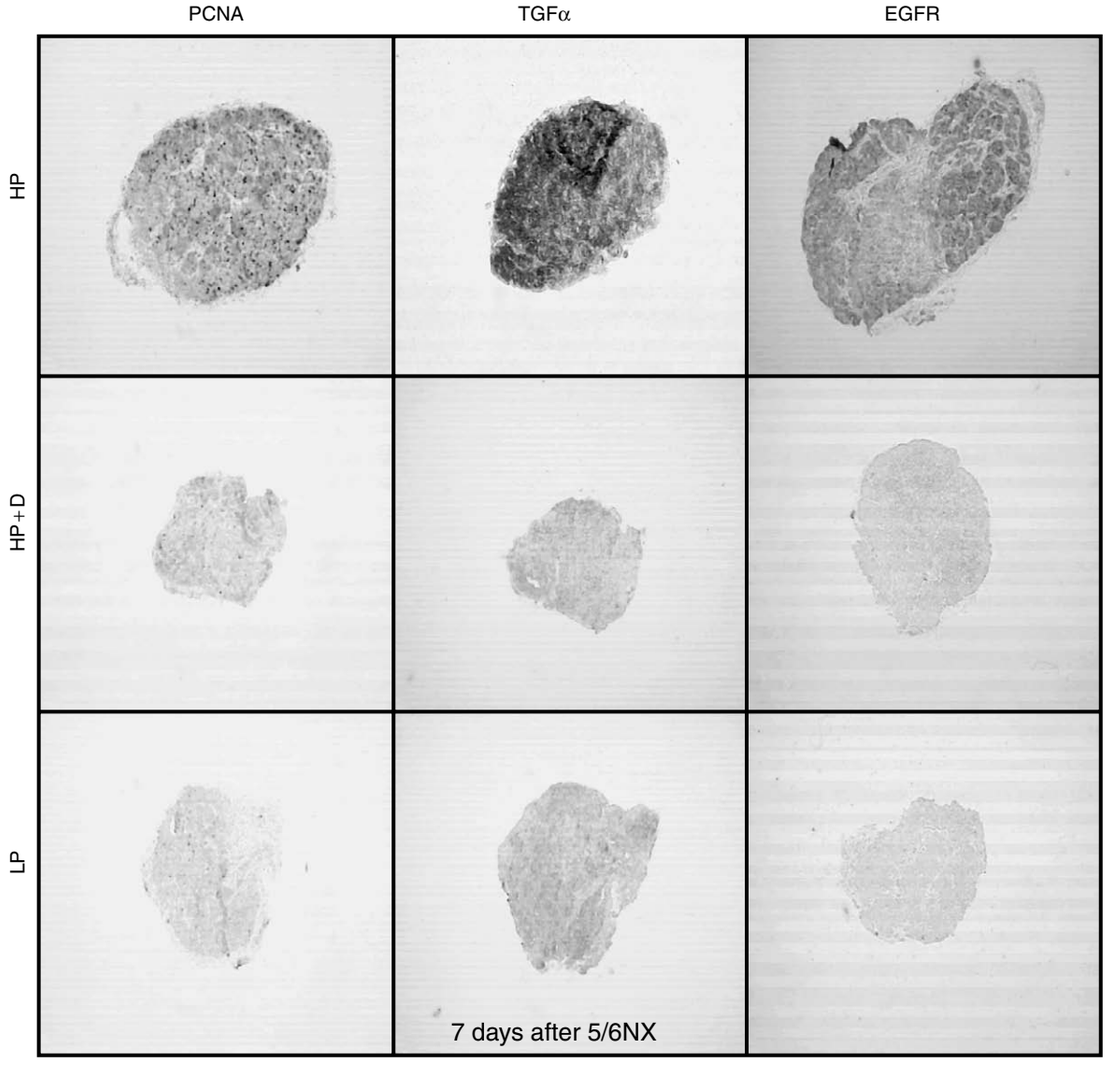

FIGURE 7 Enhanced co-expression of TGFα and EGFR contributes to parathyroid hyperplasia in CKD. Parathyroid expression of the potent TGFα/EGFR-growth loop correlates directly with both high proliferating activity [Proliferating Cell Nuclear Antigen (PCNA) expression] and enhanced parathyroid gland size. Representative immunohistochemical staining of PCNA, TGFα and EGFR in parathyroid glands from 5/6 nephrectomized rats fed either a high phosphate (HP: 0.9% P per g of diet) or a low phosphate diet (LP: 0.2% P per g of diet). Whereas, high dietary phosphate worsens uremia-induced parathyroid hyperplasia, phosphate restriction prevents uremia-induced parathyroid hyperplasia. The antiproliferative properties of prophylactic calcitriol administration (D; 4 ng of calcitriol, i.p, every other day for one week) are associated with the prevention of the increases in TGFα and EGFR induced by uremia and high dietary P.

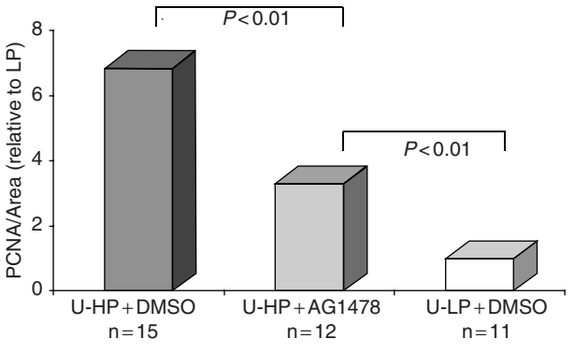

FIGURE 8 Enhanced parathyroid TGFα/EGFR co-expression is a major contributor to parathyroid hyperplasia in early renal failure. Prophylactic administration of the highly specific EGFR tyrosine-kinase inhibitor, AG1478, prevents the high parathyroid-proliferating activity [Nuclei staining positive for PCNA/Gland Area (PCNA/Area)] induced by uremia (u) and high dietary P (HP: 1.2% P per g of diet), by 7 days after the onset of renal failure by 5/6 nephrectomy. The potency of anti-EGFR therapy to inhibit PCNA is not down to the level of P restriction (LP: 0.2 % P per g of diet; arbitrarily assigned a value of 1).

specific inhibitors of EGFR-tyrosine kinase, which abolish growth signals from ligand-activated EGFR, prevent uremia- and high phosphate-induced parathyroid cell proliferation (see Fig. 8) [113].

Calcitriol treatment effectively controls parathyroid hyperplasia in early renal failure and also in established secondary hyperparathyroidism. In early stages of kidney disease in rats, prophylactic vitamin D administration [calcitriol or its analog 19-nor-1,25-dihydroxyvitamin D_2 (19-nor)] counteracts parathyroid hyperplasia through dual mechanisms: Prevention of uremia-induced increases in parathyroid TGFα and EGFR expression, which occur within the first week after inducing kidney failure by 5/6 nephrectomy (see Fig. 7, bottom panels) [112], and induction of the expression of the cell-cycle inhibitor p21 [112].

The efficacy of calcitriol and/or analog therapy [19-nor and 22-oxacalcitriol (OCT)] in preventing further enlargement of the parathyroid gland in established secondary hyperparathyroidism (high TGFα/EGFR overexpression) suggested that calcitriol antiproliferative properties could involve down-regulation of the potent mitogenic signals emerging from TGFα/EGFR overexpression. In fact, in normal and carcinogenic cell lines, overexpressing EGFR, the potent vitamin D–antiproliferative actions involve inhibition of EGFR-growth promoting signals from the plasma membrane, as well as EGFR-transactivation of the cyclin D1 gene [114]. Increased expression of parathyroid cyclin D1 is a common feature in secondary hyperparathyroidism in humans [115]. The time of exposure to calcitriol required to suppress parathyroid expression of TGFα and EGFR *in vivo* and/or TGFα/EGFR growth signals *in vitro* [114] suggests the involvement of the VDR rather than rapid, nongenomic calcitriol actions. It is unclear at present whether these novel antiproliferative properties of the calcitriol/VDR complex involve direct transcriptional regulation.

In addition to inhibiting the expression and signaling of the TGFα/EGFR-growth loop, calcitriol antiproliferative actions in hyperplastic parathyroid glands involve induction of the cyclin-dependent kinase inhibitors p21 and p27 [112,116]. A strong, direct correlation exists between parathyroid VDR levels and p21 and p27 content in human secondary hyperparathyroidism. The p21 gene is under direct transcriptional induction by the calcitriol/VDR complex [117]. Calcitriol reduction of c-myc expression was also postulated as an underlying mechanism for calcitriol antiproliferative properties in secondary hyperparathyroidism in humans [118].

As renal disease progresses, the low serum calcitriol levels lead to a reduction in parathyroid VDR, thus rendering the parathyroid gland more resistant to the suppression of cell growth and PTH synthesis in response to calcitriol therapy. The ability of calcitriol or its less calcemic analogs to increase parathyroid VDR levels in uremic rats [54] indicates that calcitriol deficiency is, in itself, a determinant of parathyroid resistance in advanced kidney disease.

In addition to direct suppression of PTH gene expression, calcitriol appears to be critical for the response of the parathyroid gland to calcium. In rats, parathyroid levels of the calcium sensing receptor (CaSR) mRNA were decreased by 40% by vitamin D deficiency [119] and enhanced by calcitriol treatment in a time and dose-dependent manner. See Chapter 31 for discussion of CaSR. Functional vitamin D responsive elements were identified in both promoters of the human CaSR gene [84]. In advanced renal failure, a strong association exists between defective levels of parathyroid CaSR and low VDR in areas of high proliferative activity [120]. Up-regulation of parathyroid CaSR by calcitriol treatment could explain the decrease in the set point for PTH suppression by calcium [121], and also, the higher levels of the CaSR in surgically removed parathyroid glands from patients receiving calcitriol compared to those from untreated patients [122].

Figure 9 summarizes the multiple direct effects of low serum calcitriol on the parathyroid glands leading to parathyroid hyperplasia and secondary hyperparathyroidism. It is clear from these actions of calcitriol that early interventions with low doses of calcitriol or its less calcemic analogs should prevent the decreases in VDR and CaSR responsible for the reduced sensitivity

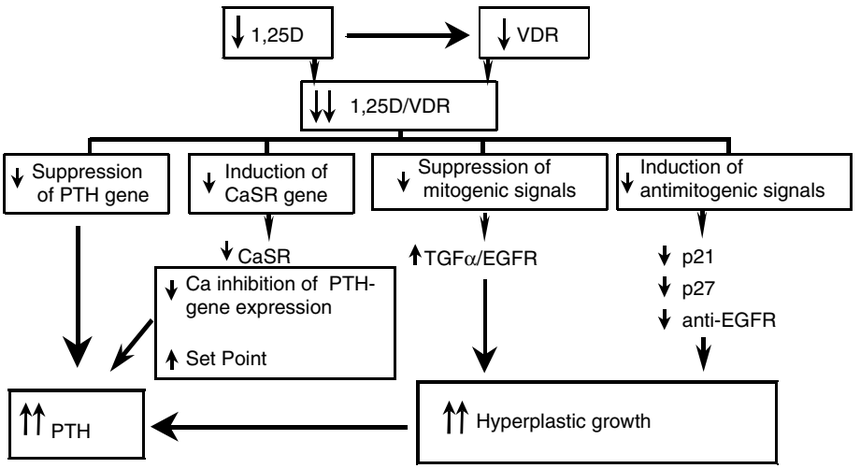

FIGURE 9 Role of calcitriol deficiency in the pathogenesis of secondary hyperparathyroidism in chronic kidney disease.

of hyperplastic parathyroid glands to control PTH synthesis and cell growth in response to calcium, calcitriol, or analog therapy. Low-dose calcitriol administration at early stages in CKD should prevent the onset of the most aggressive forms of secondary hyperparathyroidism.

3. BONE

Vitamin D is essential for the development and maintenance of a mineralized skeleton (see Chapters 32, 37, and 40). However, studies in the VDR knock-out mice [96] and children with hereditary Vitamin D resistant rickets (Chapter 72) demonstrated that vitamin D is not mandatory for the ossification process. Calcitriol induces bone mineralization by increasing intestinal absorption and therefore serum levels of calcium and phosphate. In fact, only a fraction of renal failure patients show evidence of defective mineralization, despite the low serum calcitriol levels [123].

In renal failure, calcitriol deficiency causes decreased intestinal calcium absorption and hypocalcemia, a potent stimulus for parathyroid gland hyperplasia and consequently, increases in circulating PTH. High serum PTH levels are the main determinant of osteoclastogenesis and osteoclast activation, causing osteitis fibrosa and bone loss in patients with CKD. The direct effects of calcitriol deficiency leading to hyperparathyroidism and renal osteodystrophy were extensively discussed in the previous section.

From these pathogenic mechanisms, it is clear why treatment with calcitriol or its less calcemic analogs is the therapy of choice to control the skeletal abnormalities caused by hyperparathyroidism in advance kidney disease. An important consideration in the use of calcitriol/analog therapy is to avoid oversuppression of PTH with the consequent decrease in bone turnover to abnormally low levels that cause adynamic bone disease. The recommendations for therapy by the National Kidney Foundation of the USA are specified in the last section of this chapter.

4. KIDNEY

Vitamin D actions in the kidney are the focus of Chapter 29. Because of their major impact on serum calcitriol levels and calcium homeostasis, the most important actions of calcitriol in the kidney are the suppression of 1α-hydroxylase and the stimulation of 24-hydroxylase. In addition to decreasing 1α-hydroxylase activity by reducing serum PTH, calcitriol induction of the expression of the CaSR in the proximal tubules could also mediate the sensitivity of the renal 1α-hydroxylase to calcium.

Calcitriol involvement in the renal handling of calcium and phosphate continues to be controversial due to the simultaneous effects of calcitriol on intestinal calcium and phosphate absorption, which affects the filtered load of both ions, and on serum PTH. Calcitriol enhances renal calcium reabsorption and calbindin expression, and accelerates PTH-dependent calcium transport in the distal tubule [124], the kidney site with the highest VDR content and the determinant of the final excretion of calcium into the urine. The epithelial calcium channel (ECaC or LVRT5) is an important target in calcitriol-mediated calcium reabsorption. Several putative VDR binding sites have been located in the human promoter of the renal epithelial calcium channel. More importantly for the calcitriol deficiency that associates with kidney disease, decreases in circulating

levels of calcitriol resulted in a marked decline in the expression of the channel at the protein and mRNA levels [125].

B. Nonclassical Calcitriol Responsive Systems

Hypertension, diabetes, immunological disorders, as well as neuromuscular defects, are important co-morbid conditions in CKD. This section addresses the potential contribution of calcitriol deficiency and/or resistance to calcitriol to the onset of these pathologic features in renal failure patients. It is important to emphasize that all of these pathological processes are multifactorial since no obvious hematopoietic, immunological, or neurological abnormalities were found in the VDR null mice.

1. Renin/Angiotensin System

Hypertension is a common complication in CKD. The renin/angiotensin system plays a central role in the regulation of blood pressure, electrolyte, and volume homeostasis. Several epidemiological and clinical studies suggested an association between inadequate sunlight exposure or low-serum calcitriol with high blood pressure and/or high plasma renin activity [85,126]. As fully described in Chapter 54, calcitriol acts as a negative endocrine regulator of the renin/angiotensin system. In the VDR null mice, marked increases in renin expression and plasma-angiotensin II production caused hypertension, cardiac hypertrophy, and increased water intake. Further support for an association between calcitriol deficiency and hypertension came from studies in the wild type mice. Whereas inhibition of calcitriol synthesis also led to an increase in renin expression, calcitriol administration suppressed renin production through a VDR-mediated mechanism unrelated to changes in serum calcium.

2. Pancreas

Vitamin D deficiency is associated with impaired glucose-mediated insulin secretion that can be reversed by calcitriol repletion [127]. Calcitriol, through a VDR-mediated modulation of calbindin expression, controls intracellular calcium flux in the islet cells, which in turn affect insulin release [128].

In CKD, there is abnormal insulin secretion, a blunted response of the pancreatic β cell to glucose challenge, and insulin resistance [129–131]. Calcitriol deficiency mediates the abnormal regulation of insulin secretion independently of alterations in VDR levels in pancreatic β cells. Also, calcitriol administration corrects the abnormal insulin secretion independently of changes in serum levels of calcium or PTH [132,133].

In experimental animals, vitamin D deficiency is also associated with an earlier and more aggressive onset of diabetes (see Chapter 99), a finding that raises the possibility that prolonged calcitriol deficiency in renal failure could accelerate the development of diabetes in kidney disease patients.

3. Immune System

Calcitriol is an important immunomodulatory steroid, as extensively reviewed in Chapters 36, 98, and 99. Calcitriol modulates the function of antigen-presenting cells and T lymphocytes.

The immune system functions abnormally in CKD—increased cases of infections, decreased response to vaccines, and reduced skin and homograft survival being noted [134]. Serum from CKD patients has been shown to inhibit cell-mediated immune responses *in vitro,* including lymphocyte blastogenesis, interferon production by normal lymphocytes, and monocyte phagocytosis [134].

The contribution of calcitriol deficiency to abnormal immune cell function was suggested by the alterations in the immune system in vitamin D–deficient rickets. The decreased neutrophil phagocytosis [135] and decreased polymorphonuclear leukocyte and macrophage activity [136] in these patients can be corrected by treatment of cells with $1,25(OH)_2D_3$ *in vitro* [135,136].

In CKD, calcitriol therapy greatly improved the low lymphocyte counts [137,138], the chemotactic response of polymorphonuclear leukocytes [139], and defects in T-cell-mediated immune responses. It also increased the ratio of T helper to T suppressor cells [140] and enhanced the depressed natural killer cell activity [141]. Mitogen activation of lymphocytes from hemodialysis patients is attenuated, but can be restored by prior treatment of the patients with 1α–hydroxyvitamin D_3, a precursor of calcitriol [142].

The production of calcitriol by activated macrophages was postulated as an autocrine/paracrine system that inhibits further T-cell activation and lymphokine production, thus preventing a potentially self-destructive response [135]. As mentioned in the section on the abnormal delivery of substrate to renal and extrarenal 1α-hydroxylases, in CKD, 25OHD uptake was markedly impaired in peripheral blood monocytes from hemodialysis patients and could be normalized by calcitriol treatment. Abnormalities in local calcitriol synthesis induced by calcitriol deficiency could contribute to the abnormal T-cell function in renal failure. In fact, calcitriol blocks the proliferation of activated T-cells *in vitro* [143] and normalizes the decreased

production of interleukin-2, a T-cell growth factor, by lymphocytes from dialysis [144], an effect independent of changes in serum calcium, phosphate, and PTH levels.

4. MUSCLE

Skeletal muscle weakness and atrophy, with electrophysiological abnormalities in muscle contraction and relaxation occur in vitamin D deficiency (see Chapter 102), in calcitriol deficiency due to CKD, and with the prolonged use of anticonvulsant drugs that decrease serum 25OHD levels. Although these defects were originally attributed to low calcium, there is evidence of direct calcitriol action on skeletal muscle [145]. In the heart, calcitriol controls hypertrophy in cardiac myocytes [146] and the synthesis and release of atrial natriuretic factor [147] (see Chapter 55).

In end-stage renal disease, therapy with 25OHD or calcitriol improves the left ventricular function in patients with cardiomyopathies and the skeletal muscle weakness. The mechanisms involved are unclear. *In vitro*, vitamin D analogs elicit a differential potency to regulate muscle-cell metabolism and growth [148], which suggests their therapeutic potential in ameliorating the CKD-associated myopathies.

5. NERVOUS SYSTEM

Calcitriol actions in the nervous system include induction of VDR content (VDR is expressed in the brain and on several regions of the central and peripheral nervous system) [149], as well as increases in the conductance velocity of motor neurons, and induction of the synthesis of neurotrophic factors, such as nerve growth factors and neurotrophins, that prevent the loss of injured neurons [150,151]. Calcitriol also enhances the expression of glial cell line-derived neurotrophic factor, a potential candidate to treat Parkinson's disease [152]. The impact of calcitriol deficiency in CKD on VDR expression and the neurological abnormalities in these patients is unclear at present. See Chapter 100 for a discussion of Vitamin D action in the central nervous system.

V. VITAMIN D THERAPY IN CHRONIC RENAL FAILURE

The ultimate goal of calcitriol therapy is the treatment of osteitis fibrosa, the most common form of renal osteodystrophy, resulting from sustained secondary hyperparathyroidism. Although most patients maintained on chronic dialysis have very low levels of calcitriol, the majority of them do not show on bone biopsy the presence of osteomalacia secondary to vitamin D deficiency. Thus, as discussed in this section, not all patients with advanced renal insufficiency or even those maintained on chronic hemodialysis or peritoneal dialysis require calcitriol treatment. The therapeutic approaches differ for patients before and after they enter a dialysis program and thus are discussed separately.

A. Prevention and Treatment of Secondary Hyperparathyroidism in Patients with Chronic Renal Failure before Treatment with Dialysis (Chronic Kidney Disease Stages 3 and 4)

As described above, the serum levels of calcitriol decrease with the progression of renal disease, and the majority of patients have low levels when the GFR is less than 50 ml/min. Therefore, it would seem appropriate to replace this hormone. However, the use of calcitriol in patients with moderate to advanced CRF is not completely free of side effects, and many physicians do not prescribe calcitriol until there are overt manifestations of secondary hyperparathyroidism and bone disease. Although the concern for potential aggravation of renal insufficiency is understandable, careful administration of calcitriol has been beneficial for the majority of patients.

Baker and collaborators [153] studied 16 patients with CKD (creatinine clearance 20–59 ml/min). The patients received either calcitriol at a dose 0.25 to 0.5 μg/daily or placebo. Bone biopsies were performed before entrance into the study and after 12 months of treatment. Bone histology was abnormal in all patients. Calcitriol treatment was associated with a significant fall in serum phosphorus and alkaline phosphatase concentrations, as well as with histological evidence of improvement of hyperparathyroid effects in bone. Over the 12 months of study, there was no significant deterioriation of renal function attributable to the treatment. It was recommended that long-term calcitriol administration in patients with moderate renal failure should be given only to those patients that have high levels of PTH, and that these patients should be followed closely by their physicians. Hypercalcemia arising from calcitriol treatment could further aggravate the abnormality in renal function. Thus, control of serum calcium and phosphorus and frequent monitoring of PTH levels are imperative in order to prevent potential side effects induced by administration of calcitriol.

Nordal and collaborators [154] studied 13 patients with moderate to terminal renal failure with low doses (average 0.36 ug/day) of calcitriol up to the time of renal transplantation. All patients who started calcitriol treatment and had a creatinine clearance about 30 ml/min had normal bone histology at the time of renal transplantation. This was not observed, however, when calcitriol treatment was started with a creatinine clearance below 30 ml/min. The study suggests that the full benefit of calcitriol at the bone level is obtained only if prophylactic administration is started early in the course of renal failure. It is important to emphasize that these investigators used a small dose of calcitriol. It is possible that such a low dose may not have been sufficient to control secondary hyperparathyroidism and osteitis fibrosa in patients with advanced renal insufficiency.

To obtain further information, Hamdy and collaborators [155] treated a large number of patients with 1α-hydroxyvitamin D_3 [1αOHD_3; alfa-calcidol] in a double-blind, prospective, randomised, placebo-controlled study. They studied 176 patients with mild to advanced CKD (creatinine clearance 15–50 ml/min) and with no clinical, biochemical, or radiographic evidence of bone disease. Two years of 1αOHD_3 therapy was initiated at a dose of 0.25 μg per day and was titrated according to serum calcium concentration. A total of 132 patients had histological evidence of bone disease at the start of the study; 89 patients received 1αOHD_3, and 87 control patients received placebo. After treatment, PTH concentrations had increased by 126% in controls and had not changed in patients given 1αOHD_3 ($p < 0.001$). Although hypercalcemic episodes occurred in 10 patients, they resolved rapidly after the dose of 1αOHD_3 was decreased. Histological indices of high bone turnover significantly improved in patients given 1αOHD_3 and significantly deteriorated in controls. There was no difference in the rate of progression of renal failure between the two groups. The investigators concluded that early administration of 1αOHD_3 can safely and beneficially alter the natural course of renal bone disease in patients with mild to advanced renal failure.

Until recently, there was not a uniform approach for the treatment of mineral and bone metabolism in patients with different degrees of renal insufficiency, and precise recommendations had not been developed. The United States National Kidney Foundation (NKF) has now established a classification for stages of CKD ranging from insignificant to end-stage renal disease. CKD Stage 1 includes GFR > 90 ml/min/1.73 m^2; Stage 2, GFR from 60 to 89; Stage 3, from 30 to 59; Stage 4, from 29 to 15; Stage 5, GFR < 15 or dialysis. Since secondary hyperparathyroidism and alterations in bone and mineral metabolism are early manifestations of chronic renal failure, the NKF established the Kidney Disease Outcomes Quality Interactive K/DOQI. These recommendations indicate that treatment for the alterations mentioned above should start early in patients with CKD Stage 3 (GFR from 30 to 59 ml/min/1.73 m^2). At this stage of kidney disease, if the intact PTH is greater than 70 pg/ml after correction of serum phosphorus (less than 4.6 mg/dl) and serum calcium (9.5 mg/dl), patients should receive oral calcitriol at the dose of 0.25 μg/day or alfacalcidol at the dose of 0.25 to 0.5 μg/day or doxercalciferol at the dose of 2.5 μg three times per week. It is critical that the patients have levels of 25OHD greater than 30 ng/ml. If the levels are lower, ergocalciferol should be prescribed to the patients. Usually, 50,000 IU every two weeks for one month, and then once a month for six months. Physicians should monitor the levels of 25OHD and adjust the dose accordingly. For patients with CKD Stage 4 (GFR: 15–29 ml/min /1.73 m^2), similar recommendations apply. However, treatment with calcitriol or analogs should be instituted if the PTH is greater than 100 pg/ml. Regardless of the way the patient is treated, serum calcium and phosphorus levels should be measured frequently to prevent metastatic calcification, nephrocalcinosis, and acceleration of renal disease. The levels of PTH should be kept between 40–70 pg/ml for CKD Stage 3 and 70–100 pg/ml for patients with CKD Stage 4. Lower values for serum PTH may predispose the patients to develop adynamic bone disease. On the other hand, if the levels of PTH are allowed to increase to higher levels, the patients may develop severe osteitis fibrosa.

B. Treatment of Secondary Hyperparathyroidism in Patients with Chronic Renal Failure Maintained on Hemodialysis (CKD Stage 5)

Despite dietary phosphate restriction, the use of phosphate binders, the choice of appropriate levels of calcium in the dialysate, and adequate intake of dietary calcium, a significant number of uremic patients still develop features of osteitis fibrosa. The knowledge of the major pathogenic role of calcitriol deficiency to renal osteodystrophy has created interest in the use of calcitriol in such patients.

When patients show evidence of secondary hyperparathyroidism (e.g., bone erosions, high intact PTH levels (greater than 300 pg/ml), and increased alkaline phosphatase), adequate treatment with calcitriol often leads to improvement. Results of numerous studies indicate the efficacy of calcitriol treatment in patients with symptomatic renal osteodystrophy. These clinical

evaluations have shown an improvement in muscle strength and bone pain. In addition, biochemical markers, such as plasma alkaline phosphatase, have decreased along with a fall in the levels of serum PTH. Bone histology has shown a decrease in marrow fibrosis and other features of secondary hyperparathyroidism, such as increased bone resorption and number of osteoclasts. The dose of oral calcitriol utilized in these trials has varied from 0.25 to 1.0 μg/day, and the major side effect of such treatment is the appearance of hypercalcemia. Hypercalcemia may occur only after many weeks or months of treatment, or it may appear sooner in patients with aluminum-induced osteomalacia or in adynamic-bone disease. There is substantial degradation of calcitriol in the intestine, and it is possible that the oral administration of the vitamin D sterols augments calcium absorption very effectively; however, the delivery of calcitriol to other target organs may be substantially less. In fact, studies by Maung and collaborators [156] have demonstrated that oral administration of 1alpha-vitamin D_2 is more calcemic and phosphatemic than the intravenous route.

Slatopolsky and collaborators [157] studied the effects of intravenous administration of calcitriol in patients maintained on chronic hemodialysis. Twenty patients were given calcitriol intravenously at the end of each dialysis. The dose was initially 0.5 μg and was gradually increased to a maximum of 4.0 μg per dialysis. Calcitriol was discontinued after eight weeks of treatment, and blood samples were obtained for an additional three weeks. In all patients, there was a substantial decrease in the levels of PTH during the period of intravenous calcitriol treatment, with a mean decrement of $71 \pm 3.2\%$. After calcitriol was discontinued, PTH increased rapidly in all patients. There was a significant correlation between the increase in ionized calcium and the decrease in PTH level, showing a crucial role for calcium in the suppression of PTH. However, in addition to the calcemic effect, calcitriol directly modified the secretion of PTH. The decrease in PTH levels was observed before there was an increase in ionized calcium. The effects of oral and intravenous administration of calcitriol were compared, and it was shown that the intravenous calcitriol had a greater suppressive effect on the release of PTH than when calcitriol was administered by the oral route.

The intravenous administration of calcitriol may allow a greater delivery to peripheral tissues such as the parathyroid glands and thereby generate greater expression of biological effects at these sites. Similar results were found by Andress and collaborators [158]; they studied 12 patients on hemodialysis who were not responding to oral calcitriol and were being considered for parathyroidectomy at the time. All patients exhibited baseline bone formation rates that were above normal, and the rates fell by a mean of 59% during treatment. The results indicate that intravenous administration of calcitriol is also effective in ameliorating osteitis fibrosa in patients who have moderate to severe secondary hyperparathyroidism. As mentioned before, oral calcitriol failed to suppress the secretion of PTH adequately in these patients. Parathyroidectomy, originally considered for these patients, became unnecessary due to the implementation of intravenous calcitriol.

Since the original study, more than 75 reports on the effect of intravenous calcitriol therapy in hemodialyzed CKD patients have been published, including information on more than 1000 patients. In several studies $1\alpha OHD_3$ was administered. Interestingly, Delmez and collaborators [121], in a study of the effect of calcitriol in a group of patients maintained on hemodialysis, found significant decreases in the levels of serum PTH and an improvement in the calcium set point for PTH secretion after two weeks of intravenous calcitriol therapy (Fig. 10). Similar results were found by Dunlay *et al.* [159] in a group of nine patients maintained on hemodialysis. The patients received 2 μg intravenously after each dialysis. Intravenous calcitriol resulted in a significant decrease within two weeks and continued decrease of the high serum PTH levels by the end of 10 weeks. Although these investigators did not find a change in the calcium set point, they found a shift in the calcium-PTH sigmoidal curve toward normal. Thus, both studies [121,159] show an increase in sensitivity of the parathyroid glands to serum calcium after the administration of intravenous calcitriol.

In other countries, owing to an inability to obtain the intravenous preparation of calcitriol, several clinical trials investigated the use of high doses of calcitriol given orally in an intermittent manner (oral pulse therapy). Several studies in adults and children with renal failure have been published. Most of the results show significant improvement in the suppression of PTH [160,161]. However, in many cases this approach had to be discontinued because of the development of hypercalcemia and hyperphosphatemia. Quarles *et al.* [162] published the results of a prospective trial of oral pulse versus intravenous calcitriol in the treatment of secondary hyperparathyroidism in hemodialysis patients. These investigators found that episodes of hypercalcemia and hyperphosphatemia were similar in both treatment groups and limited the dose of calcitriol that could be administered. In this study, they found that intermittent calcitriol therapy, regardless of the route of administration, was poorly tolerated, failed to correct parathyroid gland size and functional abnormalities, and had a limited ability to sustain serum

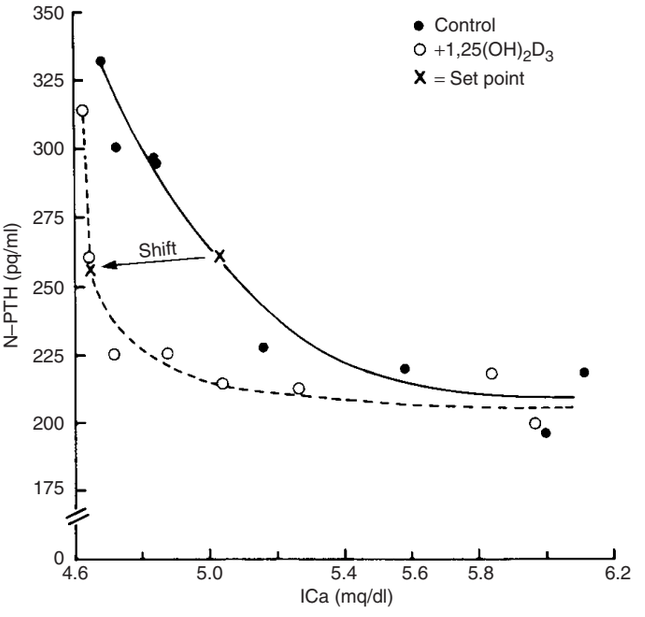

FIGURE 10 Effects of intravenous calcitriol on PTH secretion during calcium infusion in a representative patient. During the control infusion (•) the set point (x) of serum ionized calcium (ICa) was 5.04 mg/dl. After two weeks of intravenous calcitriol (○), the PTH levels decreased, despite a lower ICa value, and the set point decreased to 4.64 mg/dl. From Delmez *et al*. [121].

PTH reductions in end-stage renal failure in patients with severe hyperparathyroidism.

Unfortunately, the development of severe hyperphosphatemia in many patients interferes with the beneficial effects of calcitriol. On the other hand, Cannella *et al.* [163] found significant healing of secondary hyperparathyroidism in chronic hemodialysis patients treated with long-term intravenous calcitriol. These investigators followed a group of patients for approximately 35 weeks; the initial dose of calcitriol was 30 ng/kg of body weight intravenously three times a week after each dialysis. The mean pretreatment serum PTH concentration was 966 ± 160 pg/ml, and the values decreased significantly by the first week and fell by an average of 80% by week 35 (Fig. 11). The ionized calcium concentration was 4.76 ± 0.4 mg/dl and rose slightly to 5.36 mg/dl by the fourth week. There were significant decreases in all bone-morphometric indices of secondary hyperparathyroidism. These investigators clearly demonstrated, contrary to the studies of Quarles and collaborators [162], that intravenous calcitriol is very effective in suppressing secondary hyperparathyroidism. It is of utmost importance to emphasize that Cannella and collaborators [163] were able to control the levels of serum phosphorus, allowing them to provide calcitriol on a more sustained basis. They also indicated that, in some resistant patients, higher doses of calcitriol (4 to 6 μg/treatment) were able to reduce the levels of PTH.

Llach and collaborators [164] also demonstrated the importance in dosing intravenous calcitriol in dialysis

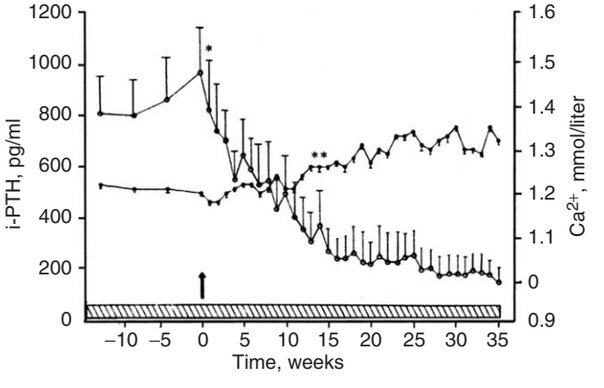

FIGURE 11 Weekly values (means ± SE) for plasma concentrations of intact PTH (○) and ionized calcium (•) in 8 hemodialysis patients, before and after the start (arrow) of intravenous calcitriol therapy (30 ng/kg body weight thrice weekly for 8 months). Asterisks indicate significant results versus time 0: *, $p < 0.05$; **, $p < 0.01$. Dashed area is the normal PTH range. Used with permission from *Kidney International,* vol 46, p. 1124, 1994.

patients with severe hyperparathyroidism. They studied 10 patients with severe hyperparathyroidism (PTH > 1200 pg/ml and serum phosphorus < 6.5 mg/dl). Ten patients with a mean PTH of 1826 ± 146 pg/ml, were treated for a minimum of 48 weeks with an intravenous dose of calcitriol commensurate with the levels of PTH. The initial calcitriol dose had to be increased in seven patients. The mean maximum dose of calcitriol was 3.8 μg three times a week. The authors concluded that patients with severe hyperparathyroidism respond well to intravenous calcitriol; however, the dose of this vitamin D metabolite should be adjusted according to PTH levels, and hyperphosphatemia should be kept under control. Thus, it seems that although there is no agreement with regard to the route and frequency of administration, the oral pulse and intravenous administration are the most accepted therapies. However, the meta-analysis of four trials that compared intermittent intravenous calcitriol with oral calcitriol, in randomized controlled studies [165,166] or cross-over trials [167,168], indicated that intravenous therapy was more effective than oral treatment (either daily or "pulse" treatment) for the suppression of intact PTH levels.

Beneficial results also have been observed in patients maintained on continuous ambulatory peritoneal dialysis. Salusky *et al.* [169] studied the pharmokinetics of calcitriol in continuous ambulatory and cycling peritoneal dialysis patients. The kinetics of calcitriol was evaluated after a single dose of 60 ng/kg body weight (equivalent to 4.2 ug for a 70-kg man) given orally, intravenously, or intraperitoneally in six patients. The area under the curve for the increment of serum calcitriol concentration above baseline levels for the 24 hr after a single dose of calcitriol was 62% greater following intravenous injection (2340 ± 115 pg/ml) than after either oral (1442 ± 191 pg/ml) or intraperitoneal (1562 ± 195 pg/ml) administration. These investigators, using a radioisotope tracer of calcitriol, found that 30 to 40% of the hormone adheres to plastic components of the peritoneal dialysate delivery system. By modifying the technique of intraperitoneal calcitriol administration, the authors found that they could obtain a dosage effect comparable to intravenous administration. Thus, it would seem that intraperitoneal administration of calcitriol also is very effective in the control of secondary hyperparathyroidism if precautions to prevent adherence to plastic are taken.

It is recommended now that in patients with CKD Stage 5 (GFR < 15 ml/min/1.73 m^2) or dialysis, serum PTH be determined at least every three months and serum calcium and phosphorus once a month. The ideal serum intact PTH levels should be 150 to 300 pg/ml. Patients with serum intact PTH greater than 300 pg/ml should receive calcitriol or vitamin D analogs, providing that the serum phosphorus is less than 5.5 mg/dl and the serum calcium less than 9.8 mg/dl. Ideally, the serum phosphorus should be 3.5 to 5.5 mg/dl, and the Ca × P product less than 55 $mg^2 \times dl^2$. The administration of calcitriol or its analogs to patients with CKD Stage 5 depends on the levels of circulating intact PTH. For patients with circulating levels of intact PTH of 300–600 pg/ml, the dose of intravenous calcitriol should be 0.5 to 1.5 μg I.V. × 3 per week. For those patients with more severe secondary hyperparathyroidism (PTH 600–1200), the dose of calcitriol should be 2 to 4 μg I.V. three times per week.

C. Use of New Less Calcemic Analogs of Calcitriol

In an effort to utilize the actions of vitamin D on the parathyroid gland and minimize the toxicities of such therapy, structural alterations of the vitamin D molecule were undertaken to try to develop vitamin D analogs that may retain the effects on the parathyroid glands but have a lesser effect on the calcium and phosphate metabolism [170]. These analogs would be relatively selective in suppressing parathyroid hyperfunction and therefore, more useful therapeutic agents. This subject is extensively discussed in Chapters 80–88. Currently, there is experimental and clinical evidence for the efficacy of four of such vitamin D analogs, which have been approved for the treatment of secondary hyperparathyroidism.

Two of these analogs have been developed in Japan, 22-oxacalcitriol and falecalcitriol and the other two in the United States, 19-nor-1,25$(OH)_2D_2$ and 1-alpha hydroxy D_2. 22-Oxacalcitriol (OCT) differs from calcitriol by an oxygen substitution at position 22 (see Chapter 86). This structure modification appears to reduce the affinity of OCT for the vitamin D receptor, as well as for DBP. The decreased affinity for DBP results in rapid clearance from the circulation, and this may be a mechanism that accounts for low calcemic and phosphatemic effect of OCT [171]. Falecalcitriol is an analog in which the hydrogens of carbons 26 and 27 have been substituted by fluorine atoms. This vitamin D analog has greater activity than calcitriol and is considerably more calcemic and more potent in calcifying epiphyseal cartilage in rats [172]. The increased potency is likely due to a decreased catabolism of this sterol. In patients with chronic renal failure, falecalcitriol was effective in decreasing PTH and appeared to be somewhat more effective than alfacalcidol in suppressing secondary hyperparathyroidism [173].

In the United States, 19-nor-1,25$(OH)_2D_2$ (paricalcitol) has been released into the market with the name

of Zemplar®. This vitamin D analog lacks the carbon at position 19. Zemplar® has been studied extensively and demonstrated to suppress PTH secretion *in vitro* as potently as calcitriol. Studies in experimental animals have shown that 19-nor-1,25$(OH)_2D_2$ is effective in suppressing PTH levels with less hypercalcemia and hyperphosphatemia than that observed with calcitriol treatment. Indeed 19-nor-1,25$(OH)_2D_2$ is approximately 10 times less active than calcitriol in mobilizing calcium and phosphate from bone [174]. This vitamin D analog is in widespread clinical use in patients on hemodialysis in the United States, and has been demonstrated to be effective in suppressing PTH levels. Thus, while 3 times more 19-nor-1,25$(OH)_2D_2$ than calcitriol is required to achieve equivalent suppression of PTH in animals, studies in patients indicate that a ratio of 3 to 4 is required [175–177]. Similarly, while paricalcitol is 10 times less calcemic and phosphatemic than calcitriol in animals studies, in patients with end-stage renal disease on a low calcium diet, at least 8 times more paricalcitol is required to achieve a similar increment in serum calcium, presumably representing calcium mobilized from bone [178]. Sprague and collaborators demonstrated less severe hyperphosphatemia in patients treated with paricalcitol compared to calcitriol [176]. Recent studies indicate a 16% decrease in mortality in a retrospective study over a period of three years, in a large group of patients treated with paricalcitol (29,025) when compared to those receiving calcitriol (38,378) [179]. Moreover, the two-year survival rate among patients who switched from calcitriol to paricalcitriol was 73% as compared to 64% among those who switched from paricalcitol to calcitriol. The exact mechanism for this effect is not clear at the present time.

Another analog of vitamin D is 1-alpha hydroxy D_2, commercially known as *Hectorol*®. This analog is used in the United States for the treatment of secondary hyperparathyroidism. This compound is considered a prohormone since it lacks the 25-hydroxyl group, and it is 25-hydroxylated in the liver to 1,25$(OH)_2D_2$. Comparative studies in normal and uremic animals (see Fig. 12) have shown [180] that 1-alpha hydroxy D_2 is more hypercalcemic and hyperphosphatemic than 19-nor-1,25$(OH)_2D_2$. Further studies in patients are necessary to corroborate this initial experimental observation.

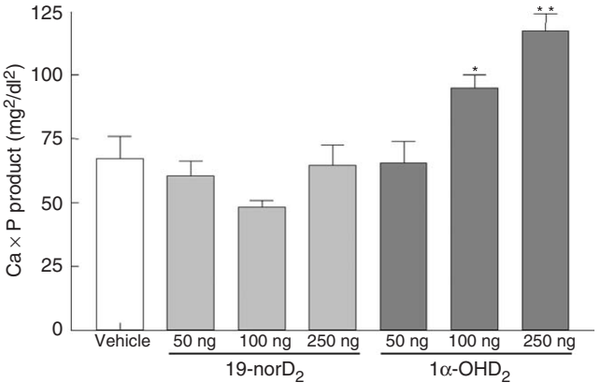

FIGURE 12 Effects of vehicle, 19-nor-1,25$(OH)_2D_2$ [19-nor; 50, 100, or 250 ng; n = 10] and 1α$(OH)D_2$ [1α$(OH)D_2$; 50, 100, or 250 ng; n = 9] on the Ca × P product in uremic rats. Rats were treated three times a week for two weeks. * and ** indicate $p < 0.01$ or $p < 0.001$ versus control rats. Adapted from Slatopolsky *et al*. [180].

VI. SUMMARY

PTH and calcitriol are the major factors responsible for maintaining extracellular calcium homeostasis within narrow limits despite the large bidirectional fluxes of calcium across the intestine, bone, and especially the kidney.

In chronic kidney disease, the progressive reduction in kidney function not only causes defective renal handling of calcium and phosphorus, but a decrease in renal calcitriol synthesis, which is proportional to the reduction in functional renal mass. In addition to reduced renal 1α-hydroxylase, the enzyme synthesizing calcitriol, several mechanisms contribute to worsen renal calcitriol synthesis. These mechanisms include: impaired substrate availability to renal 1α-hydroxylase; inhibition of renal 1α-hydroxylase activity by hyperphosphatemia, metabolic acidosis and accumulation of uremic toxins; and a blunted response to PTH induction of 1α-hydroxylase activity.

The low serum calcitriol levels result in a marked reduction in intestinal calcium absorption with concomitant hypocalcemia, as well as a proportional reduction in VDR levels in critical targets, such as the parathyroid glands.

Chronic kidney disease also impairs the activity of the calcitriol/VDR-complex as a transcriptional regulator of the expression of calcitriol responsive genes. Two mechanisms were identified: A reduction in the cellular levels of the VDR-transcriptional partner, the retinoid-X-receptor, RXR, and impaired VDR/RXR-DNA–binding interactions. Accumulation of uremic toxins; hypocalcemia- and/or hyperphosphatemia-induced increases in nuclear calreticulin; and uremia-induced activation of transcription factors from VDR-unrelated pathways reduce VDR binding to vitamin D responsive elements on the DNA.

In the parathyroid glands, the reduction in calcitriol/VDR expression and transcriptional activity results in a defective inhibition of PTH synthesis as well as the potent mitogenic signals emerging from overexpression of TGFα and EGFR; impaired induction of the antiproliferative molecules p21 and p27; and defective induction of the CaSR. These defects cause parathyroid hyperplasia, high serum PTH, and reduced sensitivity of the parathyroid gland to suppress growth and PTH secretion in response to calcitriol or calcium. High serum PTH levels lead to osteitis fibrosa and bone loss and systemic toxicities, all of which increase the morbidity and mortality in patients with CKD.

Early therapeutic interventions with calcitriol are recommended to delay the onset of calcitriol resistance by preventing the decreases in parathyroid-VDR and CaSR content. In established secondary hyperparathyroidism, the new less-calcemic vitamin D analogs are the treatment of choice. Four of these analogs are available in the USA and Japan. Although not all analog formulations are equally effective in controlling hypercalcemia and hyperphosphatemia in patients with advanced kidney disease, they offer a wider therapeutic window to counteract vitamin D resistance without causing adynamic bone disease. In the case of paricalcitol, there is a survival advantage over exclusive calcitriol therapy.

The 2003 recommendations by the National Kidney Foundation of the USA provide optimal dosage of calcitriol and its less calcemic analogs for the different stages of CKD, as well as the corrections in the therapeutic approach based on a close control of serum PTH, P and Ca levels to maximize the efficacy of treatment avoiding adynamic bone disease and the risk of vascular calcifications.

References

1. Brown AJ, Dusso A, Slatopolsky E 1999 Vitamin D. Am J Physiol **277**(2 Pt 2):F157–75.
2. Slatopolsky E, Brown A, Dusso A 1999 Pathogenesis of secondary hyperparathyroidism. Kidney Int Suppl **73**:S14–S19.
3. Martinez I, Saracho R, Montenegro J, Llach F 1996 A deficit of calcitriol synthesis may not be the initial factor in the pathogenesis of secondary hyperparathyroidism. Nephrol Dial Transplant **11**(Suppl 3):22–8.
4. Mason RS, Lissner D, Wilkinson M, Posen S 1980 Vitamin D metabolites and their relationship to azotaemic osteodystrophy. Clin Endocrinol (Oxf) **13**(4):375–85.
5. Christiansen C, Christensen MS, Melsen F, Rodbro P, DeLuca HF 1981 Mineral metabolism in chronic renal failure with special reference to serum concentrations of 1,25(OH)2D and 24,25(OH)2D. Clin Nephrol **15**(1):18–22.
6. Juttmann JR, Buurman CJ, De Kam E, Visser TJ, Birkenhager JC 1981 Serum concentrations of metabolites of vitamin D in patients with chronic renal failure (CRF). Consequences for the treatment with 1-alpha-hydroxy-derivatives. Clin Endocrinol (Oxf) **14**(3):225–36.
7. Tessitore N, Venturi A, Adami S, Roncari C, Rugiu C, Corgnati A, Bonucci E, Maschio G 1987 Relationship between serum vitamin D metabolites and dietary intake of phosphate in patients with early renal failure. Miner Electrolyte Metab **13**(1):38–44.
8. Wilson L, Felsenfeld A, Drezner MK, Llach F 1985 Altered divalent ion metabolism in early renal failure: role of $1,25(OH)_2D$. Kidney Int **27**(3):565–73.
9. Halloran BP, Schaefer P, Lifschitz M, Levens M, Goldsmith RS 1984 Plasma vitamin D metabolite concentrations in chronic renal failure: effect of oral administration of 25-hydroxyvitamin D_3. J Clin Endocrinol Metab **59**(6):1063–9.
10. Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, Melsen F, Christensen EI, Willnow TE 1999 An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. Cell **96**(4):507–15.
11. Takemoto F, Shinki T, Yokoyama K, Inokami T, Hara S, Yamada A, Kurokawa K, Uchida S 2003 Gene expression of vitamin D hydroxylase and megalin in the remnant kidney of nephrectomized rats. Kidney Int **64**(2):414–20.
12. Liu W, Yu WR, Carling T, Juhlin C, Rastad J, Ridefelt P, Akerstrom G, Hellman P 1998 Regulation of gp330/megalin expression by vitamins A and D. Eur J Clin Invest **28**(2):100–7.
13. Gallieni M, Kamimura S, Ahmed A, Bravo E, Delmez J, Slatopolsky E, Dusso A 1995 Kinetics of monocyte 1 alpha-hydroxylase in renal failure. Am J Physiol **268**(4 Pt 2): F746–53.
14. Dusso A, Lopez-Hilker S, Rapp N, Slatopolsky E 1988 Extra-renal production of calcitriol in chronic renal failure. Kidney Int **34**(3):368–75.
15. Kohno M, Takahashi S, Oida K, Suzuki J, Tamai T, Yamamoto T, Nakai T 1997 1 alpha,25-dihydroxyvitamin D3 induces very low-density lipoprotein receptor mRNA expression in HL-60 cells in association with monocytic differentiation. Atherosclerosis **133**(1):45–9.
16. Jouni ZE, Winzerling JJ, McNamara DJ 1995 1,25-Dihydroxyvitamin D3–induced HL-60 macrophages: regulation of cholesterol and LDL metabolism. Atherosclerosis **117**(1):125–38.
17. Haussler MR, Baylink DJ, Hughes MR, Brumbaugh PF, Wergedal JE, Shen FH, Nielsen RL, Counts SJ, Bursac KM, McCain TA 1976 The assay of 1alpha,25-dihydroxyvitamin D3: physiologic and pathologic modulation of circulating hormone levels. Clin Endocrinol (Oxf) **5** (Suppl):151S–65S.
18. Garabedian M, Holick MF, Deluca HF, Boyle IT 1972 Control of 25-hydroxycholecalciferol metabolism by parathyroid glands. Proc Natl Acad Sci USA **69**(7):1673–6.
19. Ritz E, Seidel A, Ramisch H, Szabo A, Bouillon R 1991 Attenuated rise of 1,25 (OH)2 vitamin D_3 in response to parathyroid hormone in patients with incipient renal failure. Nephron **57**(3):314–18.
20. Henry HL, Norman AW 1984 Vitamin D: metabolism and biological actions. Annu Rev Nutr **4**:493–520.
21. Murayama A, Takeyama K, Kitanaka S, Kodera Y, Hosoya T, Kato S 1998 The promoter of the human 25-hydroxyvitamin D_3 1 alpha-hydroxylase gene confers positive and negative responsiveness to PTH, calcitonin, and 1 alpha,25(OH)2D3. Biochem Biophys Res Commun **249**(1):11–16.
22. Brenza HL, Kimmel-Jehan C, Jehan F, Shinki T, Wakino S, Anazawa H, Suda T, DeLuca HF 1998 Parathyroid hormone

activation of the 25-hydroxyvitamin D_3-1alpha-hydroxylase gene promoter. Proc Natl Acad Sci USA **95**(4):1387–91.
23. Bellorin-Font E, Humpierres J, Weisinger JR, Milanes CL, Sylva V, Paz-Martinez V 1985 Effect of metabolic acidosis on the PTH receptor-adenylate cyclase system of canine kidney. Am J Physiol **249**(4 Pt 2):F566–72.
24. Shinki T, Shimada H, Wakino S, Anazawa H, Hayashi M, Saruta T, DeLuca HF, Suda T 1997 Cloning and expression of rat 25-hydroxyvitamin D_3-1alpha-hydroxylase cDNA. Proc Natl Acad Sci USA **94**(24):12920–5.
25. Beck N, Kim HP, Kim KS 1975 Effect of metabolic acidosis on renal action of parathyroid hormone. Am J Physiol **228**(5):1483–88.
26. Prince RL, Hutchison BG, Kent JC, Kent GN, Retallack RW 1988 Calcitriol deficiency with retained synthetic reserve in chronic renal failure. Kidney Int **33**(3):722–8.
27. Portale AA, Halloran BP, Morris RC Jr 1989 Physiologic regulation of the serum concentration of 1,25-dihydroxyvitamin D by phosphorus in normal men. J Clin Invest **83**(5):1494–9.
28. Gray RW 1981 Control of plasma 1,25-(OH)2-vitamin D concentrations by calcium and phosphorus in the rat: effects of hypophysectomy. Calcif Tissue Int **33**(5):485–8.
29. Zhang MY, Wang X, Wang JT, Compagnone NA, Mellon SH, Olson JL, Tenenhouse HS, Miller WL, Portale AA 2002 Dietary phosphorus transcriptionally regulates 25OHD-1alpha-hydroxylase gene expression in the proximal renal tubule. Endocrinology **143**(2):587–95.
30. Yoshida T, Yoshida N, Monkawa T, Hayashi M, Saruta T 2001 Dietary phosphorus deprivation induces 25-hydroxyvitamin D_3 1alpha-hydroxylase gene expression. Endocrinology **142**(5):1720–6.
31. Lucas PA, Brown RC, Woodhead JS, Coles GA 1986 1,25-dihydroxycholecalciferol and parathyroid hormone in advanced chronic renal failure: effects of simultaneous protein and phosphorus restriction. Clin Nephrol **25**(1):7–10.
32. Lopez-Hilker S, Dusso AS, Rapp NS, Martin KJ, Slatopolsky E 1990 Phosphorus restriction reverses hyperparathyroidism in uremia independent of changes in calcium and calcitriol. Am J Physiol **259**(3 Pt 2):F432–7.
33. Tenenhouse HS, Martel J, Gauthier C, Zhang MY, Portale AA 2001 Renal expression of the sodium/phosphate cotransporter gene, Npt2, is not required for regulation of renal 1 alpha-hydroxylase by phosphate. Endocrinology **142**(3):1124–9.
34. Portale AA, Booth BE, Halloran BP, Morris RC Jr 1984 Effect of dietary phosphorus on circulating concentrations of 1,25-dihydroxyvitamin D and immunoreactive parathyroid hormone in children with moderate renal insufficiency. J Clin Invest **73**(6):1580–9.
35. Gafter U, Kraut JA, Lee DB, Silis V, Walling MW, Kurokawa K, Haussler MR, Coburn JW 1980 Effect of metabolic acidosis in intestinal absorption of calcium and phosphorus. Am J Physiol **239**(6):G480–4.
36. Bushinsky DA, Favus MJ, Schneider AB, Sen PK, Sherwood LM, Coe FL 1982 Effects of metabolic acidosis on PTH and 1,25(OH)2D3 response to low calcium diet. Am J Physiol **243**(6):F570–5.
37. Kraut JA, Gordon EM, Ransom JC, Horst R, Slatopolsky E, Coburn JW, Kurokawa K 1983 Effect of chronic metabolic acidosis on vitamin D metabolism in humans. Kidney Int **24**(5):644–8.
38. Bushinsky DA, Riera GS, Favus MJ, Coe FL 1985 Response of serum 1,25(OH)2D3 to variation of ionized calcium during chronic acidosis. Am J Physiol **249**(3 Pt 2):F361–5.
39. Langman CB, Bushinsky DA, Favus MJ, Coe FL 1986 Ca and P regulation of 1,25(OH)2D3 synthesis by vitamin D–replete rat tubules during acidosis. Am J Physiol **251**(5 Pt 2): F911–8.
40. Hsu CH, Patel S 1992 Uremic plasma contains factors inhibiting 1 alpha-hydroxylase activity. J Am Soc Nephrol **3**(4):947–52.
41. Maierhofer WJ, Gray RW, Adams ND, Smith GA, Lemann J Jr 1981 Synthesis and metabolic clearance of 1,25-dihydroxyvitamin D as determinants of serum concentrations: a comparison of two methods. J Clin Endocrinol Metab **53**(3):472–5.
42. Henry HL 1981 Insulin permits parathyroid hormone stimulation of 1,25-dihydroxyvitamin D3 production in cultured kidney cells. Endocrinology **108**(2):733–5.
43. Hsu CH, Patel S, Buchsbaum BL 1991 Calcitriol metabolism in patients with chronic renal failure. Am J Kidney Dis **17**(2):185–90.
44. Hsu CH, Patel S, Young EW, Simpson RU 1987 Production and degradation of calcitriol in renal failure rats. Am J Physiol **253**(5 Pt 2):F1015–9.
45. Dusso A, Lopez-Hilker S, Lewis-Finch J, Grooms P, Brown A, Martin K, Slatopolsky E 1989 Metabolic clearance rate and production rate of calcitriol in uremia. Kidney Int **35**(3):860–4.
46. Koyama H, Inaba M, Nishizawa Y, Ishimura E, Imanishi Y, Hini M, Furuyama T, Takagi H, Morii H 1994 Potentiated 1,25(OH)2D3-induced 24-hydroxylase gene expression in uremic rat intestine. Am J Physiol **267**(6 Pt 2):F926–30.
47. Lee SW, Russell J, Avioli LV 1977 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol: conversion impaired by systemic metabolic acidosis. Science **195**(4282):994–6.
48. Costa EM, Feldman D 1986 Homologous up-regulation of the 1,25 (OH)2 vitamin D3 receptor in rats. Biochem Biophys Res Commun **137**(2):742–7.
49. Wiese RJ, Uhland-Smith A, Ross TK, Prahl JM, DeLuca HF 1992 Up-regulation of the vitamin D receptor in response to 1,25-dihydroxyvitamin D3 results from ligand-induced stabilization. J Biol Chem **267**(28):20082–6.
50. Fukuda N, Tanaka H, Tominaga Y, Fukagawa M, Kurokawa K, Seino Y 1993 Decreased 1,25-dihydroxyvitamin D3 receptor density is associated with a more severe form of parathyroid hyperplasia in chronic uremic patients. J Clin Invest **92**(3): 1436–43.
51. Korkor AB 1987 Reduced binding of [3H]1,25-dihydroxyvitamin D3 in the parathyroid glands of patients with renal failure. N Engl J Med **316**(25):1573–7.
52. Merke J, Hugel U, Zlotkowski A, Szabo A, Bommer J, Mall G, Ritz E 1987 Diminished parathyroid 1,25(OH)2D3 receptors in experimental uremia. Kidney Int **32**(3):350–3.
53. Brown AJ, Dusso A, Lopez-Hilker S, Lewis-Finch J, Grooms P, Slatopolsky E 1989 1,25-(OH)2D receptors are decreased in parathyroid glands from chronically uremic dogs. Kidney Int **35**(1):19–23.
54. Denda M, Finch J, Brown AJ, Nishii Y, Kubodera N, Slatopolsky E 1996 1,25-dihydroxyvitamin D3 and 22-oxacalcitriol prevent the decrease in vitamin D receptor content in the parathyroid glands of uremic rats. Kidney Int **50**(1):34–9.
55. Koyama H, Nishizawa Y, Inaba M, Hino M, Prahl JM, DeLuca HF, Morii H 1994 Impaired homologous up-regulation of vitamin D receptor in rats with chronic renal failure. Am J Physiol **266**(5 Pt 2):F706–12.
56. Reinhardt TA, Horst RL 1990 Parathyroid hormone down-regulates 1,25-dihydroxyvitamin D receptors (VDR) and VDR messenger ribonucleic acid *in vitro* and blocks homologous up-regulation of VDR *in vivo*. Endocrinology **127**(2):942–8.

57. Patel SR, Ke HQ, Hsu CH 1994 Regulation of calcitriol receptor and its mRNA in normal and renal failure rats. Kidney Int **45**(4):1020–27.
58. Goff JP, Reinhardt TA, Beckman MJ, Horst RL 1990 Contrasting effects of exogenous 1,25-dihydroxyvitamin D [1,25-(OH)2D] versus endogenous 1,25-(OH)2D, induced by dietary calcium restriction, on vitamin D receptors. Endocrinology **126**(2):1031–35.
59. Sandgren ME, DeLuca HF 1990 Serum calcium and vitamin D regulate 1,25-dihydroxyvitamin D3 receptor concentration in rat kidney *in vivo*. Proc Natl Acad Sci USA **87**(11): 4312–14.
60. Uhland-Smith A, DeLuca HF 1993 The necessity for calcium for increased renal vitamin D receptor in response to 1,25-dihydroxyvitamin D. Biochim Biophys Acta **1176**(3): 321–6.
61. Brown AJ, Zhong M, Finch J, Ritter C, Slatopolsky E 1995 The roles of calcium and 1,25-dihydroxyvitamin D3 in the regulation of vitamin D receptor expression by rat parathyroid glands. Endocrinology **136**(4):1419–25.
62. Huang YC, Lee S, Stolz R, Gabrielides C, Pansini-Porta A, Bruns ME, Bruns DE, Miffin TE, Pike JW, Christakos S 1989 Effect of hormones and development on the expression of the rat 1,25-dihydroxyvitamin D3 receptor gene. Comparison with calbindin gene expression. J Biol Chem **264**(29):17454–61.
63. Martinez J, Olmos JM, de Francisco AL, Amado JA, Riancho JA, Gonzalez-Macias J 1994 1,25-Dihydroxyvitamin D3 receptors in peripheral blood mononuclear cells from patients with primary and secondary hyperparathyroidism. Bone Miner **27**(1):25–32.
64. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA 1994 Prediction of bone density from vitamin D receptor alleles. Nature **367**(6460):284–7.
65. Koshiyama H, Sone T, Nakao K 1995 Vitamin D–receptor gene polymorphism and bone loss. Lancet **345**(8955):990–1.
66. Eisman JA 1999 Genetics of osteoporosis. Endocr Rev **20**(6):788–804.
67. Gomez Alonso C, Naves Diaz ML, Diaz-Corte C, Fernandez Martin JL, Cannata Andia JB 1998 Vitamin D receptor gene (VDR) polymorphisms: effect on bone mass, bone loss, and parathyroid hormone regulation. Nephrol Dial Transplant **13**(Suppl 3):73–7.
68. Carling T, Kindmark A, Hellman P, Lundgren E, Ljunghall S, Rastad J, Akerstrom G, Melhus H 1995 Vitamin D receptor genotypes in primary hyperparathyroidism. Nat Med **1**(12):1309–11.
69. Kontula K, Valimaki S, Kainulainen K, Viitanen AM, Keski-Oja J 1997 Vitamin D receptor polymorphism and treatment of psoriasis with calcipotriol. Br J Dermatol **136**(6): 977–8.
70. Fernandez E, Fibla J, Betriu A, Piulats JM, Almirall J, Montoliu J 1997 Association between vitamin D receptor gene polymorphism and relative hypoparathyroidism in patients with chronic renal failure. J Am Soc Nephrol **8**(10):1546–52.
71. Akiba T, Ando R, Kurihara S, Heishi M, Tazawa H, Marumo F 1997 Is the bone mass of hemodialysis patients genetically determined? Kidney Int Suppl **62**:S69–71.
72. Borras M, Torregrossa V, Oliveras A, Almirall J, Ma Paz M, Betriu A, Martin M, Muray S, Fibla J, Fernandez E 2003 BB genotype of the vitamin D receptor gene polymorphism postpones parathyroidectomy in hemodialysis patients. J Nephrol **16**(1):116–20.
73. Nagaba Y, Heishi M, Tazawa H, Tsukamoto Y, Kobayashi Y 1998 Vitamin D receptor gene polymorphisms affect secondary hyperparathyroidism in hemodialyzed patients. Am J Kidney Dis **32**(3):464–9.
74. Yokoyama K, Shigematsu T, Tsukada T, Ogura Y, Takemoto F, Hara S, Yamada A, Kawaguchi Y, Hosoya T 1998 Apa I polymorphism in the vitamin D receptor gene may affect the parathyroid response in Japanese with end-stage renal disease. Kidney Int **53**(2):454–8.
75. Giannini S, D'Angelo A, Nobile M, Carraro G, Rigotti P, Silva-Netto F, Pavan S, Marchini F, Zaninotto M, Dalla Carbonare L, Sartori L, Crepaldi G 2002 The effects of vitamin D receptor polymorphism on secondary hyperparathyroidism and bone density after renal transplantation. J Bone Miner Res **17**(10):1768–73.
76. Yokoyama K, Shigematsu T, Kagami S, Tsukada T, Arai T, Hara S, Yamada A, Kawaguchi Y, Hosoya T 2001 Vitamin D receptor gene polymorphism detected by digestion with Apa I influences the parathyroid response to extracellular calcium in Japanese chronic dialysis patients. Nephron **89**(3):315–20.
77. Whitfield GK, Remus LS, Jurutka PW, Zitzer H, Oza AK, Dang HT, Haussler CA, Galligan MA, Thatcher ML, Encinas Dominguez C, Haussler MR 2001 Functionally relevant polymorphisms in the human nuclear vitamin D receptor gene. Mol Cell Endocrinol **177**(1–2):145–9.
78. Crofts LA, Hancock MS, Morrison NA, Eisman JA 1998 Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. Proc Natl Acad Sci USA **95**(18):10529–34.
79. Yamamoto H, Miyamoto K, Li B, Taketani Y, Kitano M, Inoue Y, Morita K, Pike JW, Takeda E 1999 The caudal-related homeodomain protein Cdx-2 regulates vitamin D receptor gene expression in the small intestine. J Bone Miner Res **14**(2):240–7.
80. Arai H, Miyamoto KI, Yoshida M, Yamamoto H, Taketani Y, Morita K, Kubota M, Yoshida S, Ikeda M, Watabe F, Kanemasa Y, Takeda E 2001 The polymorphism in the caudal-related homeodomain protein Cdx-2 binding element in the human vitamin D receptor gene. J Bone Miner Res **16**(7):1256–64.
81. Verroust PJ, Christensen EI 2002 Megalin and cubilin—the story of two multipurpose receptors unfolds. Nephrol Dial Transplant **17**(11):1867–71.
82. Segersten U, Correa P, Hewison M, Hellman P, Dralle H, Carling T, Akerstrom G, Westin G 2002 25OHD(3)-1alpha-hydroxylase expression in normal and pathological parathyroid glands. J Clin Endocrinol Metab **87**(6):2967–72.
83. Teramoto T, Endo K, Ikeda K, Kubodera N, Kinoshita M, Yamanaka M, Ogata E 1995 Binding of vitamin D to low-density-lipoprotein (LDL) and LDL receptor-mediated pathway into cells. Biochem Biophys Res Commun **215**(1): 199–204.
84. Canaff L, Hendy GN 2002 Human calcium-sensing receptor gene. Vitamin D response elements in promoters P1 and P2 confer transcriptional responsiveness to 1,25-dihydroxyvitamin D. J Biol Chem **277**(33):30337–50.
85. Li YC, Kong J, Wei M, Chen ZF, Liu SQ, Cao LP 2002 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system. J Clin Invest **110**(2):229–38.
86. Sawaya BP, Koszewski NJ, Qi Q, Langub MC, Monier-Faugere MC, Malluche HH 1997 Secondary hyperparathyroidism and vitamin D receptor binding to vitamin D response elements in rats with incipient renal failure. J Am Soc Nephrol **8**(2):271–78.

87. MacDonald PN, Ritter C, Brown AJ, Slatopolsky E 1994 Retinoic acid suppresses parathyroid hormone (PTH) secretion and PreproPTH mRNA levels in bovine parathyroid cell culture. J Clin Invest **93**(2):725–30.
88. Hsu CH, Patel SR 1995 Altered vitamin D metabolism and receptor interaction with the target genes in renal failure: calcitriol receptor interaction with its target gene in renal failure. Curr Opin Nephrol Hypertens **4**(4):302–6.
89. Patel SR, Ke HQ, Vanholder R, Koenig RJ, Hsu CH 1995 Inhibition of calcitriol receptor binding to vitamin D response elements by uremic toxins. J Clin Invest **96**(1):50–9.
90. Sela-Brown A, Russell J, Koszewski NJ, Michalak M, Naveh-Many T, Silver J 1998 Calreticulin inhibits vitamin D's action on the PTH gene *in vitro* and may prevent vitamin D's effect *in vivo* in hypocalcemic rats. Mol Endocrinol **12**(8):1193–200.
91. Vidal M, Ramana CV, Dusso AS 2002 Stat1-vitamin D receptor interactions antagonize 1,25-dihydroxyvitamin D transcriptional activity and enhance stat1-mediated transcription. Mol Cell Biol **22**(8):2777–87.
92. Jurutka PW, Whitfield GK, Hsieh JC, Thompson PD, Haussler CA, Haussler MR 2001 Molecular nature of the vitamin D receptor and its role in regulation of gene expression. Rev Endocr Metab Disord **2**(2):203–16.
93. Rachez C, Freedman LP 2001 Mediator complexes and transcription. Curr Opin Cell Biol **13**(3):274–80.
94. Rachez C, Freedman LP 2000 Mechanisms of gene regulation by vitamin D(3) receptor: a network of coactivator interactions. Gene **246**(1–2):9–21.
95. May P, Bock HH, Herz J 2003 Integration of endocytosis and signal transduction by lipoprotein receptors. Sci STKE **2003**(176):PE12.
96. Li YC, Amling M, Pirro AE, Priemel M, Meuse J, Baron R, Delling G, Demay MB 1998 Normalization of mineral ion homeostasis by dietary means prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia in vitamin D receptor-ablated mice. Endocrinology **139**(10):4391–96.
97. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, Stockmans I, Van Herck E, Kato S, Bindels RJ, Collen D, Carmeliet P, Bouillon R, Carmeliet G 2001 Duodenal calcium absorption in vitamin D receptor knockout mice: functional and molecular aspects. Proc Natl Acad Sci USA **98**(23):13324–29.
98. Bouillon R, Van Cromphaut S, Carmeliet G 2003 Intestinal calcium absorption: Molecular vitamin D–mediated mechanisms. J Cell Biochem **88**(2):332–9.
99. van Abel M, Hoenderop JG, van der Kemp AW, van Leeuwen JP, Bindels RJ 2003 Regulation of the epithelial Ca2+ channels in small intestine as studied by quantitative mRNA detection. Am J Physiol Gastrointest Liver Physiol **285**(1):G78–85.
100. Brown AJ, Finch J, Slatopolsky E 2002 Differential effects of 19-nor-1,25-dihydroxyvitamin D(2) and 1,25-dihydroxyvitamin D(3) on intestinal calcium and phosphate transport. J Lab Clin Med **139**(5):279–84.
101. Ogg CS 1968 The intestinal absorption of 47Ca by patients in chronic renal failure. Clin Sci **34**(3):467–71.
102. Recker RR, Saville PD 1971 Calcium absorption in renal failure: its relationship to blood urea nitrogen, dietary calcium intake, time on dialysis, and other variables. J Lab Clin Med **78**(3):380–8.
103. Wong RG, Norman AW, Reddy CR, Coburn JW 1972 Biologic effects of 1,25-dihydroxycholecalciferol (a highly active vitamin D metabolite) in acutely uremic rats. J Clin Invest **51**(5):1287–91.
104. Hartenbower DL, Coburn JW, Reddy CR, Norman AW 1974 Calciferol metabolism and intestinal calcium transport in the chick with reduced renal function. J Lab Clin Med **83**(1):38–45.
105. Brickman AS, Coburn JW, Norman AW 1972 Action of 1,25-dihydroxycholecalciferol, a potent, kidney-produced metabolite of vitamin D, in uremic man. N Engl J Med **287**(18):891–95.
106. Walling MW, Kimberg DV, Wasserman RH, Feinberg RR 1976 Duodenal active transport of calcium and phosphate in vitamin D–deficient rats: effects of nephrectomy, cestrum diurnum, and 1alpha,25-dihydroxyvitamin D3. Endocrinology **98**(5):1130–34.
107. Juttmann JR, Hagenouw-Taal JC, Lameyer LD, Ruis AM, Birkenhager JC 1978 Intestinal calcium absorption, serum phosphate, and parathyroid hormone in patients with chronic renal failure and osteodystrophy before and during hemodialysis. Calcif Tissue Res **26**(2):119–26.
108. Chanard JM, Drueke T, Zingraff J, Man NK, Russo-Marie F, Funck-Brentano JL 1976 Effects of haemodialysis on fractional intestinal absorption of calcium in uremia. Eur J Clin Invest **6**(3):261–4.
109. Slatopolsky E, Brown A, Dusso A 2001 Role of phosphorus in the pathogenesis of secondary hyperparathyroidism. Am J Kidney Dis **37**(1 Suppl 2):S54–7.
110. Dusso AS, Pavlopoulos T, Naumovich L, Lu Y, Finch J, Brown AJ, Morrissey J, Slatopolsky E 2001 p21(WAF1) and transforming growth factor-alpha mediate dietary phosphate regulation of parathyroid cell growth. Kidney Int **59**(3):855–65.
111. Gogusev J, Duchambon P, Stoermann-Chopard C, Giovannini M, Sarfati E, Drueke TB 1996 *De novo* expression of transforming growth-factor alpha in parathyroid gland tissue of patients with primary or secondary uremic hyperparathyroidism. Nephrol Dial Transplant **11**(11):2155–62.
112. Cozzolino M, Lu Y, Finch J, Slatopolsky E, Dusso AS 2001 p21WAF1 and TGF-alpha mediate parathyroid growth arrest by vitamin D and high calcium. Kidney Int **60**(6):2109–17.
113. Cozzolino MLY, Slatopolsky E, Dusso A 2002 Specific inhibiton of EGFR-signaling prevents high phosphorus-induced parathyroid hyperplasia in renal failure. J Am Soc Nephrol **13**:193A.
114. Cordero JB, Cozzolino M, Lu Y, Vidal M, Slatopolsky E, Stahl PD, Barbieri MA, Dusso A 2002 1,25-Dihydroxyvitamin D down-regulates cell membrane growth—and nuclear growth—promoting signals by the epidermal growth factor receptor. J Biol Chem **277**(41):38965–71.
115. Tominaga Y, Tsuzuki T, Uchida K, Haba T, Otsuka S, Ichimori T, Yamada K, Numano M, Tanaka Y, Takagi H 1999 Expression of PRAD1/cyclin D1, retinoblastoma gene products, and Ki67 in parathyroid hyperplasia caused by chronic renal failure versus primary adenoma. Kidney Int **55**(4):1375–83.
116. Tokumoto M, Tsuruya K, Fukuda K, Kanai H, Kuroki S, Hirakata H 2002 Reduced p21, p27, and vitamin D receptor in the nodular hyperplasia in patients with advanced secondary hyperparathyroidism. Kidney Int **62**(4):1196–207.
117. Liu M, Lee MH, Cohen M, Bommakanti M, Freedman LP 1996 Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev **10**(2):142–53.
118. Kremer R, Bolivar I, Goltzman D, Hendy GN 1989 Influence of calcium and 1,25-dihydroxycholecalciferol on proliferation and proto-oncogene expression in primary cultures of bovine parathyroid cells. Endocrinology **125**(2):935–41.

119. Brown AJ, Zhong M, Finch J, Ritter C, McCracken R, Morrissey J, Slatopolsky E 1996 Rat calcium-sensing receptor is regulated by vitamin D but not by calcium. Am J Physiol **270**(3 Pt 2):F454–60.
120. Kifor O, Moore FD Jr Wang P, Goldstein M, Vassilev P, Kifor I, Hebert SC, Brown EM 1996 Reduced immunostaining for the extracellular Ca2+-sensing receptor in primary and uremic secondary hyperparathyroidism. J Clin Endocrinol Metab **81**(4):1598–06.
121. Delmez JA, Tindira C, Grooms P, Dusso A, Windus DW, Slatopolsky E 1989 Parathyroid hormone suppression by intravenous 1,25-dihydroxyvitamin D. A role for increased sensitivity to calcium. J Clin Invest **83**(4):1349–55.
122. Shiraishi K, Tsuchida M, Wada T, Yoshihiro S, Takai K, Suga A, Kaneda Y, Naito K 2001 22-Oxacalcitriol upregulates p21(WAF1/Cip1) in human parathyroid glands. A preliminary report. Am J Nephrol **21**(6):507–11.
123. Malluche HH, Ritz E, Lange HP, Kutschera L, Hodgson M, Seiffert U, Schoeppe W 1976 Bone histology in incipient and advanced renal failure. Kidney Int **9**(4):355–62.
124. Friedman PA, Gesek FA 1993 Vitamin D3 accelerates PTH-dependent calcium transport in distal convoluted tubule cells. Am J Physiol **265**(2 Pt 2):F300–08.
125. Hoenderop JG, Muller D, Van Der Kemp AW, Hartog A, Suzuki M, Ishibashi K, Imai M, Sweep F, Willems PH, Van Os CH, Bindels RJ 2001 Calcitriol controls the epithelial calcium channel in kidney. J Am Soc Nephrol **12**(7):1342–9.
126. Li YC 2003 Vitamin D regulation of the renin-angiotensin system. J Cell Biochem **88**(2):327–31.
127. Chertow BS, Sivitz WI, Baranetsky NG, Clark SA, Waite A, Deluca HF 1983 Cellular mechanisms of insulin release: the effects of vitamin D deficiency and repletion on rat insulin secretion. Endocrinology **113**(4):1511–8.
128. Christakos S, Friedlander EJ, Frandsen BR, Norman AW 1979 Studies on the mode of action of calciferol. XIII. Development of a radioimmunoassay for vitamin D–dependent chick intestinal calcium-binding protein and tissue distribution. Endocrinology **104**(5):1495–503.
129. Lowrie EG, Soeldner JS, Hampers CL, Merrill JP 1970 Glucose metabolism and insulin secretion in uremic, prediabetic, and normal subjects. J Lab Clin Med **76**(4):603–15.
130. Allegra V, Mengozzi G, Martimbianco L, Vasile A 1990 Glucose-induced insulin secretion in uremia: effects of aminophylline infusion and glucose loads. Kidney Int **38**(6):1146–50.
131. Alvestrand A, Mujagic M, Wajngot A, Efendic S 1989 Glucose intolerance in uremic patients: the relative contributions of impaired beta-cell function and insulin resistance. Clin Nephrol **31**(4):175–83.
132. Quesada JM, Martin-Malo A, Santiago J, Hervas F, Martinez ME, Castillo D, Barrio V, Aljama P 1990 Effect of calcitriol on insulin secretion in uremia. Nephrol Dial Transplant **5**(12):1013–7.
133. Allegra V, Luisetto G, Mengozzi G, Martimbianco L, Vasile A 1994 Glucose-induced insulin secretion in uremia: role of 1 alpha,25(HO)2-vitamin D3. Nephron **68**(1):41–7.
134. Goldblum SE, Reed WP 1980 Host defenses and immunologic alterations associated with chronic hemodialysis. Ann Intern Med **93**(4):597–613.
135. Reichel H, Koeffler HP, Norman AW 1989 The role of the vitamin D endocrine system in health and disease. N Engl J Med **320**(15):980–91.
136. Bar-Shavit Z, Noff D, Edelstein S, Meyer M, Shibolet S, Goldman R 1981 1,25-dihydroxyvitamin D3 and the regulation of macrophage function. Calcif Tissue Int **33**(6):673–6.
137. Park K, Ha SK, Han DS 1988 Studies on lymphocyte subpopulations and cell-mediated immunity in patients with chronic renal failure. Yonsei Med J **29**(2):109–16.
138. Lin CY, Huang TP 1988 Serial cell-mediated immunological changes in terminal uremic patients on continuous ambulatory peritoneal dialysis therapy. Am J Nephrol **8**(5):355–62.
139. Venezio FR, Kozeny GA, DiVincenzo CA, Hano JE 1988 Effects of 1,25 dihydroxyvitamin D3 on leukocyte function in patients receiving chronic hemodialysis. J Infect Dis **158**(5):1102–5.
140. Bargman JM, Silverman ED, Klein MH 1989 Effect of 1,25-dihydroxyvitamin D3 *in vivo* on circulating T lymphocytes. Miner Electrolyte Metab **15**(6):359–64.
141. Quesada JM, Serrano I, Borrego F, Martin A, Pena J, Solana R 1995 Calcitriol effect on natural killer cells from hemodialyzed and normal subjects. Calcif Tissue Int **56**(2):113–17.
142. Tabata T, Suzuki R, Kikunami K, Matsushita Y, Inoue T, Okamoto T, Miki T, Nishizawa Y, Morii H 1986 The effect of 1 alpha-hydroxyvitamin D3 on cell-mediated immunity in hemodialyzed patients. J Clin Endocrinol Metab **63**(5): 1218–21.
143. Lemire JM, Adams JS, Kermani-Arab V, Bakke AC, Sakai R, Jordan SC 1985 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity *in vitro*. J Immunol **134**(5):3032–5.
144. Tabata T, Shoji T, Kikunami K, Matsushita Y, Inoue T, Tanaka S, Hino M, Miki T, Nishizawa Y, Morii H 1988 *In vivo* effect of 1 alpha-hydroxyvitamin D3 on interleukin-2 production in hemodialysis patients. Nephron **50**(4):295–298.
145. Boland R 1986 Role of vitamin D in skeletal muscle function. Endocr Rev **7**(4):434–48.
146. Wu J, Garami M, Cheng T, Gardner DG 1996 1,25(OH)2 vitamin D3 and retinoic acid antagonize endothelin-stimulated hypertrophy of neonatal rat cardiac myocytes. J Clin Invest **97**(7):1577–88.
147. Wu J, Garami M, Cao L, Li Q, Gardner DG 1995 1,25(OH)2D3 suppresses expression and secretion of atrial natriuretic peptide from cardiac myocytes. Am J Physiol **268**(6 Pt 1):E1108–13.
148. Selles J, Massheimer V, Santillan G, Marinissen MJ, Boland R 1997 Effects of calcitriol and its analogues, calcipotriol (MC 903) and 20-epi-1alpha,25-dihydroxyvitamin D3 (MC 1288), on calcium influx and DNA synthesis in cultured muscle cells. Biochem Pharmacol **53**(12):1807–1014.
149. Brown J, Bianco JI, McGrath JJ, Eyles DW 2003 1,25-dihydroxyvitamin D3 induces nerve growth factor, promotes neurite outgrowth, and inhibits mitosis in embryonic rat hippocampal neurons. Neurosci Lett **343**(2):139–43.
150. Cai Q, Tapper DN, Gilmour RF Jr deTalamoni N, Aloia RC, Wasserman RH 1994 Modulation of the excitability of avian peripheral nerves by vitamin D: relation to calbindin-D28k, calcium status, and lipid composition. Cell Calcium **15**(5):401–10.
151. Cantorna MT, Hayes CE, DeLuca HF 1996 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. Proc Natl Acad Sci U S A **93**(15):7861–4.
152. Sanchez B, Lopez-Martin E, Segura C, Labandeira-Garcia JL, Perez-Fernandez R 2002 1,25-Dihydroxyvitamin D(3) increases striatal GDNF mRNA and protein expression in adult rats. Brain Res Mol Brain Res **108**(1–2):143–6.
153. Baker LR, Abrams L, Roe CJ, Faugere MC, Fanti P, Subayti Y, Malluche HH 1989 1,25(OH)2D3 administration in moderate renal failure: a prospective double-blind trial. Kidney Int **35**(2):661–9.

154. Nordal KP, Dahl E, Halse J, Attramadal A, Flatmark A 1995 Long-term, low-dose calcitriol treatment in predialysis chronic renal failure: can it prevent hyperparathyroid bone disease? Nephrol Dial Transplant **10**(2):203–6.
155. Hamdy NA, Kanis JA, Beneton MN, Brown CB, Juttmann JR, Jordans JG, Josse S, Meyrier A, Lins RL, Fairey IT 1995 Effect of alfacalcidol on natural course of renal bone disease in mild to moderate renal failure. Bmj **310**(6976):358–63.
156. Maung HM, Elangovan L, Frazao JM, Bower JD, Kelley BJ, Acchiardo SR, Rodriguez HJ, Norris KC, Sigala JF, Rutkowski M, Robertson JA, Goodman WG, Levine BS, Chesney RW, Mazess RB, Kyllo DM, Douglass LL, Bishop CW, Coburn JW 2001 Efficacy and side effects of intermittent intravenous and oral doxercalciferol (1alpha-hydroxyvitamin D(2)) in dialysis patients with secondary hyperparathyroidism: a sequential comparison. Am J Kidney Dis **37**(3):532–43.
157. Slatopolsky E, Weerts C, Thielan J, Horst R, Harter H, Martin KJ 1984 Marked suppression of secondary hyperparathyroidism by intravenous administration of 1,25-dihydroxycholecalciferol in uremic patients. J Clin Invest **74**(6):2136–43.
158. Andress DL, Norris KC, Coburn JW, Slatopolsky EA, Sherrard DJ 1989 Intravenous calcitriol in the treatment of refractory osteitis fibrosa of chronic renal failure. N Engl J Med **321**(5):274–9.
159. Dunlay R, Rodriguez M, Felsenfeld AJ, Llach F 1989 Direct inhibitory effect of calcitriol on parathyroid function (sigmoidal curve) in dialysis. Kidney Int **36**(6):1093–8.
160. Tsukamoto Y, Nomura M, Takahashi Y, Takagi Y, Yoshida A, Nagaoka T, Togashi K, Kikawada R, Marumo F 1991 The "oral 1,25-dihydroxyvitamin D3 pulse therapy" in hemodialysis patients with severe secondary hyperparathyroidism. Nephron **57**(1):23–8.
161. Tsukamoto Y, Mariyo R, Nomura Y, Sato N, Faugere MC, Malluche HH 1993 Long-term effect of oral calcitriol pulse therapy on bone in hemodialysis patients. Bone **14**(3):421–5.
162. Quarles LD, Yohay DA, Carroll BA, Spritzer CE, Minda SA, Bartholomay D, Lobaugh BA 1994 Prospective trial of pulse oral versus intravenous calcitriol treatment of hyperparathyroidism in ESRD. Kidney Int **45**(6):1710–21.
163. Cannella G, Bonucci E, Rolla D, Ballanti P, Moriero E, De Grandi R, Augeri C, Claudiani F, Di Maio G 1994 Evidence of healing of secondary hyperparathyroidism in chronically hemodialyzed uremic patients treated with long-term intravenous calcitriol. Kidney Int **46**(4):1124–32.
164. Llach F, Hervas J, Cerezo S 1995 The importance of dosing intravenous calcitriol in dialysis patients with severe hyperparathyroidism. Am J Kidney Dis **26**(5):845–51.
165. Bacchini G, Fabrizi F, Pontoriero G, Marcelli D, Di Filippo S, Locatelli F 1997 "Pulse oral" versus intravenous calcitriol therapy in chronic hemodialysis patients. A prospective and randomized study. Nephron **77**(3):267–72.
166. Indridason OS, Quarles LD 2000 Comparison of treatments for mild secondary hyperparathyroidism in hemodialysis patients. Durham Renal Osteodystrophy Study Group. Kidney Int **57**(1):282–92.
167. Fischer ER, Harris DC 1993 Comparison of intermittent oral and intravenous calcitriol in hemodialysis patients with secondary hyperparathyroidism. Clin Nephrol **40**(4): 216–20.
168. Liou HH, Chiang SS, Huang TP, Shieh SD, Akmal M 1994 Comparative effect of oral or intravenous calcitriol on secondary hyperparathyroidism in chronic hemodialysis patients. Miner Electrolyte Metab **20**(3):97–102.
169. Salusky IB, Goodman WG, Horst R, Segre GV, Kim L, Norris KC, Adams JS, Holloway M, Fine RN, Coburn JW 1990 Pharmacokinetics of calcitriol in continuous ambulatory and cycling peritoneal dialysis patients. Am J Kidney Dis **16**(2):126–32.
170. Slatopolsky E, Brown AJ 2002 Vitamin D analogs for the treatment of secondary hyperparathyroidism. Blood Purif **20**(1):109–12.
171. Dusso AS, Negrea L, Gunawardhana S, Lopez-Hilker S, Finch J, Mori T, Nishii Y, Slatopolsky E, Brown AJ 1991 On the mechanisms for the selective action of vitamin D analogs. Endocrinology **128**(4):1687–92.
172. Tanaka Y, DeLuca HF, Kobayashi Y, Ikekawa N 1984 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D3: a highly potent, long-lasting analog of 1,25-dihydroxyvitamin D3. Arch Biochem Biophys **229**(1):348–54.
173. Akiba T, Marumo F, Owada A, Kurihara S, Inoue A, Chida Y, Ando R, Shinoda T, Ishida Y, Ohashi Y 1998 Controlled trial of falecalcitriol versus alfacalcidol in suppression of parathyroid hormone in hemodialysis patients with secondary hyperparathyroidism. Am J Kidney Dis **32**(2):238–46.
174. Finch JL, Brown AJ, Slatopolsky E 1999 Differential effects of 1,25-dihydroxyvitamin D3 and 19-nor-1,25-dihydroxyvitamin D2 on calcium and phosphorus resorption in bone. J Am Soc Nephrol **10**(5):980–5.
175. Llach F, Yudd M 2001 Paricalcitol in dialysis patients with calcitriol-resistant secondary hyperparathyroidism. Am J Kidney Dis **38**(5 Suppl 5):S45–50.
176. Sprague SM, Lerma E, McCormmick D, Abraham M, Batlle D 2001 Suppression of parathyroid hormone secretion in hemodialysis patients: comparison of paricalcitol with calcitriol. Am J Kidney Dis **38**(5 Suppl 5):S51–56.
177. Martin KJ, Gonzalez EA, Gellens ME, Hamm LL, Abboud H, Lindberg J 1998 Therapy of secondary hyperparathyroidism with 19-nor-1alpha,25-dihydroxyvitamin D2. Am J Kidney Dis **32**(2 Suppl 2):S61–6.
178. Coyne DW, Grieff M, Ahya SN, Giles K, Norwood K, Slatopolsky E 2002 Differential effects of acute administration of 19-nor-1,25-dihydroxyvitamin D2 and 1,25-dihydroxyvitamin D3 on serum calcium and phosphorus in hemodialysis patients. Am J Kidney Dis **40**(6):1283–8.
179. Teng M, Wolf M, Lowrie E, Ofsthun N, Lazarus JM, Thadhani R 2003 Survival of patients undergoing hemodialysis with paricalcitol or calcitriol therapy. N Engl J Med **349**(5):446–56.
180. Slatopolsky E, Cozzolino M, Finch JL 2002 Differential effects of 19-nor-1,25-(OH)(2)D(2) and 1alpha-hydroxyvitamin D(2) on calcium and phosphorus in normal and uremic rats. Kidney Int **62**(4):1277–84.

CHAPTER 77

Idiopathic Hypercalciuria and Nephrolithiasis

MURRAY J. FAVUS Section of Endocrinology, The University of Chicago Pritzker School of Medicine, Chicago, Illinois

FREDRIC L. COE Nephrology Section, The University of Chicago Pritzker School of Medicine, Chicago, Illinois

I. INTRODUCTION

This chapter focuses on idiopathic hypercalciuria (IH) as a major cause of hypercalciuria and nephrolithiasis and the potential role of vitamin D. Less frequent causes of hypercalcemia and hypercalciuria may also promote renal stone formation and are discussed in Chapters 78 and 79. IH is found in 5 to 7% of the adult population, is responsible for 50% of calcium oxalate nephrolithiasis, and is the most common 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] excess state.

Idiopathic hypercalciuria is characterized by normocalcemia in the absence of known systemic causes of hypercalciuria. Increased intestinal calcium (Ca) absorption is almost always increased, and serum $1,25(OH)_2D$ levels are elevated in one-third to one-half of patients. Serum parathyroid hormone (PTH) levels are elevated in less than 5%. The pathogenesis of IH is unknown but several models have been offered from observations in patients including: a primary increase in intestinal Ca absorption; a primary overproduction of $1,25(OH)_2D$; and a primary renal tubular Ca transport defect or "renal leak" of Ca. Evidence for each model can be found in some patients with IH, suggesting the disorder may be heterogeneous. There is also evidence that IH is a state of $1,25(OH)_2D$ excess. As already mentioned one-third to one-half of IH patients have elevated serum $1,25(OH)_2D$ levels. The remaining 50% with normal serum $1,25(OH)_2D$ levels cannot be distinguished from those with elevated levels because intestinal Ca absorption is just as high, and negative Ca balance may develop during low Ca intake. Of interest is the observation that all of the changes in Ca metabolism characteristic of IH can be induced by the administration of small doses of $1,25(OH)_2D_3$ to healthy volunteers.

An animal model of genetic hypercalciuria has been developed in Sprague-Dawley rats by breeding hypercalciuric male and female animals. The hypercalciuria in genetic hypercalciuric stone forming (GHS) rats is due to an increase in intestinal Ca absorption and bone resorption and decreased renal Ca reabsorption. Elevated vitamin D receptor (VDR) content in intestinal mucosa, renal tubules, and bone cells strongly supports the concept that hypercalciuria is a state of vitamin D receptor-mediated excess. A post-transcriptional dysregulation of VDR is suggested by decreased VDR mRNA and increased accumulation of normal VDR protein that has normal binding affinity for $1,25(OH)_2D_3$. The nature of the genetic defect in GHS rats and in human IH that permit hypercalciuria remains unknown.

II. IDIOPATHIC HYPERCALCIURIA

A. Overview

Hypercalciuria is common among patients with Ca oxalate nephrolithiasis and is thought to contribute to stone formation by increasing the state of urine supersaturation with respect to Ca and oxalate. Flocks [1] first commented on the frequency of hypercalciuria among patients with Ca stones; however, it was not until the mid-1950s that Albright and Henneman [2,3] defined the condition of IH as hypercalciuria with normal serum Ca, no systemic illness, and no clinical skeletal disease. The definition of hypercalciuria is arbitrary and based on the distribution of urine Ca excretion values among unselected populations of healthy men

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TABLE I Causes of Normocalcemic Hypercalciuria

Paget's disease
Sarcoidosis
Hyperthyroidism
Renal tubular acidosis
Cushing's syndrome
Immobilization
Malignant tumor
Furosemide administration

and women in Western countries [4,5]. The distribution of urine Ca forms a continuum that is clustered about a mean with a long tail of higher values. IH patients are those whose urine Ca exceeds the arbitrary upper limit of normal, which is most commonly defined as greater than 300 mg/24 hr for men, greater than 250 mg/24 hr for women, or greater than 4 mg/kg body weight or 140 mg Ca per gram urine creatinine for either sex [6]. Using these definitions, hypercalciuria is found in about 50% of calcium oxalate stone formers [6,7] and is the most common cause of normocalcemic hypercalciuric stone formation [7]. The diagnosis of IH requires the exclusion of the known causes of normocalcemic hypercalciuria (see Table I).

Surveys of stone formers attending kidney stone clinics report a high proportion with kidney stone formation among first-degree relatives [8,9]. A genetic basis of IH was further suggested by subsequent surveys [10–12] that revealed a strong familial occurrence of IH with high rates of vertical and horizontal penetrance (see Fig. 1) consistent with an autosomal dominant mode of inheritance. IH also occurs in children with the same frequency of occurrence as in adults [13]. That hypercalciuria can have a genetic origin has been clearly demonstrated by breeding experiments in which the offspring of spontaneously hypercalciuric male and female Sprague-Dawley rats are intensely hypercalciuric [14–16]. Other human hypercalciuric genetic disorders have been described, but they differ from IH in

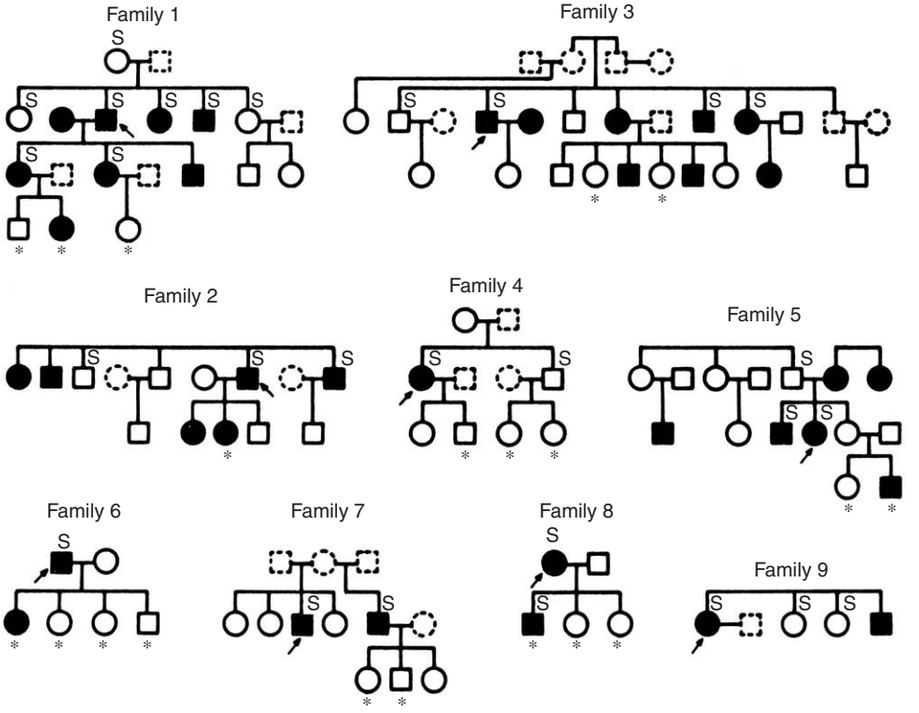

FIGURE 1 Family pedigrees of nine probands with idiopathic hypercalciuria. Solid circles and solid squares are females and males with hypercalciuria; S is stone formation; * indicates children (younger than age 20). Arrows indicate probands from each family. Dashed symbols are relatives who were not studied. Hypercalciuria occurred in 11 of 24 siblings, 7 of 16 offspring, and 1 of 3 parents of probands. Reprinted by permission of *The New England Journal of Medicine* (Coe FL, Parks JH, Moore ES. Familial idiopathic hypercalciuria N Engl J Med **300**:337–340). Copyright 1979, Massachusetts Medical Society.

having either a renal phosphate leak [17] that may lead to rickets (Chapter 69), renal tubular acidosis [18], or X-linked recessive stone formation with early renal failure [19].

Idiopathic hypercalciuria is a common disorder that affects 5 to 7% of otherwise healthy men and women [4]. If 50% of stone formers have IH [6,7], and the frequency of stone disease among adults is 0.5%, then 80 to 90% of IH is asymptomatic and never associated with stone formation. The increased frequency of osteopenia (see Section B.4) suggests that hypercalciuria may be an important pathogenetic factor for development of low bone mass even among those who do not form stones.

B. Pathogenesis of Human Idiopathic Hypercalciuria

1. Renal Histopathology in Calcium Oxalate Nephrolithiasis

Interstitial crystal deposition at or near the tips of papillae are found in 100% of kidneys of Ca oxalate stone formers who have IH and no systemic cause of hypercalciuria or other cause of stone formation (Table I). Less frequently (43%), nonstone formers may have such papillary depositions [20]. These lesions first described by Randall [21] have recently been found to be composed of calcium phosphate (apatite) and contain no Ca oxalate [22]. The plaques originate in the basement membrane of the thin loops of Henle and spread from there through the interstitium to just beneath the urothelium. There is no Ca phosphate or Ca oxalate crystal depositions within the renal tubule lumen. Rather, the Ca phosphate plaque may serve as a site of heterogeneous nucleation of Ca oxalate crystals that subsequently grow and form Ca oxalate kidney stones [22,23]. The role of hypercalciuria in the development of the Ca phosphate interstitial lesions of Randall's plaques remains unknown; however, the Ca phosphate plaques are rather specific for Ca oxalate stone formers, as the interstitial lesions are absent in intestinal bypass patients who form Ca oxalate stones [22].

2. Increased Intestinal Calcium Absorption

Normally, the quantity of Ca absorbed is determined by dietary Ca intake and the efficiency of intestinal Ca absorption [24]. Absorption of Ca across the intestine is the sum of two transepithelial transport processes: a nonsaturable paracellular pathway and a saturable, cellular active transport system [25,26] (also see Chapters 24, 25). Absorption via the paracellular path is diffusional and driven by the lumen-to-blood Ca gradient [24]. The cellular pathway is vitamin D–dependent and is regulated by the ambient concentration of $1,25(OH)_2D$. Thus, intraluminal Ca concentration and tissue $1,25(OH)_2D$ levels are the driving forces for Ca translocation via the paracellular and cellular pathways, respectively.

Increased intestinal Ca absorption has been found in most patients with IH [27–35]. Using either a single oral dose of Ca isotope to measure fecal isotope excretion or double Ca isotope administration in which the intravenous dose adjusts for isotope distribution, IH patients were shown to have an increase in the Ca absorptive flux (Fig. 2). External Ca balance studies conducted while IH patients and normal nonstone formers ingested diets containing comparable amounts of Ca show net intestinal Ca absorption rates to be greater in IH patients [36]. Biopsies of proximal intestine obtained following oral Ca isotopic administration reveal increased mucosal accumulation of isotope compared to normocalciuric nonstoneformers [37]. Thus, by all techniques used, IH is characterized by increased intestinal Ca absorption.

3. Elevated $1,25(OH)_2D$

Kaplan and colleagues [33] first reported elevated serum levels of $1,25(OH)_2D$ in a group of patients with IH. Subsequently, others have confirmed that, on average, serum $1,25(OH)_2D$ levels are higher in IH (Fig. 3). Increased *in vivo* conversion of tritiated 25-hydroxyvitamin D_3 (^{3}H-25OHD$_3$) to ^{3}H-$1,25(OH)_2D_3$ with normal metabolic clearance [38] in a group of IH patients with elevated serum $1,25(OH)_2D$ levels indicate that the increase in serum $1,25(OH)_2D$ levels in some IH patients is the result of increased production. Of note is the considerable overlap of serum $1,25(OH)_2D$ levels between IH patients and nonstone formers (Fig. 3).

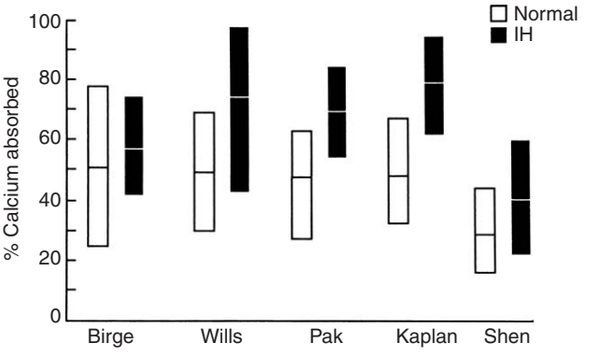

FIGURE 2 Intestinal Ca absorption in healthy volunteers and patients with IH. Absorption rates are expressed as percentages of dietary Ca absorbed as calculated from the appearance of Ca isotopes in blood or fecal collections. Values are means (horizontal bar) ± 2 standard deviations. Names indicate references: Birge [28]; Wills [29]; Pak [32]; Kaplan [33]; and Shen [34].

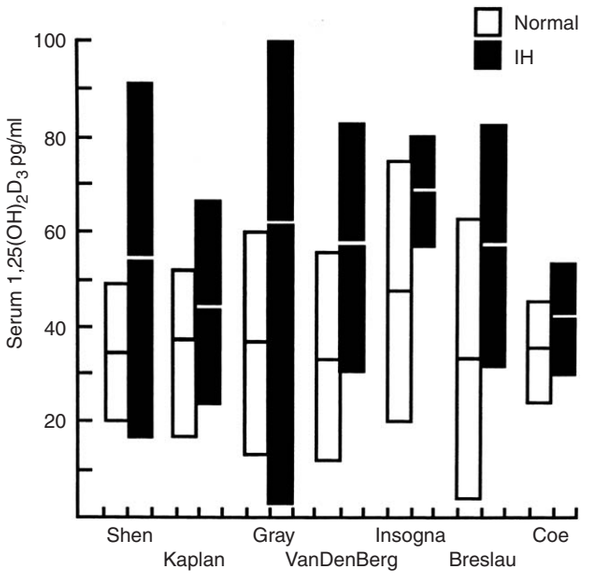

FIGURE 3 Plot of means ± 2 SD of serum $1,25(OH)_2D$ in IH patients and nonstone formers. Horizontal bar is mean of group. Names indicate references: Kaplan [33]; Shen [34]; Insogna [38]; Coe [40]; Gray [42]; Van Den Berg [63]; and Breslau [64].

Thus, for about 50% of IH patients, increased intestinal Ca transport may be caused by increased circulating $1,25(OH)_2D$. For the remainder, other mechanisms of increased Ca absorption must be considered.

The mechanism whereby $1,25(OH)_2D$ production is increased in IH is unknown. The major regulators of renal proximal tubule mitochondrial 25-hydroxyvitamin D 1-hydroxylase (1-hydroxylase) activity include PTH, phosphate depletion, and insulin-like growth factor-I (IGF-I) (see Chapter 5). However, only 5% of IH patients have elevated circulating PTH levels [32,39], and urinary cAMP levels, a surrogate measure of PTH, are also normal in most patients [32,40,41]. Mild hypophosphatemia with reduced renal tubular phosphate reabsorption has been described in as many as one-third of IH patients [33,34,39,42]. A strong inverse association between serum $1,25(OH)_2D$ levels and renal tubular phosphate reabsorption has been reported [34,41]. As elevated PTH or hypophosphatemia accompany elevated serum $1,25(OH)_2D$ in only a minority of patients; the cause of increased serum $1,25(OH)_2D$ in most patients with IH remains unknown. Detailed studies of IGF-I have not been performed.

The recent description of sequences of mutations in the q23.3-q24 region of the first chromosome in three kindred with absorptive IH [43] involves a region containing a gene that is analogous with the rat soluble adenylate cyclase gene. This first description of specific base pair substitutions suggests the possibility of a gene defect associated with IH that may involve altered receptor signaling. Whether this mutation alters functions related to the regulation of the 1-hydroxylase remains to be determined. However, caution has been expressed in accepting this report as conclusively demonstrating that the substitutions or a mutation of this gene causes IH [44].

Serum $1,25(OH)_2D$ values in normals and IH patients overlap extensively in each series reported (Fig. 3). Kaplan *et al*. [33] found that in patients with absorptive IH [defined as normal fasting urine Ca and normal or elevated serum $1,25(OH)_2D$, intestinal Ca absorption measured by fecal excretion of orally administered ^{47}Ca was increased out of proportion to the simultaneously measured serum $1,25(OH)_2D$ concentration (Fig. 4B). In contrast, a strong positive correlation between intestinal Ca absorption and serum $1,25(OH)_2D$ is found in normal volunteers, normocalciuric stone formers, patients with primary hyperparathyroidism, and IH patients with fasting hypercalciuria (Fig. 4A). The high intestinal Ca absorption rates with normal or elevated serum $1,25(OH)_2D$ levels suggest that the pathogenesis of IH is heterogeneous, with at least one phenotype resulting from $1,25(OH)_2D$ overproduction.

4. DECREASED RENAL CALCIUM REABSORPTION

A defect in the tubular reabsorption of Ca, a so-called renal leak of Ca, has been postulated as a cause of hypercalciuria in IH. Two reports [45,46] found a greater fraction of filtered Ca excreted in the urine of IH patients compared to nonstone formers. The values were calculated from *inulin* clearance or creatinine clearance and used blood ionized Ca as an estimate of ultrafilterable Ca. Although urinary sodium (Na) excretion is a major determinant of Ca excretion in normal and IH patients, there is no evidence that patients overingest or overexcrete Na. Hydrochlorothiazide and acetazolamide increase urine Ca, Na, and magnesium (Mg) excretion in IH compared to normals [47], suggesting a generalized defect in proximal tubule electrolyte and water transport in IH patients. The basis for the abnormal renal transport is not known, but increased activity of the erythrocyte plasma membrane Ca^{2+}, Mg^{2+}-ATPase in IH patients and correlation of enzyme activity with urine Ca excretion in families with IH [48] suggests a more widespread genetic defect in monovalent and divalent ion transport.

5. LOW BONE MASS

Abnormal skeletal metabolism in IH has been demonstrated by low bone mineral density of the distal radius [49,50] and lumbar spine [51–53] and by lower skeletal Ca content by neutron activation analysis [54].

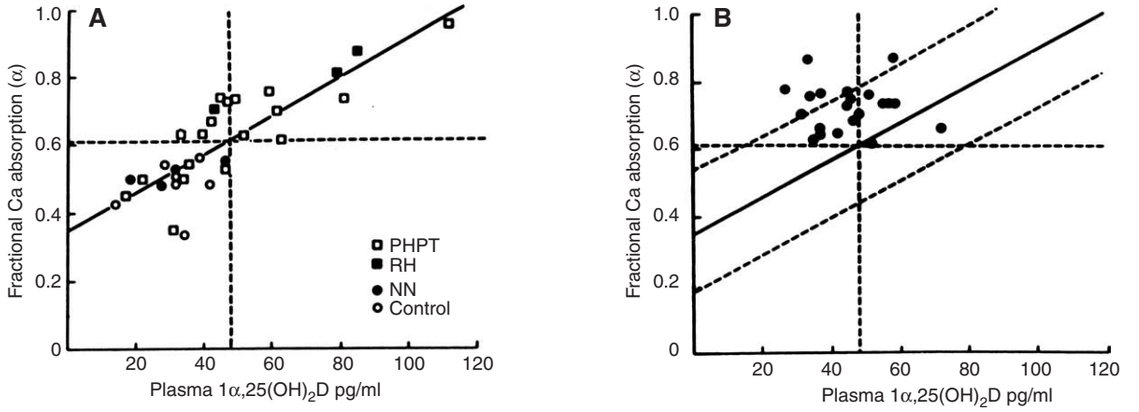

FIGURE 4 Relationship of calcium absorption to $1,25(OH)_2D$ levels. **(A)** Fractional intestinal absorption of oral ^{47}Ca versus serum $1,25(OH)_2D$ level in normal controls (open circles), normocalciuric stone formers (NN, filled circles), IH stone formers who have fasting hypercalciuria and elevated PTH (RH, filled squares), and patients with primary hyperparathyroidism (PHPT, open squares). **(B)** Fractional calcium absorption of IH patients with absorptive hypercalciuria (normal fasting urine Ca) superimposed on the 95% confidence limits for the relationship in controls. Reproduced from *The Journal of Clinical Investigation,* 1977, Vol. **59**, pp. 756–760 [33], by copyright permission of The American Society for Clinical Investigation.

Reports differ as to possible pathogenesis, with low bone density found only in those with renal leak hypercalciuria in one study [50], and low bone density in those with absorptive hypercalciuria in another study [52]. Information on bone dynamics is limited to one early study in which ^{47}Ca labeling was interpreted as increased bone turnover, with bone resorption and formation both increased [55]. Two studies of bone histology showed reduced bone apposition rate, delayed mineralization of osteoid seams, and prolonged mineralization lag time and formation period [56,57]. These observations suggest defective mineralization, which may be caused by hypophosphatemia in some patients. The observations are also consistent with a defect in osteoblastic function. Measurements of biochemical markers of bone turnover reveal increased urine hydroxyproline excretion in unselected IH patients [58] and increased serum osteocalcin in IH patients with renal but not absorptive hypercalciuria [59].

Whether the low bone density is a result of the lifelong hypercalciuria, habitual low Ca intake, or a genetic defect in osteoblast function independent of urine Ca excretion remains to be determined. In a study of 59 subjects from 11 families with at least one member a hypercalciuric Ca oxalate stoneformer [60], lumbar spine and femoral neck bone density Z scores varied inversely with urine Ca and urine ammonium in the stoneformers but not in the nonstone formers. There were no correlations of Z score for bone turnover markers or serum $1,25(OH)_2D$ levels. Ca consumption was lower in stoneformers, suggesting that the admonition to ingest a low Ca diet to avoid more stones, in fact predisposes to bone loss.

The well-documented low bone mass in IH patients is associated with increased fracture risk [61]. Reduction of urine Ca during thiazide therapy has been studied in a small number of IH patients and found to be effective in improving bone mass (see Section V.B below).

6. PROPOSED PATHOGENETIC MODELS OF IDIOPATHIC HYPERCALCIURIA

On the basis of the consistent increase in intestinal Ca absorption, normal or elevated serum $1,25(OH)_2D$ levels, and normal or elevated fasting urinary Ca, Pak and colleagues [62] separated IH into three groups: absorptive, renal, and resorptive. In the first, primary intestinal Ca hyperabsorption (Fig. 5A) would transiently raise postprandial serum Ca above normal and increase ultrafilterable Ca. Postprandial hypercalcemia would transiently suppress PTH secretion, resulting in reduced tubular Ca reabsorption and hypercalciuria. In the second, a primary renal tubular leak of Ca (Fig. 5B) would cause hypercalciuria and a transient reduction in serum Ca. Secondary hyperparathyroidism would normalize serum Ca and increase proximal tubule $1,25(OH)_2D$ synthesis, which would stimulate intestinal Ca absorption. PTH secretion would then decline to the extent that serum Ca is normalized. This scenario predicts that serum $1,25(OH)_2D$ would be elevated in renal IH and normal or elevated in absorptive IH [63,64]. A third possibility is based on a primary overproduction of $1,25(OH)_2D_3$ that increases intestinal Ca absorption and bone resorption (Fig. 5C) while PTH remains normal and fasting urine Ca excretion may be normal or elevated.

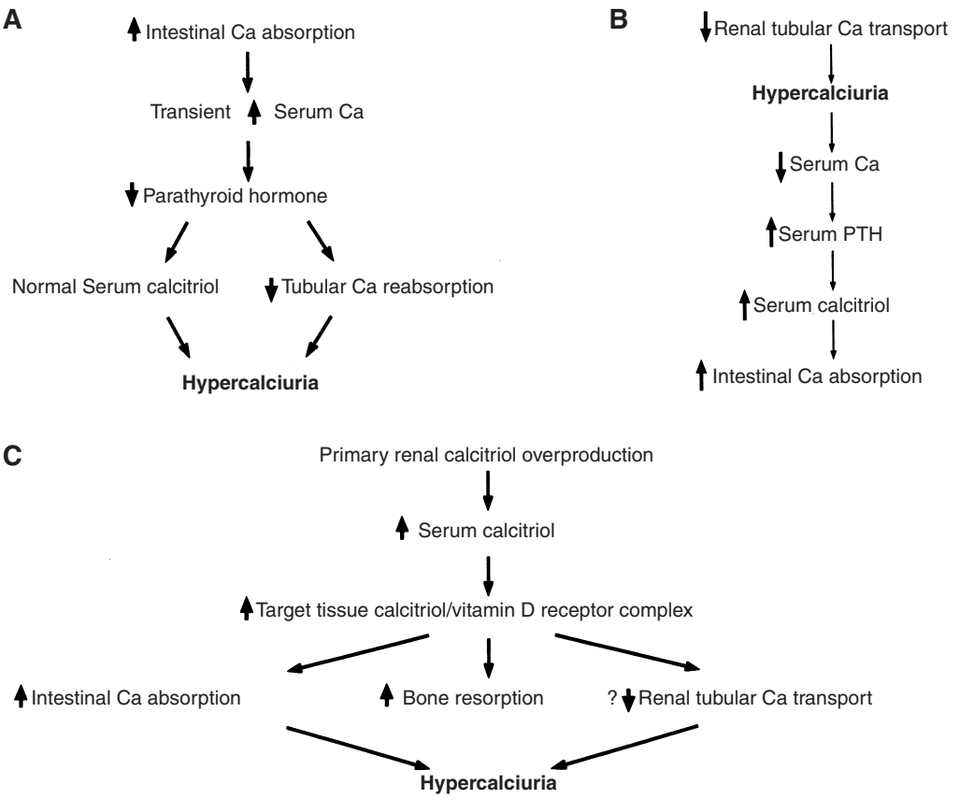

FIGURE 5 Three proposed models of IH. **(A)** Absorptive IH with primary intestinal overabsorption, postprandial hypercalcemia, suppressed PTH, and normal fasting urine Ca. Serum $1,25(OH)_2D$ is normal. **(B)** Primary renal tubular leak of Ca leads to a transient decrease in serum Ca and elevated PTH with secondary increases in serum $1,25(OH)_2D$ and intestinal Ca absorption. Fasting urine Ca is elevated. **(C)** Primary overproduction of $1,25(OH)_2D$ increases serum $1,25(OH)_2D$ and stimulates intestinal Ca absorption and bone resorption. Serum PTH is normal or decreased, and fasting urine Ca is normal or elevated.

7. TESTS OF THE MODELS

Knowledge of the pathophysiology of IH is fundamental to developing rational therapy for the prevention of recurrent kidney stones. If the model of primary intestinal overabsorption were correct, then dietary Ca restriction would reduce the amount of Ca absorbed and Ca excreted in the urine without altering bone mass. If a renal leak of Ca were the primary event, or if urinary Ca originates from bone rather than diet, then restricting dietary Ca will have little effect on urinary Ca excretion, while worsening Ca balance and promoting bone loss. Testing certain predictions has assessed the accuracy of the absorptive and renal models.

a. Fasting Serum PTH, Urine Ca If repeated episodes of postprandial hypercalcemia suppress PTH secretion sufficiently to cause chronic hypoparathyroidism, fasting serum PTH and urine cAMP would be low and fasting urine Ca elevated. Transient suppression of PTH would permit normal serum PTH, urine cAMP, and fasting urine Ca. In contrast, renal IH requires increased PTH and urine cAMP and increased fasting urine Ca [65]. As existing PTH radioimmunoassays do not differentiate normal from low values, most IH patients have been found to have PTH levels in the normal range. Although normal fasting urine Ca is not unusual among IH patients, only 5% have elevated PTH and, therefore, fail to meet the criteria for renal IH [32,62]. Thus, a primary renal leak of Ca with a secondary increase in PTH cannot account for fasting hypercalciuria in a majority of patients. About 24% of patients meet the criteria of absorptive hypercalciuria by reducing urine Ca during fasting to maintain neutral Ca balance [63].

b. External Ca Balance The relationship between net intestinal Ca absorption and 24-hr urine Ca excretion calculated from 6-day balance studies is different

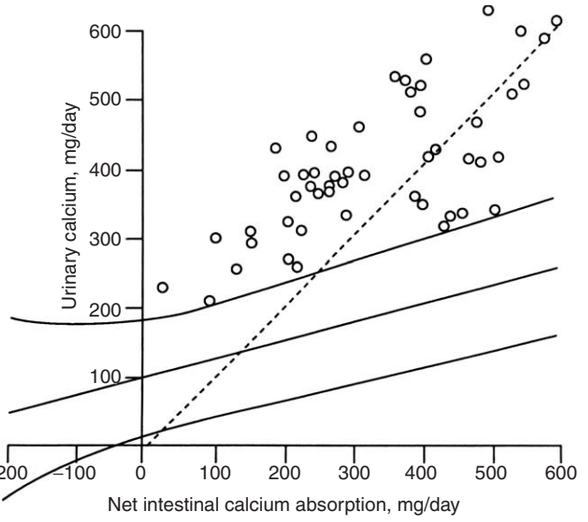

FIGURE 6 Urinary Ca excretion as a function of net intestinal Ca absorption. Data are derived from 6-day external mineral balance studies. Solid lines indicate the 95% confidence limits about the mean regression line derived from the data on 195 adult nonstone formers. Individual balance studies performed on 51 patients with IH are shown as open circles. The dashed line represents equivalent rates of urinary Ca excretion and net intestinal Ca absorption (the line of identity). Normal values are from [47,58,68,69]. Values from patients are from References [47,57,71–74], and J. Lemann (personal communication, 1992). Adapted from Asplin *et al.* [95].

in IH patients compared to normal subjects (Fig. 6) [66–72]. In nonstone formers, urinary Ca excretion is positively correlated with net absorption, and overall Ca balance is positive when net absorption is greater than 200 mg per 24 hr (see 95% confidence limits calculated from balance studies on normal subjects in Fig. 6). Net Ca absorption tends to be greater in IH patients, and for every level of net absorption, 24-hr urine Ca excretion is higher in the patients compared to healthy subjects. In IH patients, a greater portion of absorbed Ca is excreted in the urine. In normal subjects, net Ca absorption exceeds urine Ca excretion, and balance is positive when net absorption exceeds 200 mg/24 hr. In contrast, almost 50% of the IH patients have urine Ca excretion in excess of net absorption and are in negative Ca balance, even when allowance is made for some variability in the balance data (±50 mg). Thus, at all levels of net Ca absorption, negative Ca balance (above the zero balance or above the line of identity) is common in IH patients but not in healthy subjects. Negative Ca balance in the presence of adequate Ca intake is incompatible with a primary hyperabsorption of dietary Ca or absorptive hypercalciuria and cannot, by itself, account for the hypercalciuria.

c. Urine Ca and Ca Balance during Low Ca Diet The hypothesis of primary intestinal Ca overabsorption predicts that dietary Ca restriction would reduce the amount of Ca absorbed and would therefore reduce urinary Ca excretion. Like normal subjects, IH patients would be in positive or neutral Ca balance when net absorption is above 200 mg/24 hr (Fig. 6). A low Ca diet would reduce urine Ca excretion through an increase in PTH secretion, which would promote distal tubular Ca reabsorption. In contrast, patients with a primary renal Ca leak would be unable to conserve urine Ca at any level of Ca intake and would maintain an excessive or inappropriately high urine Ca excretion even during low Ca diet. As a result, Ca balance during low Ca diet would shift from positive or neutral to negative or become more negative. Serum PTH would be expected to increase to high levels during a low Ca diet.

To test whether the responses of IH patients fit these predictions, Coe *et al.* [40] fed a low Ca diet (2 mg/kg/day) to nine normal volunteers and 26 unselected IH stone formers. After 10 days on the diet, urine Ca excretion decreased to 2.0 mg/kg body weight or less in both patients and controls, but 17 of the 26 IH patients (Fig. 7) showed values greater than the highest value in normal controls. In patients, urine Ca excretion (CaE) ranged from normal to persistently high levels. It exceeded Ca intake (CaI) (Fig. 7, CaI – CaE) in 11 of the 26 patients and none of the nonstone-forming controls. Thus, almost 50% of the patients had more Ca in the urine than what was provided by the diet and were clearly in negative Ca balance.

The results indicate that a chronic low Ca diet may be detrimental for some patients, as the inability to conserve urine Ca would eventually lead to clinically detectable bone loss. The data also suggest that some patients with IH may have diet-dependent hypercalciuria, whereas others have diet-independent hypercalciuria. The two proposed mechanisms cannot be readily distinguished by any clear discontinuity in the distribution of urine Ca values, and serum PTH and $1,25(OH)_2D$ levels do not predict the urine Ca responses during a low Ca diet. Patients with the highest urine Ca and most extreme negative Ca balance had serum PTH and 1,25 $(OH)_2D$ levels that were not different from patients who conserved Ca to the levels found in normal subjects.

d. Role of 1,25(OH)$_2$D Excess The majority of patients are classified as having absorptive hypercalciuria [62,65], yet negative Ca balance during low Ca diet [40] without elevated PTH, or $1,25(OH)_2D$ is not predicted by the absorptive model (Fig. 5A). Patients who meet the criteria of renal hypercalciuria tend to

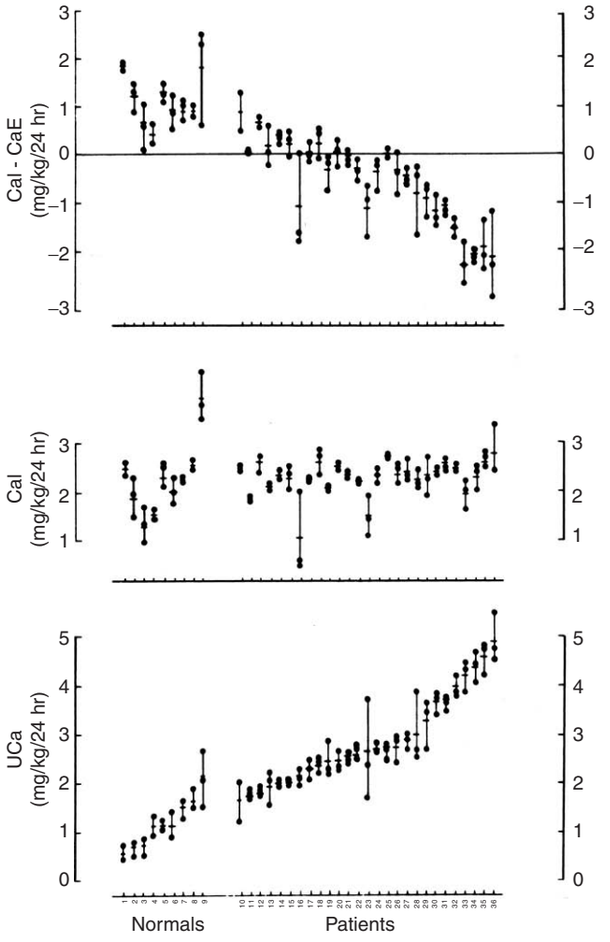

FIGURE 7 Calcium intake and urinary excretion in patients with IH and normal subjects. Urine Ca excretion (UCa) and Ca intake (Cal) are during a low Ca diet. Mean Ca intakes for patients and controls (2.29 ± 0.15 versus 2.31 ± 0.05 mg/kg/day) were not different. Mean urine excretion rates during low Ca intake and values of Cal–CaE (an index of Ca balance) differed significantly between normals and IH patients. Subjects and patients are arranged in ascending order of urinary Ca excretion. Reprinted by permission of the publisher from Coe *et al.* [40]. "Effects of low-calcium diet on urine calcium excretion, parathyroid function and serum $1,25(OH)_2D_3$ levels in patients with idiopathic hypercalciuria and in normal subjects," *American Journal of Medicine,* Vol. **72**, pp. 25–32. Copyright 1982 by Excerpta Medica Inc.

have higher serum $1,25(OH)_2D$ levels, but only a small portion have elevated PTH levels. Further, serum $1,25(OH)_2D$ levels do not predict whether patients will be classified as absorptive or renal, and at least one-third of patients have normal serum $1,25(OH)_2D$ levels despite intestinal Ca hyperabsorption. For them, the mechanism of intestinal Ca hyperabsorption remains unexplained.

The model of primary vitamin D excess (Fig. 5C) is supported by elevated $1,25(OH)_2D$ production rates and enhanced biological actions of $1,25(OH)_2D$, including increased intestinal Ca absorption and bone resorption. Creation of a mild form of $1,25(OH)_2D$ excess was achieved by the administration of pharmacological doses of $1,25(OH)_2D_3$ (3.0 ug/day) to healthy men for 10 days while Ca intake varied from low (160 mg) to normal (372 mg) or high (880 mg) [73–75]. Increased urine Ca excretion and net intestinal Ca absorption led to negative Ca balance as calculated from 6-day metabolic balance studies (Fig. 8). Dietary Ca strongly influenced the response to $1,25(OH)_2D_3$, as Ca balance was more negative during low Ca intake, and the increase in urine Ca resulted primarily from accelerated bone resorption. At low-normal or normal Ca intake, $1,25(OH)_2D_3$ administration increased urine Ca and net intestinal Ca absorption, and Ca balance remained neutral. During normal Ca diet, $1,25(OH)_2D_3$ administration maintained neutral or positive Ca balance. Thus, 3 ug/day of $1,25(OH)_2D_3$, which was insufficient to cause hypercalcemia, during the 10-day study has profound effects on intestinal Ca absorption, urine Ca excretion, and Ca balance. Further, $1,25(OH)_2D_3$ administration caused negative Ca balance only during low Ca intake. Thus, $1,25(OH)_2D_3$ induced changes in Ca balance in normal subjects similar to that observed in IH patients on comparable levels of Ca intake.

In other experiments, ketoconazole administration to IH patients inhibited renal $1,25(OH)_2D$ biosynthesis [64] and decreased serum $1,25(OH)_2D$ levels, intestinal Ca absorption, and urine Ca excretion. The results of the effects of $1,25(OH)_2D_3$ treatment and the response to ketoconazole provide further support for a primary $1,25(OH)_2D$ excess in at least some patients with IH. The nature of the disordered regulation of renal $1,25(OH)_2D$ production or action remains to be determined, as neither elevated PTH nor hypophosphatemia were present in responders or were absent in nonresponders to ketoconazole.

III. GENETIC HYPERCALCIURIC RATS

Tests of the absorptive, renal, and vitamin D excess models of IH have been complicated by difficulty in controlling for potential variables such as inheritance and environmental factors that may influence dietary patterns. The availability of an animal model of IH would permit the testing of the three hypotheses under conditions that exclude genetic and dietary influences. The strong familial occurrence of IH in humans and the high frequency of elevated urine Ca in adult men

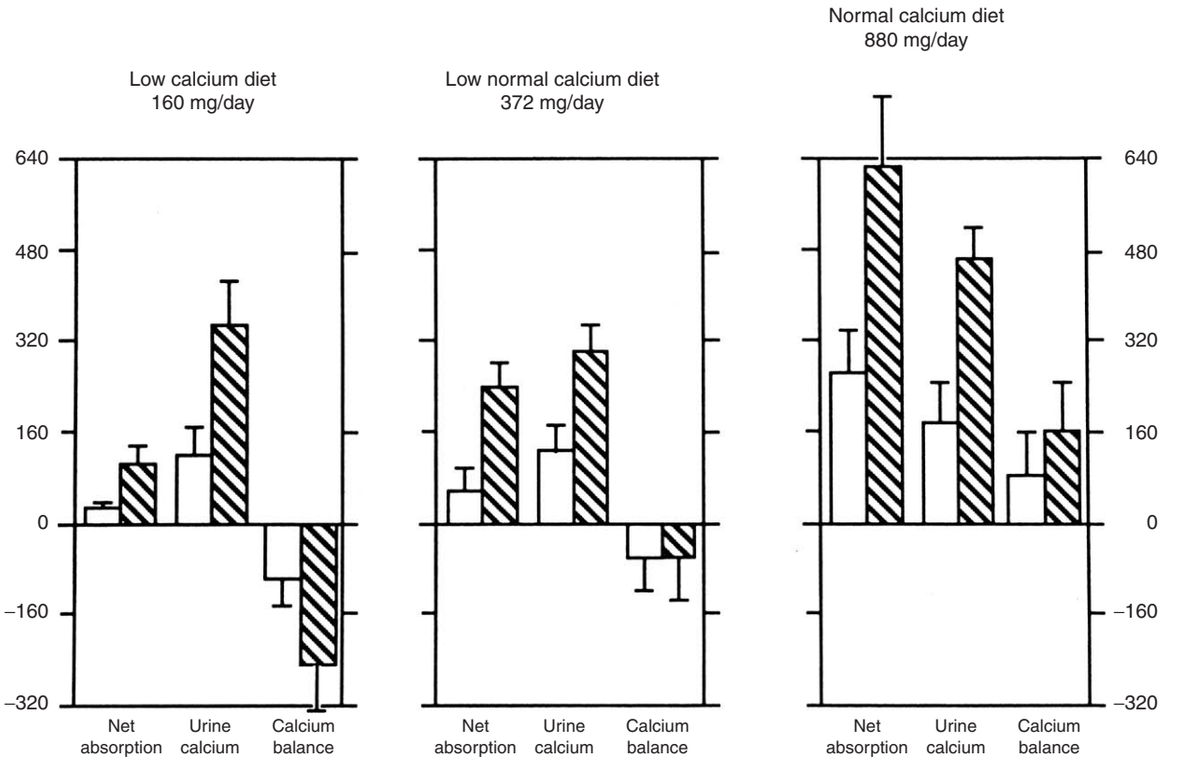

FIGURE 8 Intestinal Ca absorption, urine Ca excretion, and Ca balance in normal men receiving $1,25(OH)_2D_3$ (hatched bars) or controls (open bars) at varying levels of dietary Ca. Values (mg/24 hr) are means ± SEM for 6 men per group. For Ca balance, values above the horizontal line indicate positive balance and those below the line, negative balance. Data from Maierhofer *et al.* [76] and Adams *et al.* [77]. Reprinted with permission from Coe FL, Parks JH 1988 Nephrolithiasis: Pathogenesis and Treatment, Second Edition, Year Book Publishers: Chicago 1988 [75].

and women suggested that spontaneous hypercalciuria might also be found in animals.

A. Establishment of a Colony of Genetically Hypercalciuric Rats

The distribution of urine Ca excretion in a population of male Sprague-Dawley rats fed a normal Ca diet (0.8% Ca) was similar to that found in a population of healthy humans in that it followed a nonGaussian distribution, with values clustering about the mean and a long tail of higher values [14]. Using an arbitrary definition of hypercalciuria as urine Ca greater than two standard deviations above the mean value, about 5 to 10% of male and female rats were hypercalciuric. Mating males and females with the most severe hypercalciuria resulted in offspring with hypercalciuria. The most hypercalciuric offspring were used for repeated matings, leading to a colony with hypercalciuria that has increased in intensity and frequency with each successive generation [15]. By the twentieth generation, over 95% of males and females were hypercalciuric. By the fortieth generation, mean urine Ca excretion was 7.0 ± 0.3 mg/24 hr compared to the stable mean excretion of less than 0.75 mg/24 hr by wild-type rats [77]. Hypercalciuria is lifelong and may be detected as soon as the animals are weaned (about 50 g body weight). Weight and growth of the hypercalciuric rats have been comparable to wild-type Sprague-Dawley rats obtained from the same supplier that provided the original spontaneously hypercalciuric animals. No anatomical or structural abnormalities have been identified; however, by 18 weeks of age 100% of the animals have grossly evident Ca-containing kidney stones in the upper and lower urinary tracts [78]. No stones are found in the kidney or urinary tract of wild-type rats.

B. Serum and Urine Chemistries

Serum Ca and Mg are within the normal range in the genetic hypercalciuric stone-forming (GHS) male and female rats [15]. Serum phosphate is lower in

female rats, and there is no difference between GHS and wild-type males and females. Serum PTH levels in GHS rats are not different from controls. Urine volumes are greater in the GHS rats.

C. Mineral Balance

Six-day external balance studies performed while the animals were fed a normal Ca diet showed the animals to be in positive balance for Ca, Mg, and phosphorus [15] with greater net Ca absorption in GHS rats. The GHS rats maintained positive Ca balance because the increased urine Ca excretion was matched by a greater net intestinal Ca absorption.

D. Intestinal Calcium Transport

To investigate the mechanism of the increased Ca absorption, segments of duodenum were mounted *in vitro* in modified Ussing chambers, and transepithelial bidirectional fluxes of Ca were measured in the absence of electrochemical gradients [22]. Under these conditions, [15], duodenal segments from GHS rats had a fivefold increase in the mucosal-to-serosal (absorptive) transepithelial flux of Ca (J_{ms}), whereas the secretory flux of Ca from serosa to mucosa (J_{sm}) was only mildly increased compared to wild type (Table II). As Ca J_{ms} was 10 to 12 times higher than Ca J_{sm}, changes in J_{sm} had a nonsignificant effect on net Ca absorption.

E. Serum 1,25(OH)$_2$D

Circulating 1,25(OH)$_2$D levels were lower in the fourth generation GHS rats; however, the differences disappeared by the tenth generation [at 190 g, mean ± SD serum 1,25(OH)$_2$D was 135 ± 12 versus 174 ± 19 pg/ml, nonsignificant], and no subsequent differences in serum 1,25(OH)$_2$D levels have been observed [16]. *In vitro* duodenal net flux (J_{net}, equal to $J_{ms} - J_{sm}$) for Ca was positively correlated with serum 1,25(OH)$_2$D in normocalciuric and GHS male and female rats (Fig. 9). However, the regression coefficients were different for the wild-type and GHS rats, with the latter having a steeper slope. The greater Ca J_{net} in GHS rats with serum 1,25(OH)$_2$D levels comparable to the wild-type rats strongly suggests that duodenal Ca-transporting cells in GH rats are more sensitive to 1,25(OH)$_2$D.

TABLE II *In Vitro* Bidirectional Duodenal Calcium Active Transport*

Flux	NM	GHM	NF	GHF
J_{ms}	51 ± 12	264 ± 27	29 ± 9	258 ± 40
J_{sm}	11 ± 2	19 ± 2	14 ± 2	23 ± 2
J_{net}	40 ± 11	245 ± 28	14 ± 8	235 ± 40

*Values are means ± SE for 5 to 11 rats per group. NM and NF are normocalciuric (wild-type) male and female rats, respectively. GHM and GHF are genetic hypercalciuric male and female rats, respectively. J_{ms} and J_{sm} are mucosal-to-serosal and serosal-to-mucosal fluxes of Ca, respectively. J_{net} is net Ca absorption, where $J_{net} = J_{ms} - J_{sm}$. Adapted from Li *et al.* [16] and reproduced with permission from *The Journal of Clinical Investigation,* 1993, Vol. **91**, pp. 661–667, by copyright permission of The American Society for Clinical Investigation.

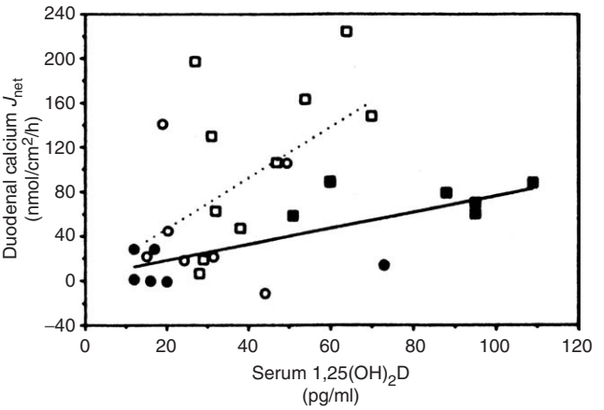

FIGURE 9 Duodenal Ca net flux (J_{net}) as a function of serum 1,25(OH)$_2$D for hypercalciuric and normocalciuric male (open and filled squares, respectively) and female (open and filled circles, respectively) rats. J_{net} and serum 1,25(OH)$_2$D were correlated for male and female normocalciuric rats ($r = 0.789$, $n = 12$, $p < 0.001$, solid line) and for male and female GHS rats ($r = 0.500$, $n = 17$, $p < 0.03$, dotted line). The regressions were different (F ratio = 5.469, $p < 0.015$). Reproduced from *The Journal of Clinical Investigation*, 1988, Vol. **82**, pp. 1585–1591 [15], by copyright permission of The American Society for Clinical Investigation.

F. Role of the Vitamin D Receptor

The increased intestinal Ca transport and normal serum 1,25(OH)$_2$D levels in GHS rats suggested either that Ca transport was being stimulated by an unidentified, vitamin D–independent process or that 1,25(OH)$_2$D action was being amplified at the level of vitamin D target tissues. As 1,25(OH)$_2$D stimulates Ca transport by binding to the vitamin D receptor (VDR) to up-regulate vitamin D–dependent genes that encode for proteins involved in transepithelial Ca transport, and because the biological actions of 1,25(OH)$_2$D are directly related to the tissue VDR content [79–81], VDR binding in intestinal epithelial cells was measured. Duodenal cytosolic

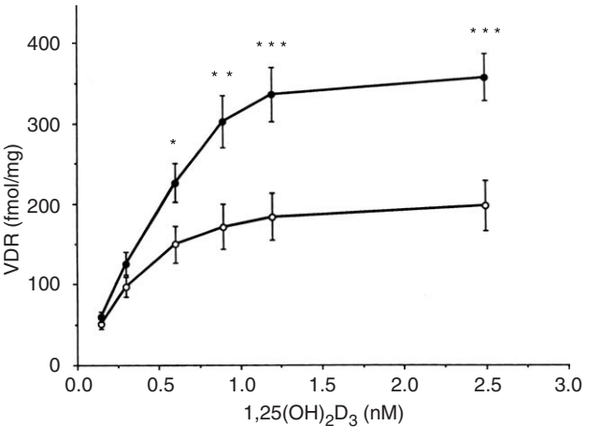

FIGURE 10 Specific binding of ^{3}H-1,25$(OH)_2D_3$ to duodenal cytosolic fractions (VDR) prepared from GHS rats (filled circles) and wild-type controls (open circles) while fed a normal Ca diet. Values are means ± SEM for four observations per concentration point. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ vs controls. Reproduced from *The Journal of Clinical Investigation,* 1993, Vol. **91**, pp. 661–667 [16], by copyright permission of The American Society for Clinical Investigation.

fractions prepared in high potassium buffer from male GHS rats bound more ^{3}H-1,25$(OH)_2D_3$ than comparable fractions from wild-type control rats [16] (Fig. 10). Cytosolic fractions from kidney cortex and from splenic monocytes also exhibited greater specific binding of 1,25$(OH)_2D_3$. Scatchard analysis of the specific binding curves revealed a single class of VDR binding sites in tissues from both wild-type and GHS rats. The number of VDR binding sites in GHS rat duodenal cells was double that found in cells from wild-type rats (536 ± 73 versus 243 ± 42 fmol/mg protein; $n = 8$ and $n = 14$; $p < 0.001$), with comparable affinity of the receptor for its ligand (0.33 ± 0.01 versus 0.49 ± 0.01 nM; nonsignificant). A twofold increase in VDR binding sites was also found in GHS rat renal cortical homogenates [16].

Using Western blotting, homogenates of duodenal mucosa from GHS rats contained a band at 50 kDa that comigrated with duodenal extracts from wild-type rats and with recombinant human VDR. The bands from the GHS rat tissues were more intense compared to controls, confirming that the increase in specific ^{3}H-1,25$(OH)_2D_3$ binding was due to an increase in VDR protein. Northern analysis of RNA extracts from GHS and wild-type rat tissues revealed a single species of VDR mRNA at 4.4 kb with no difference in migration between the two groups [16]. Duodenal extracts from GHS rats contained less VDR mRNA than controls. Estimates of duodenal cell transcription rates using standard nuclear run-on assays found no clear difference between GHS rats and controls [16]. The *in vivo* half-life of the VDR mRNA in GHS rat duodenum was comparable to that of controls (6 hr). Administration of a small dose of 1,25$(OH)_2D_3$ (30 ng as a single dose) resulted in a significant elevation of VDR message and prolongation of message half-life in GHS rats but not controls [82]. Thus, in GHS rat intestine, the increased VDR level is not due to an increase in VDR gene transcription. The data are consistent with either an increase in VDR mRNA translation efficiency or changes that result in a prolongation of the VDR half-life. The increased accumulation of the vitamin D–dependent calbindin-D_{9K} found in GHS rat duodenum [16] is evidence that the increased level of VDR is functional and that the increased Ca transport is likely a vitamin D–mediated process.

Major questions remain as to the genetic basis of the increased VDR activity. However, sequence of a cDNA prepared from GHS rat duodenal VDR mRNA failed to reveal any difference compared to the sequence of VDR cDNA prepared from wild-type duodenum. The cumulative evidence suggests that the primary genetic defect does not directly involve the VDR gene.

G. Increased Bone Resorption

In vitro studies of bone resorption using neonatal calvariae from normal and GHS rats show that Ca efflux (a measure of bone resorption) increases in a dose-dependent manner in the presence of 1,25$(OH)_2D_3$ or PTH [83]. The dose-response curve is much steeper for 1,25$(OH)_2D_3$ in calvariae from GHS rats, whereas the dose-response curves for PTH-stimulated Ca efflux are not different between control and GHS calvariae. Western blotting showed a fourfold increase in VDR protein from GHS neonatal rat calvariae [83]. Thus, the increase in target tissue VDR exerts biological actions that increase l,25$(OH)_2$D3–dependent bone resorption, which likely contributes to the hypercalciuria.

H. Response to Low Calcium Diet

To test whether the hypercalciuria in GHS rats is the result of a primary overabsorption of dietary Ca, GHS and wild-type control rats were fed diets either normal (0.6% Ca) or low (0.02% Ca) with respect to Ca. During the low Ca diet, urine Ca excretion decreased in both groups (Fig. 11); however, urine Ca remained higher in GHS rats and resulted in negative Ca balance [84]. The inability of GHS rats to conserve Ca during low Ca intake excludes overabsorption of dietary Ca as the sole cause of hypercalciuria in GHS rats.

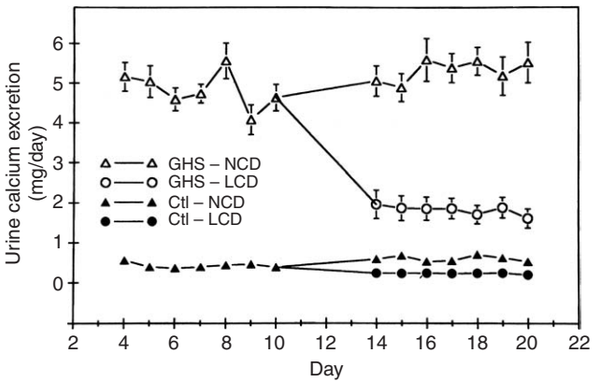

FIGURE 11 Daily urine Ca excretion in nineteenth-generation GHS rats (open symbols) or wild-type control rats (filled symbols) fed a normal Ca diet (NCD, 0.6% Ca, triangles) during days 1–10 followed by either continuation of the NCD (triangles) or feeding of a low Ca diet (LCD, 0.02% Ca, circles). Rats were pair-fed to 13 g of diet per day. Reprinted with permission from Kim *et al.* [86].

I. Summary of Pathogenesis in the Genetic Hypercalciuric Rat

Figure 12 summarizes current knowledge of the pathogenesis of hypercalciuria in the GHS rats. Breeding by selection for hypercalciuria has emphasized a trait in the offspring that likely involves the expression of several genes for full phenotypic expression. To date, none of the genes has been identified. Studies implicate the increased VDR concentration as part of the primary event(s) and a cause of the hypercalciuria; however, a secondary adaptive increase in VDR to compensate for urinary Ca losses has not been excluded. Further information is required regarding the renal handling of Ca in GHS rats and whether the GHS genotype results in a primary defect in renal Ca transport.

IV. CURRENT VIEW OF HUMAN GENETIC HYPERCALCIURIA

Striking similarities in Ca metabolism between GHS rats, IH patients, and human volunteers treated with $1,25(OH)_2D_3$ (Table III) strongly support a primary role of excess $1,25(OH)_2D$ biological action in the pathogenesis of human IH. When deprived of dietary Ca, few patients conserve Ca to the extent that normals do (Fig. 7). The renal IH model predicts ongoing urinary losses of Ca independent of Ca intake, and negative Ca balance during a low calcium diet. However, most patients have normal, not elevated PTH, as renal IH would require. Therefore, the absorptive and renal models of hypercalciuria cannot explain the response of most patients to a low Ca diet. In nonstone formers, $1,25(OH)_2D_3$ administration changes urine Ca and Ca balance to those observed in a majority of IH patients who have either normal or elevated serum $1,25(OH)_2D$ levels. For some patients, elevated serum $1,25(OH)_2D_3$ increases in intestinal Ca hyperabsorption and urine Ca excretion, and causes negative Ca balance during low Ca intake. The source of $1,25(OH)_2D$ excess is more elusive in patients with normal serum $1,25(OH)_2D$ levels. They may be more similar to the GHS rats in that both have normal serum $1,25(OH)_2D$, increased

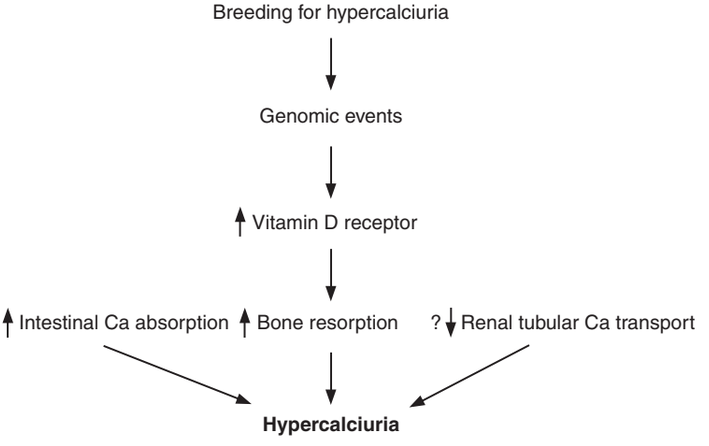

FIGURE 12 Proposed series of events that result from breeding selection for hypercalciuric rats. The renal handling of Ca by GHS rats and the role of increased VDR content, if any, in the transport process remain unknown.

TABLE III Pathophysiology of Genetic Hypercalciuria

Parameter	Human	Human	GHS rats
Serum Ca	N	N	N
Serum phosphate	N	N-D	N
Serum $1,25(OH)_2D$	I	N-I	N
Urinary Ca on NCD	I	I	I
Urinary Ca on LCD	I	N-I	I
Intestinal Ca absorption	I	I	I
Ca balance on NCD	Pos-N	N-Neg	Pos
Ca balance on LCD	N-Neg	N-Neg	Neg

Values for human controls are responses to treatment with 3 ug $1,25(OH)_2D_3$ daily for 7 days compared to pretreatment. GHS, genetic hypercalciuric stone-forming; NCD, normal Ca diet; LCD, low Ca diet; N, normal; I, increased; D, decreased; Pos, positive; Neg, negative.

intestinal absorption and bone resorption during a low Ca diet, and low bone density. Whether these changes in human IH are due to increased intestinal, renal, and bone cell VDR content that can amplify the biological actions of normal circulating $1,25(OH)_2D$ levels remains to be determined.

V. THERAPEUTICS OF IDIOPATHIC HYPERCALCIURIA AND EFFECTS ON CALCIUM METABOLISM

A. Dietary Calcium Restriction

Hypercalciuria promotes urine calcium oxalate supersaturation and increases spontaneous crystal formation [85]. The goals of preventive therapy are to reduce Ca oxalate supersaturation by increasing urine volume and decreasing urine Ca excretion. If the pathophysiological role of $1,25(OH)_2D$ excess or VDR excess is borne out, then ideal therapy may eventually include either a specific $1,25(OH)_2D$ antagonist or an inhibitor of VDR function. In the absence of such agents, therapies will continue to concentrate on lowering urine Ca through indirect means.

Since the description of IH, dietary Ca restriction has been recommended to lower urine Ca. Dietary Ca restriction or the use of Ca-binding resin to prevent absorption [86] could be efficacious for patients with primary intestinal Ca hyperabsorption (absorptive hypercalciuria). However, it appears that many patients develop negative Ca balance during low Ca intake. For them, chronic dietary Ca restriction and negative Ca balance would eventually cause bone loss, osteoporosis, and increased fracture risk. Reports of lower bone density in IH patients suggest that Ca restriction may only worsen the existing reduction in bone mass. Therefore, treatment with Ca restriction requires knowledge that the patient will normally conserve urine Ca and not develop negative Ca balance.

B. Thiazides

Thiazide and the related chlorthalidone diuretics reduce urine Ca excretion by inducing a NaCl diuresis, which causes volume contraction and decreased Ca delivery to the distal tubule segments [87]. These agents also stimulate distal tubule Ca reabsorption through a direct interaction with the tubule cells [86–88]. Thiazides may decrease or have no effect [31,35,89] on intestinal Ca transport in IH patients, and serum $1,25(OH)_2D$ and PTH levels are not changed by thiazide. In one study, IH patients treated with chlorthalidone for six months improved Ca balance to or toward positive by decreasing both urine Ca and intestinal Ca absorption [90], with urine Ca declining to a greater extent than intestinal absorption. The epidemiological studies suggesting that chronic thiazide therapy reduces fracture risk [91,92] may result from drug-induced improvement in Ca balance [89] and reduced bone turnover and improved mineralization [59].

The effects of thiazide on urine Ca and bone metabolism are accompanied by a decrease in new Ca stone formation compared to placebo controls [93]. The beneficial effect of thiazide is evident during the second and third year of therapy, when stone recurrence is reduced by about 50 to 80%. The reduction in new stone formation is due to a decrease in urine Ca oxalate supersaturation, as urine Ca declines while oxalate is unchanged. As thiazides can reduce urine Ca excretion and stone formation rates in all forms of IH [94], knowledge of the pathogenesis of IH in each patient may not be required prior to selecting thiazide therapy.

VI. RISK OF STONE FORMATION USING VITAMIN D ANALOGS

A growing research interest in the cell differentiation and immune modulator effects of vitamin D and analogs may result in their use in a variety of disorders [95–97] (also see chapters in Sections VIII and IX). However, the development of hypercalciuria and hypercalcemia may limit the use of the naturally occurring vitamin D metabolites, as well as synthetic analogs [98,99]. While some vitamin D analogs are reported to have little or no hypercalcemic action, hypercalcemia and hypercalciuria may appear at higher doses through

the classic vitamin D actions on intestine, kidney, and bone [97,98]. Low Ca diets have had only modest beneficial effects to limit hypercalciuria and hypercalcemia and could promote bone loss. It remains to be determined whether the newer vitamin D analogs with less calcemic activity will, in practice, cause less calciuria and a lower risk of kidney stone formation.

Until such actions of the vitamin D analogs are known, standard approaches to minimize stone formation should be followed. These include (1) assuring sufficient fluid intake to maintain at least 1.5 liters urine output per day; (2) if necessary, increasing urine citrate excretion to normal in those with low citrate [85]; and (3) discontinuing or reducing treatment if significant hypercalciuria develops. The addition of a thiazide may avoid or minimize hypercalciuria, but hypercalcemia may occur because of thiazide-induced Ca retention.

VII. SUMMARY

Idiopathic hypercalciuria is the most common cause of Ca oxalate kidney stone formation, and is the most common cause of $1,25(OH)_2D_3$ excess. Several models of hypercalciuria incorporate intestinal Ca hyperabsorption, increased bone resorption, and decreased renal tubule Ca reabsorption, all of which can be accounted for by the elevated serum $1,25(OH)_2D_3$ found in about 50% of patients. There is evidence for a defect in the regulation of the 1-hydroxylase, but the nature of the dysregulation remains unknown. In those patients with normal serum $1,25(OH)_2D_3$ levels, the possibility of vitamin D receptor excess as found in GHS rats, offers a testable hypothesis.

References

1. Flocks RH 1939 Calcium and phosphorus excretion in the urine of patients with renal or ureteral calculi. JAMA **13**:1466–1471.
2. Albright F, Henneman P, Benedict PH, Forbes HR 1953 Idiopathic hypercalciuria. A preliminary report. Proc R Soc Med Lond (Biol) **46**:1077.
3. Henneman PH, Benedict PH, Forbes AP Dudley HR 1958 Idiopathic hypercalciuria. N Engl J Med **259**:802–807.
4. Hodgkinson A, Pyrah LN 1958 The urinary excretion of calcium and inorganic phosphate in 344 patients with calcium stones of renal origin. Br J Surg **46**:10–18.
5. Robertson WG, Morgan DB 1972 The distribution of urinary calcium excretions in normal persons and stone-formers. Clin Chim Acta **37**:503–508.
6. Coe FL, Parks JH, Asplin JR 1992 The pathogenesis and treatment of kidney stones. N Engl J Med **327**:1141–1152.
7. Coe FL 1977 Treated and untreated recurrent calcium nephrolithiasis in patients with idiopathic hypercalciuria, hyperuricosuria, or no metabolic disorder. Ann Intern Med **87**:404–410.
8. McGeown MG 1960 Heredity in renal stone disease. Clin Sci **19**:465–471.
9. Resnick M, Pridgen DB, Goodman HO 1968 Genetic predisposition of calcium oxalate renal calculi. N Engl J Med **278**:1313–1318.
10. Coe FL, Parks JH, Moore ES 1979 Familial idiopathic hypercalciuria. N Engl J Med **300**:337–340.
11. Pak CYC, McGuire J, Peterson R, Britton F, Harrod MJ 1981 Familial absorptive hypercalciuria in a large kindred. J Urol **126**:717–719.
12. Mehes K, Szelid Z 1980 Autosomal dominant inheritance of hypercalciuria. Eur J Pediatr **133**:239–242.
13. Moore ES, Coe FL, McMann BJ, Favus MJ 1978 Idiopathic hypercalciuria in children: Prevalence and metabolic characteristics. J Pediatr **92**:906–910.
14. Coe FL, Favus MJ 1981 Hypercalciuric states. Miner Electrolyte Metab **5**:183–200.
15. Bushinsky DA, Favus MJ 1988 Mechanism of hypercalciuria in genetic hypercalciuric rats. Inherited defect in intestinal calcium transport. J Clin Invest **82**:1585–1591.
16. Li X-Q, Tembe V, Horwitz GM, Bushinsky DA, Favus MJ 1993 Increased intestinal vitamin D receptor in genetic hypercalciuric rats: A cause of intestinal calcium hyperabsorption. J Clin Invest **91**:661–667.
17. Broadus AE, Insogna KL, Lang R, Mallette LE, Oren DA, Gertner JM, Kliger AS, Ellison AF 1984 A consideration of the hormonal basis and phosphate leak hypothesis of absorptive hypercalciuria. J Clin Endocrinol Metab **58**:161–169.
18. Buckalew VM, Purvis ML, Shulman MG, Herndon CN, Rudman D 1974 Hereditary renal tubular acidosis. Medicine **53**:229–254.
19. Frymoyer PA, Scheinman SJ, Dunham PB, Jones DB, Hueber P, Schroeder ET 1991 X-linked recessive nephrolithiasis with renal failure. N Engl J Med **325**:681–686.
20. Low RK, Stoller ML 1997 Endoscopic mapping of renal papillae for Randall's plaques in patients with urinary stone disease. J Urol **158**:2062–2064.
21. Randall A 1937 The origin and growth of renal calculi. Ann Surg **105**:1009–1027.
22. Evan AP, Lingeman JE, Coe FL, Parks JH, Bledsoe SB, Shao Y, Sommer AJ, Paterson RF, Kuo RL, Grynpas M 2003 Randall's plaque of patients with nephrolithiasis begins in basement membranes of thin loops of Henle. J Clin Invest **111**:607–616.
23. Bushinsky DA 2003 Nephrolithiasis: site of the initial solid phase. J Clin Invest **111**:602–605.
24. Klugman VA, Favus MJ 1996 Intestinal absorption of calcium, magnesium, and phosphorus. In: Coe FL, Favus MJ, Pak CYC, Parks JH, Preminger GM (eds) Kidney Stones: Medical and Surgical Management, 1st Ed. Lippincott-Raven: Philadelphia, Pennsylvania, pp. 210–221.
25. Bronner F, Pansu D, Stein WD 1986 An analysis of intestinal calcium transport across the rat intestine. Am J Physiol **250**:G561–G569.
26. Favus MJ 1985 Factors that influence absorption and secretion of calcium in the small intestine and colon. Am J Physiol **248**:G147–G157.
27. Caniggia A, Gennari C, Cesari L 1965 Intestinal absorption of ^{45}Ca in stone-forming patients. Br Med J **1**:427–429.
28. Birge SJ, Peck WA, Berman M, Wheadon GD 1969 Study of calcium absorption in man: A kinetic analysis and physiologic model. J Clin Invest **48**:1705–1713.

29. Wills MR, Zisman E, Wortsman J, Evens RG, Pak CYC, Bartter FC 1970 The measurement of intestinal calcium absorption in nephrolithiasis. Clin Sci **39**:95–106.
30. Pak CYC, East DA, Sanzenbacher LJ, Delea CS, Bartter FC 1972 Gastrointestinal calcium absorption in nephrolithiasis. J Clin Endocrinol Metab **35**:261–270.
31. Ehrig U, Harrison JE, Wilson DR 1974 Effect of long-term thiazide therapy on intestinal calcium absorption in patients with recurrent renal calculi. Metabolism **23**:139–149.
32. Pak CYC, Ohata M, Lawrence EC, Snyder W 1974 The hypercalciurias: Causes, parathyroid functions, and diagnostic criteria. J Clin Invest **54**:387–400.
33. Kaplan RA, Haussler MR, Deftos LJ, Bone H, Pak CYC 1977 The role of 1,25-dihydroxyvitamin D in the mediation of intestinal hyperabsorption of calcium in primary hyperparathyroidism and absorptive hypercalciuria. J Clin Invest **59**:756–760.
34. Shen FH, Baylink DJ, Nielsen RL, Sherrard DJ, Ivey JL, Haussler MR 1977 Increased serum 1,25-dihydroxyvitamin D in idiopathic hypercalciuria. J Lab Clin Med **90**:955–962.
35. Barilla DE, Tolentino R, Kaplan RA, Pak CYC 1978 Selective effects of thiazide on intestinal absorption of calcium in absorptive and renal hypercalciurias. Metabolism **27**:125–131.
36. Lemann J Jr 1992 Pathogenesis of idiopathic hypercalciuria and nephrolithiasis. In: Coe FL, Favus MJ (eds) Disorders of Bone and Mineral Metabolism, 1st Ed. Raven: New York, pp. 685–706.
37. Duncombe VM, Watts RWE, Peters TJ 1984 Studies on intestinal calcium absorption in patients with idiopathic hypercalciuria. Q J Med **209**:69–79.
38. Insogna KL, Broadus AE, Dreyer BE, Ellison AF, Gertner JM 1985 Elevated production rate of 1,25-dihydroxyvitamin D in patients with absorptive hypercalciuria. J Clin Endocrinol Metab **61**:490–495.
39. Bataille P, Bouillon R, Fournier A, Renaud H, Gueris J, Idrissi A 1987 Increased plasma concentrations of total and free $1,25(OH)_2D_3$ in calcium stone formers with idiopathic hypercalciuria. Contrib Nephrol **58**:137–142.
40. Coe FL, Favus MJ, Crockett T, Strauss LM, Parks JH, Porat A, Gantt CL, Sherwood LM 1982 Effects of low-calcium diet on urine calcium excretion, parathyroid function, and serum $1,25(OH)_2D_3$ levels in patients with idiopathic hypercalciuria and in normal subjects. Am J Med **72**:25–32.
41. Broadus AE, Insogna KL, Lang R, Ellison AF, Dreyer BE 1984 Evidence for disordered control of 1,25-dihydroxyvitamin D production in absorptive hypercalciuria. N Engl J Med **311**:73–80.
42. Gray RW, Wilz DR, Caldas AE, Lemann J Jr 1977 The importance of phosphate in regulating plasma $l,25(OH)_2$ vitamin D levels in humans: Studies in healthy subjects, in calcium stone formers, and in patients with primary hyperparathyroidism. J Clin Endocrinol Metab **45**:299–306.
43. Reed BY, Gitomer WL, Heller HJ, Hsu MC, Lemke M, Padalino P, Pak CYC 2002 Identification and characterization of a gene with base substitutions associated with the absorptive hypercalciuria phenotype and low spinal bone density. J Clin Endocrinol Metab **87**:1476–1485.
44. Econs MJ, Foroud T 2002 Editorial: The genetics of absorptive hypercalciuria—A note of caution. J Clin Endocrinol Metab **87**:1473–1475.
45. Edwards NA, Hodgkinson A 1965 Metabolic studies in patients with idiopathic hypercalciuria. Clin Sci **29**:143–157.
46. Peacock M, Nordin BEC 1968 Tubular reabsorption of calcium in normal and hypercalciuric subjects. J Clin Pathol **21**:353–358.
47. Sutton RAL, Walker VR 1980 Responses to hydrochlorothiazide and acetazolamide in patients with calcium stones. N Engl J Med **302**:709–713.
48. Bianchi G, Vezzoli G, Cusi D, Cova T, Elli A, Soldati L, Tripodi G, Surian M, Ottaviano E, Rigatti P 1988 Abnormal red-cell calcium pump in patients with idiopathic hypercalciuria. N Engl J Med **319**:897–901.
49. Alhava EM, Juuti M, Karjalainen P 1976 Bone mineral density in patients with urolithiasis. Scand J Urol Nephrol **10**:154–156.
50. Lawoyin S, Sismilich S, Browne R, Pak CYC 1979 Bone mineral content in patients with calcium urolithiasis. Metabolism **28**:1250–1254.
51. Borgi L, Meschi T, Guerra A, Maninetti L, Pedrazzoni M, Macato A, Vescovi P, Novarini A 1991 Vertebral mineral content in diet-dependent and diet-independent hypercalciuria. J Urol **146**:1334–1338.
52. Bataille P, Achard JM, Fournier A, Boudailliez B, Westeel PF, Laval Jeantet MAL, Bouillon R, Sebert JL 1991 Diet, vitamin D, and vertebral mineral density in hypercalciuric calcium stone formers. Kidney Int **39**:1193–1205.
53. Pietschmann F, Breslau NA, Pak CYC 1992 Reduced vertebral bone density in hypercalciuric nephrolithiasis. J Bone Miner Res **7**:1383–1388.
54. Barkin J, Wilson DR, Manuel MA, Arnold B, Murray T, Harrison J 1985 Bone mineral content in idiopathic calcium nephrolithiasis. Miner Electrolyte Metab **11**:19–24.
55. Liberman UA, Sperling O, Atsmon A, Frank M, Modan M, de-Vries A 1968 Metabolic and calcium kinetic studies in idiopathic hypercalciuria. J Clin Invest **47**:2580–2590.
56. Malluche HH, Tschoepe W, Ritz E, Meyer-Sabelle W, Massry SG 1980 Abnormal bone histology in idiopathic hypercalciuria. J Clin Endocrinol Metab **50**:654–658.
57. Steiniche T, Mosekilde L, Christensen MS, Melsen F 1989 A histomorphometric determination of iliac bone remodeling in patients with recurrent renal stone formation and idiopathic hypercalciuria. APMIS **97**:309–316.
58. Sutton RAL, Walker VR 1986 Bone resorption and hypercalciuria in calcium stone formers. Metabolism **35**:465–488.
59. Urivetzky M, Anna PS, Smith AD 1988 Plasma osteocalcin levels in stone disease. A potential aid in the differential diagnosis of calcium nephrolithiasis. J Urol **139**:12–14.
60. Asplin JR, Bauer KA, Kinder J, Muller G, Coe BJ, Parks JH, Coe FL 2003 Bone mineral density and urine calcium excretion among subjects with and without nephrolithiasis. Kidney Internat **63**:662–669.
61. Melton LJ III, Crowson CS, Khosla S, Wilson DM, O'Fallon WM 1998 Fracture risk among patients with urolithiasis: A population-based cohort study. Kidney Int **53**: 459–464.
62. Pak CYC, Kaplan R, Bone H, Townsend J, Waters O 1975 A simple test for the diagnosis of absorptive, resorptive, and renal hypercalciurias. N Engl J Med **292**:497–500.
63. Van Den Berg CJ, Kumar R, Wilson DM, Heath III H, Smith LH 1980 Orthophosphate therapy decreases urinary calcium excretion and serum 1,25-dihydroxyvitamin D concentrations in idiopathic hypercalciuria. J Clin Endocrinol Metab **51**:998–1001.
64. Breslau NA, Preminger GM, Adams BV, Otey J, Pak CYC 1992 Use of ketoconazole to probe the pathogenetic importance of 1,25-dihydroxyvitamin D in absorptive hypercalciuria. J Clin Endocrinol Metab **75**:1446–1452.
65. Pak CYC, Britton F, Peterson R, Ward D, Northcutt C, Breslau NA, McGuire J, Sakahee K, Bush S, Nicar M, Norman DA,

Peters P 1980 Ambulatory evaluation of nephrolithiasis: Classification, clinical presentation and diagnostic criteria. Am J Med **69**:19–30.
66. Knapp EL 1943 Studies on the urinary excretion of calcium. Ph.D. Thesis, Department of Chemistry, State University of Iowa, Ames.
67. Lafferty FW, Pearson OH 1963 Skeletal, intestinal, and renal calcium dynamics in hyperparathyroidism. J Clin Endocrinol Metab **23**:891–902.
68. Nassim JR, Higgins BA 1965 Control of idiopathic hypercalciuria. Br Med J **1**:675–681.
69. Jackson WPU, Dancaster C 1959 A consideration of the hypercalciuria in sarcoidosis, idiopathic hypercalciuria, and that produced by vitamin D. A new suggestion regarding calcium metabolism. J Clin Endocrinol Metab **19**:658–680.
70. Harrison AR 1959 Some results of metabolic investigations in cases of renal stone. Br J Urol **31**:398.
71. Dent CE, Harper CM, Parfitt AM 1964 The effect of cellulose phosphate on calcium metabolism in patients with hypercalciuria. Clin Sci **27**:417–425.
72. Parfitt AM, Higgins BA, Nassim JR, Collins JA, Hilb A 1964 Metabolic studies in patients with hypercalciuria. Clin Sci **27**:463–482.
73. Coe FL, Parks JH 1988 Nephrolithiasis: Pathogenesis and Treatment, 2nd Ed. Year Book Publishers: Chicago, p. 113.
74. Maierhofer WJ, Lemann J Jr, Gray RW, Cheung HS 1984 Dietary calcium and serum 1,25-$(OH)_2$ vitamin D concentrations as determinants of calcium balance in healthy men. Kidney Int **26**:752–759.
75. Adams ND, Gray RW, Lemann J Jr 1979 The effects of oral $CaCO_3$ loading and dietary calcium deprivation on plasma 1,25-dihydroxyvitamin D concentration in healthy adults. J Clin Endocrinol Metab **48**:1008–1016.
76. Adams ND, Gray RW, Lemann J Jr, Cheung HS 1982 Effects of calcitriol administration on calcium metabolism in healthy men. Kidney Int **21**:90–97.
77. Bashir MA, Nakagawa Y, Riordon D, Coe FL, Bushinsky DA 1995 Increased dietary oxalate does not increase urinary calcium oxalate oversaturation in hypercalciuric rats. J Am Soc Nephrol **6**:943 (abstract).
78. Bushinsky DA, Nilsson EL, Nakagawa Y, Coe FL 1995 Stone formation in genetic hypercalciuric rats. Kidney Int **48**:1705–1713.
79. Costa EM, Hirst MA, Feldman D 1985 Regulation of 1,25-dihydroxyvitamin D_3 receptor by vitamin D analogs in cultured mammalian cells. Endocrinology **117**:2203–2210.
80. Pols HAP, Birkenhager JC, Schlite JP, Visser TJ 1988 Evidence that self-induced metabolism of 1,25-dihydroxyvitamin D_3 limits the homologous up-regulation of its receptor in rat osteosarcoma cells. Biochim Biophys Acta **970**:122–129.
81. Reinhardt TA, Horst RL 1989 Self-induction of 1,25-dihydroxyvitamin D_3 metabolism limits receptor occupancy and target tissue responsiveness. J Biol Chem **264**:15917–15921.
82. Yao J, Kathpalia P, Bushinsky DA, Favus MJ 1998 Hyperresponsiveness of vitamin D receptor gene expression to 1,25-dihydroxyvitamin D_3. A new characteristic of genetic hypercalciuric stone-forming rats. J Clin Invest **101**:2223–2232.
83. Krieger NS, Stathopoulos VM, Bushinsky DA 1996 Increased sensitivity to 1,25$(OH)_2D_3$ in bone from genetic hypercalciuric rats. Am J Physiol **271**:C130–C135.
84. Kirn M, Sessler NE, Tembe V, Favus MJ, Bushinsky DA 1993 Response of genetic hypercalciuric rats to a low calcium diet. Kidney Int **43**:189–196.
85. Parks JH, Coe FL 1996 Pathogenesis and treatment of calcium stones. Semin Nephrol **16**:398–411.
86. Wilson DR, Strauss AL, Manuel MA 1984 Comparison of medical treatments for the prevention of recurrent calcium nephrolithiasis. Urol Res **12**:39–40.
87. Edwards BR, Baer PG, Sutton RA, Dirks JH 1973 Micropuncture study of diuretic effects on sodium and calcium reabsorption in the dog nephron. J Clin Invest **52**:2418–2427.
88. Costanzo LS, Windhager EE 1978 Calcium and sodium transport by the distal convoluted tubule of the rat. Am J Physiol **235**:F492–F506.
89. Zerwekh JE, Pak CYC 1980 Selective effects of thiazide therapy on serum l-alpha,25-dihydroxyvitamin D and intestinal calcium absorption in renal and absorptive hypercalciurias. Metabolism **29**:13–17.
90. Coe FL, Parks JP, Bushinsky DA, Langman CB, Favus MJ 1988 Chlorthalidone promotes mineral retention in patients with idiopathic hypercalciuria. Kidney Int **33**:1140–1146.
91. Wasnich RD, Benfante RJ, Yano K, Heilbrun L, Vogel JM 1983 Thiazide effect on the mineral content of bone. N Engl J Med **309**:344–347.
92. LaCroix AZ, Wienpahl J, White LR, Wallace RB, Scherr PA, George LK 1990 Thiazide diuretic agents and the incidence of hip fracture. N Engl J Med **322**:286–290.
93. Asplin JR, Favus MJ, Coe FL 1996 Nephrolithiasis. In: Brenner BR (ed) The Kidney, 5th Ed. Saunders: Philadelphia, Pennsylvania, pp. 1893–1935.
94. Ohkawa M, Tokunga S, Nakashima T, Orito M, Hisazumi H 1992 Thiazide treatment for calcium nephrolithiasis in patients with idiopathic hypercalciuria. Br J Urol **69**:571–576.
95. Cheskis B, Lemon BD, Uskokovic M, Lomedico PT, Freedman LP 1995 Vitamin D_3 retinoid X receptor dimerization, DNA binding, and transactivation are differentially affected by analogs of 1,25-dihydroxyvitamin D_3. Mol Endocrinol **9**:1814–1824.
96. Skowronski RJ, Peehl DM, Feldman D 1995 Actions of vitamin D_3, analogs on human prostate cancer cell lines: Comparison with 1,25-dihydroxyvitamin D_3. Endocrinology **136**:20–26.
97. Fleet JC, Bradley J, Reddy GS, Ray R, Wood RJ 1996 1 alpha,25-$(OH)_2$ vitamin D analogs with minimal *in vivo* calcemic activity can stimulate significant transepithelial calcium transport and mRNA expression *in vitro*. Arch Biochem Biophys **329**:228–234.
98. Naveh-Many T, Silver J 1993 Effects of calcitriol, 22-oxacalcitriol, and calcipotriol on serum calcium and parathyroid hormone gene expression. Endocrinology **133**:2724–2728.
99. Brown AJ, Finch J, Grieff M, Ritter C, Kubodera N, Nishii Y, Slatopolsky E 1993 The mechanism for the disparate actions of calcitriol and 22-oxacaltriol in the intestine. Endocrinology **133**:1158–1164.

CHAPTER 78

Hypercalcemia Due to Vitamin D Toxicity

MISHAELA R. RUBIN AND SUSAN THYS-JACOBS
Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York

FREDRIECH K. W. CHAN
Department of Medicine, Queen Elizabeth Hospital, Hong Kong

LILIA M. C. KOBERLE
Health Sciences Department, Federal University, Sao Carlos, Brazil

JOHN P. BILEZIKIAN
Department of Medicine and Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, New York

I. INTRODUCTION

Vitamin D toxicity is not a common cause of hypercalcemia. In the differential diagnosis of hypercalcemia, it is often buried amid a long list of other more and less common causes (Table I). Among the more common causes, primary hyperparathyroidism and hypercalcemia of malignancy are the principal etiologies. Together, primary hyperparathyroidism and hypercalcemia of malignancy constitute the overwhelming majority of causes of hypercalcemia. They are in fact so common that the practical issue in the diagnosis of a hypercalcemic individual is to distinguish between these two etiologies first and not to consider other etiologies until these two have been ruled out.

Patients with primary hyperparathyroidism tend to be asymptomatic, whereas patients with hypercalcemia of malignancy tend to be ill. The diagnosis of primary hyperparathyroidism is established by an elevated concentration of parathyroid hormone (PTH), an association that is made in over 90% of patients with primary hyperparathyroidism. In contrast, patients with hypercalcemia of malignancy, including those whose hypercalcemia is due to the elaboration of parathyroid hormone-related protein (PTHrP), show levels of PTH that are typically suppressed. If the PTH level is suppressed, the diagnosis of primary hyperparathyroidism is ruled out. The diagnosis of malignancy, however, is not necessarily ruled in. Certainly, if a malignancy is detected that is classically associated with hypercalcemia, such as squamous cell carcinoma of the lung, the etiology becomes clear. However, the longer list of other causes of hypercalcemia is also associated, with rare exceptions, with reduced levels of PTH. This situation, namely, elevated serum calcium concentration with reduced or undetectable levels of PTH, is seen in the various forms of vitamin D toxicity. If primary hyperparathyroidism is ruled out and malignancy is not apparent, the likelihood of vitamin D toxicity looms as an important possible etiology of hypercalcemia. In that long list of other causes, vitamin D toxicity now becomes a major diagnostic consideration (Table I). This chapter reviews the various forms of vitamin D toxicity, mechanisms of hypercalcemia due to vitamin D toxicity, clinical manifestations, diagnosis, and management.

II. FORMS OF EXOGENOUS VITAMIN D TOXICITY

Vitamin D toxicity can be life threatening and associated with high morbidity, if not identified quickly. Hypervitaminosis D with hypercalcemia may be secondary to excessive intake of parent vitamin D, its metabolites 25-hydroxyvitamin D (25OHD), 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], or vitamin D

VITAMIN D, 2ND EDITION
FELDMAN, PIKE, AND GLORIEUX

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TABLE I Differential Diagnosis of Hypercalcemia

Primary hyperparathyroidism
Sporadic (adenoma, hyperplasia, or carcinoma)
Familial
Isolated
Cystic
Multiple endocrine neoplasia type I or II
Malignancy
Parathyroid hormone-related protein
Excess production of 1,25-dihydroxyvitamin D
Other factors (cytokines, growth factors)
Disorders of vitamin D
Exogenous vitamin D toxicity—parent D compound, 25OHD,1,25$(OH)_2$D
Endogenous production of 25-hydroxyvitamin D (Williams syndrome)
Endogenous production of 1,25-dihydroxyvitamin D
Granulomatous diseases
a. Sarcoidosis
b. Tuberculosis
c. Histoplasmosis
d. Coccidioidomycosis
e. Leprosy
f. Others
Lymphoma
Nonparathyroid endocrine disorders
Thyrotoxicosis
Pheochromocytoma
Acute adrenal insufficiency
Vasoactive intestinal polypeptide hormone-producing tumor (VIPoma)
Medications
Thiazide diuretics
Lithium
Estrogens/antiestrogens, testosterone in breast cancer
Milk-alkali syndrome
Vitamin A toxicity
Familial hypocalciuric hypercalcemia
Immobilization
Parenteral nutrition
Aluminum excess
Acute and chronic renal disease

analogs; to increased production of 25OHD or 1,25$(OH)_2$D from exogenous substrate; and even to topical applications of potent vitamin D analogs.

A. Vitamin D and 25-Hydroxyvitamin D Toxicity

The most common etiology of vitamin D toxicity is inadvertent or improper oral use of pharmaceutical preparations. Excessive ingestion of vitamin D (usually greater than 10,000 IU daily) can cause vitamin D intoxication that is recognized by markedly elevated levels of 25OHD (usually >150 ng/ml) in association with levels of 1,25$(OH)_2$D that are only slightly elevated. Hyperphosphatemia typically accompanies the hypercalcemia [1–3]. The hyperphosphatemia can be a clue to the etiology of the hypercalcemia as due to vitamin D toxicity. The usual setting of vitamin D toxicity is in its use as a therapy for the hypophosphatemic disorders: hypoparathyroidism, pseudohypoparathyroidism, osteomalacia, renal failure, or osteoporosis. Ingestion of excessive quantities of 25OHD, 1-alpha-hydroxyvitamin D, 1,25$(OH)_2$D, dihydrotachysterol, or exuberant use of the topical calcipotriene (Dovonex) for psoriasis can cause vitamin D intoxication [4]. Health conscious adults have been reported to ingest large doses of megavitamins from over the counter supplements, in amounts that may exceed 2 million IU of vitamin D daily [5]. Cancer patients, in particular, have been observed to consume excess nutritional supplements such as calcium, vitamin D, and shark cartilage [6]. Excessive sunlight exposure can raise serum concentrations of 25OHD to as high as 79 ng/ml (normal range 9–52 ng/ml), but there is no evidence that sunlight exposure alone can result in vitamin D toxicity and hypercalcemia in normal individuals [7]. Hypercalcemia associated with granulomatous diseases, such as sarcoidosis, can be worsened by excessive sunlight exposure.

Natural foods, in general, other than fatty fish, eggs, milk, and liver do not contain much vitamin D. Hypervitaminosis D has been associated with drinking milk when erroneously fortified with massive concentrations of vitamin D. One investigation of eight patients manifesting symptoms of nausea, vomiting, weight loss, hyperirritability, or failure-to-thrive revealed markedly elevated mean concentrations of 25OHD of 293 ± 174 ng/ml (nl: 9–52 ng/ml) [3]. Analysis of the milk production facility at the local dairy revealed excessive vitamin D fortification of milk with up to 245,840 IU per liter (232,565 IU of vitamin D_3 per quart). Usual fortification of milk in the United States is 400 IU per quart. Milk is not fortified with vitamin D in other parts of the world. Generally, milk is the only dairy product that is fortified with vitamin D in the United States. In addition to milk, vitamin D fortification of natural foods includes certain breakfast cereals, pasta, baked goods, fats, and recently orange juice oils [8]. There is no documentation that excessive ingestion of any of these other fortified foods has ever resulted in vitamin D toxicity. However, industrial contamination of table sugar with vitamin D_3 and consequent severe vitamin D toxicity (25OHD 1555 nmol/L, nl: 20–80 nmol/l) has been reported [9].

Vitamin D_2 and vitamin D_3, although used interchangeably in the treatment of metabolic bone diseases, may differ in toxic potential at higher doses. In general, vitamin D_3 appears to be somewhat more toxic than D_2. Investigations in rats, sheep, pigs, horses, and primates support differences in metabolic clearance

rates and in toxicity between the two vitamin D compounds [10]. In horses, vitamin D_2 has a lower toxicity compared to vitamin D_3 [11]. Massive doses of vitamin D_3 administered to Old World primates can cause toxicity and death, whereas equivalent doses of vitamin D_2 are better tolerated [12]. In human subjects, it has been shown that vitamin D_3 increases 25OHD levels 1.7 times more than the equivalent dose of vitamin D_2 [13].

The current, officially recommended dietary allowance (RDA) for vitamin D is 400 IU per day, but many authoritative bodies are calling for increases in the requirements to 600 units in individuals over the age of 70 [14]. Although this chapter concerns itself with vitamin D toxicity, the reason for the trend to increase recommendations for vitamin D intake is the large numbers of free-living adults who are being shown worldwide to have vitamin D deficiency (see Chapters 61–62) [15,16].

The smallest dose of parent vitamin D in adults that can produce toxicity and hypercalcemia is not known, but is clearly much higher than the RDA [17]. The threshold for vitamin D toxicity was evaluated in a study in which 61 subjects were randomized to 1000 or 4000 IU vitamin D_3 daily for 2–5 months. Increases in 25OHD were greater in the higher dose than lower dose group (96.4 ± 14.6 vs. 68.7 ± 16.9 nmol/L), but remained within the physiologic range, leading the authors to conclude that as much as 4000 IU daily was a safe dose [18].

On the other hand, in infants, daily dosages of 2000 IU or less have been associated with hypercalcemia and nephrocalcinosis [19]. Intermittent oral dosages of 15 mg or 600,000 IU to infants to prevent vitamin deficiency have been shown to be excessive during the first year of life, resulting in transient hypercalcemia and vitamin D overload [20,21,22]. Lower amounts of 5 mg (200,000 IU) every 6 months or 2.5 mg (100,000 IU) every 3 months appear to be safer and to provide better protection in high risk infants. In adults, doses of greater than 40,000–60,000 IU per day, as commonly used in the treatment of hypoparathyroidism, can be associated with significant toxicity.

Individuals manifest wide variations both in their response to hypercalcemic doses of vitamin D and in the duration of the effect. This variation in individual responsiveness might reflect differences in intestinal absorption and vitamin D metabolism, in the concentration of free vitamin D metabolites, in the rate of degradation of the metabolites and conversion to inactive metabolites, and in the capacity of storage sites for 25OHD [17]. Factors that enhance susceptibility to vitamin D toxicity and hypercalcemia include increased dietary calcium intake, reduced renal function, co-administration of vitamin A, and granulomatous disorders such as sarcoidosis that render subjects more sensitive to vitamin D (see Chapter 79) [2]. Hypercalciuria in hypervitaminosis D usually presents much earlier than hypercalcemia, but it is easily missed for the obvious reason that hypercalciuria is not routinely measured in the absence of renal symptomatology.

B. 1,25-Dihydroxyvitamin D Toxicity

The greater potency of $1,25(OH)_2D_3$ and its direct actions on target tissues have resulted in its increased use for a variety of metabolic bone diseases [23]. Its ability to inhibit PTH synthesis and secretion has also made $1,25(OH)_2D_3$ and its analogs useful agents in patients with renal osteodystrophy and secondary hyperparathyroidism. Most recently, $1,25(OH)_2D_3$ has been found to inhibit the growth of human cancer cells in vitro [24] (see below, and Section VIII of this book). As $1,25(OH)_2D_3$ is increasingly recognized for its antiproliferative, prodifferentiating, and immunomodulatory actions, its potential therapeutic use is expanding [25–27]. Thus, considerable attention has focused on possible toxic effects of $1,25(OH)_2D_3$ not usually associated with the parent vitamin D compound. The incidence of hypercalcemia and hypercalciuria with $1,25(OH)_2D_3$ use has been reported as very high, with one review citing complications in two-thirds of treated patients [28]. The mechanisms of the hypercalcemia are increased intestinal absorption and potentiation of osteoclastic activity. Dosages of $1,25(OH)_2D_3$ above 0.75 μg/day have been associated with toxicity, whereas dosages at or below 0.5 μg/day rarely result in toxicity. One investigation showed that over 90% of patients on doses of $1,25(OH)_2D_3$ between 1.0 and 2.0 μg/day became hypercalcemic, and all had hypercalciuria when calcium intake was set at 1000 mg per day [29]. Accelerated deterioration of renal function was recorded in a number of reports in patients with renal insufficiency receiving $1,25(OH)_2D_3$ therapy [30]. Compared to oral therapy, intravenous administration of $1,25(OH)_2D_3$ to renal dialysis patients induces hypercalcemia less frequently, with a smaller increment in the serum calcium concentration and a more effective reduction of PTH levels [31]. Other studies, however, suggest that intermittent oral pulse administration of $1,25(OH)_2D_3$ may be effective, though not as effective as intravenous $1,25(OH)_2D_3$, in suppressing PTH in uremic patients with secondary hyperparathyroidism [32–34] (see Chapter 76 for further discussion).

C. Toxicity Due to Synthetic Analogs

In one investigation, oral pulse therapy with 1α-hydroxyvitamin D_3 ($1\alpha OHD_3$) resulted in a rapid control of secondary hyperparathyroidism without causing hypercalcemia or hyperphosphatemia [35].

However, 1α-OHD_3 may harbor potential calcemic effects similar to 1,25$(OH)_2D_3$ in the treatment of renal osteodystrophy. Crocker *et al*. [36] investigated the comparative toxicity of vitamin D, 1α-OHD_3, and 1,25$(OH)_2D_3$ in weanling male mice at three different doses over a four-week period. 1α-OHD_3 appeared to be more toxic in the high dose group only, with significantly higher serum calcium levels, higher urinary calcium excretion, and severe nephrocalcinosis [36]. 1α-OHD_3 has been described as less potent than 1,25$(OH)_2D_3$ at low doses but equipotent at doses greater than 2.0 μg/day. At the higher doses, there is a delayed onset of action and a prolonged half-life, suggesting a potential for cumulative toxicity in renal insufficiency [37,38].

The potential for hypercalcemia, hypercalciuria, and soft tissue calcifications limits the clinical usefulness of 1α-OHD_3. Mortensen and colleagues compared the toxicity of both 1α-OHD_3 and 1,25$(OH)_2D_3$ in rats fed standard or low calcium diets. High doses of either compound resulted in severe hypercalcemia, with retarded growth, nephrosis, and structural bone changes in the rats fed the standard diet. On the low calcium diet, however, slight hypercalcemia occurred, but without growth retardation or bone changes. There was minimal effect on the kidney. Calcium restriction again proved effective in protecting the animals against the toxic effects of the vitamin D analogs. Animals fed the low calcium diet tolerated 1α-OHD_3 at dose levels up to 10 times higher than rats on the standard diets [39]. In human subjects, 1α-OHD_3 causes toxic effects at doses above 1.0 μg/day, but doses of 0.5 to 1.0 μg/day appear to be safe.

Because of the relatively narrow therapeutic window of vitamin D_3 compounds, a synthetic analog of vitamin D_2, 1α-OHD_2 (doxercalciferol) was developed with the concept that the window of therapeutic efficacy to toxicity would be wider. In postmenopausal osteopenic women, doses of doxercalciferol ranging from 1.0 to 5.0 μg/day were administered to 15 subjects. There was no evidence of vitamin D toxicity manifesting as either hypercalciuria or hypercalcemia, whereas significant therapeutic effects on osteoblastic activity were demonstrated [40]. Similar to the 1α-OHD_3, doxercalciferol requires obligatory hepatic 25-hydroxylation for activation. However, doxercalciferol is able to activate its catabolic pathway via hepatic 24-hydroxylation with a lower potential for toxicity [41]. These investigations on synthetic analogs seem to confirm earlier reports that vitamin D_2 compounds, in general, are as efficacious and somewhat better tolerated than D_3 compounds.

Because of our understanding of the nonclassic target tissue effects of vitamin D in the modulation of hormones and cytokines, and in the regulation of cellular differentiation and proliferation, newer clinical uses have been developed (see Section IX of this book). The clinical applications of these newer properties of vitamin D, however, have also been tempered by the potential for complications, such as hypercalcemia and hypercalciuria, prompting the development of other analogs to distinguish even better calcemic from antiproliferative effects [42] (see also Section VIII of this book). Depending on the chemical modification of the basic structure of vitamin D, some analogs do demonstrate reduced calcemic activity, but others have been developed with increased calcemic activity owing to enhanced intestinal calcium absorption and bone mineral mobilization. Fluorination of C-24, C-26, or C-27 apparently results in markedly increased calcemic activity resulting from reduced enzymatic degradation of the side chain. Calcemic potency of 1,25$(OH)_2D_3$ and its analogs can be also enhanced at least two- to fivefold by epimerization at the C-20 site [43].

The vitamin D analogs in use for secondary hyperparathyroidism in the United States include doxercalciferol, 22-oxacalcitriol (1,25-dihydroxy-22-oxavitamin D_3) and paricalcitol (19-nor-1,25-dihydroxyvitamin D_2). Each analog retains suppressive action on PTH and parathyroid gland growth, but has less calcemic and phosphatemic activity than calcitriol. It is unclear how the analogs compare to each other, although in rats, paricalcitol is less calcemic and phosphatemic than doxercalciferol [44]. Overall, the effect of vitamin D analogs to minimize the calcium-phosphate product might reduce vascular calcification [45] and mortality in the renal failure population [46]. Of additional potential importance is the decreased likelihood of low bone turnover, or adynamic bone disease, with the use of these agents [47–48]. The mechanism for the differential actions of vitamin D analogs is not completely understood. Oxacalcitriol, for example, has a low affinity for vitamin D–binding protein, so more of the drug circulates in the free form, allowing it to be more rapidly metabolized than calcitriol [49]. This leads to a shorter half-life, which could explain the small and transient stimulation of intestinal calcium absorption. It does not, however, seem to account for the prolonged inhibition of PTH release (see Chapter 86).

Other vitamin D analogs, such as topical calcipotriol (MC903), have proved very effective in the treatment of psoriasis (see Chapter 101). Because of its low absorption rate and rapid degradation, calcipotriol is believed to have negligible effects on systemic calcium homeostasis when administered topically. However, isolated cases of hypercalcemia and hypercalciuria have been reported, even in patients taking recommended doses [50]. In one investigation, Bourke and colleagues noted suppression of serum PTH concentrations in all patients within two weeks of treatment with calcipotriol. Mean serum and urine calcium

levels increased during treatment and fell following withdrawal [51]. The authors concluded that although this particular synthetic analog alters serum and urinary calcium with a dose-dependent effect on systemic calcium homeostasis, it is well tolerated and effective for mild to moderate chronic plaque psoriasis. However, it is potentially hazardous in extensive, unstable, exfoliative disease [52].

III. FORMS OF ENDOGENOUS VITAMIN D TOXICITY

A. Endogenous Production of 25-Hydroxyvitamin D

Hypervitaminosis D with hypercalcemia is rarely due to endogenous dysregulation of vitamin D metabolites as seen in Williams syndrome [53]. Williams syndrome, an idiopathic infantile form of hypercalcemia, is associated with late psychomotor development, selective mental deficiency, and supravalvular aortic stenosis [54]. The hypercalcemia has been reported to range widely from 12 to 19 mg/dl but usually subsides by 4 years of age. One report suggests an exaggerated production of 25OHD with small doses of vitamin D as a possible etiology of the hypervitaminosis D [53].

B. Production of 1,25-Dihydroxyvitamin D

1. Granulomatous Diseases

In contrast to the megadosages of vitamin D that are usually required to produce vitamin D toxicity, patients with granulomatous diseases can develop hypercalcemia rather easily without excessive intake of exogenous vitamin D. They are said to be hypersensitive to vitamin D. The etiology of the vitamin D toxicity in this syndrome is due to poorly regulated extrarenal synthesis of $1,25(OH)_2D$ by the granulomatous tissue itself (as described in detail in Chapter 79). In contrast to the various presentations of vitamin D toxicity described earlier, the responsible metabolite in granulomatous disease is quite different. In the case of vitamin D toxicity due to overdosage of vitamin D or 25OHD, 25OHD is the active metabolite; renal production of $1,25(OH)_2D$ in this setting is highly regulated and not excessively high. In granulomatous tissue, however, $1,25(OH)_2D$ formation is not subject to control by any recognized regulators, such as PTH, phosphorus, or calcium. Thus, this syndrome is due to ectopic production of $1,25(OH)_2D$ by the granulomatous tissue itself. The mechanisms by which hypercalcemia occurs, however, are similar to all other vitamin D toxic states, namely, increased intestinal calcium absorption and enhanced osteoclastic bone resorption [55,56]. Many studies have led to greater understanding of the pathophysiology and immunological features associated with this syndrome.

a. Sarcoidosis Abnormalities in calcium metabolism have long been noted in patients with sarcoidosis [57]. Sarcoidosis is also the most common granulomatous disease associated with hypercalcemia. Approximately 10% of patients with sarcoidosis will develop hypercalcemia, and as many as 50% will experience hypercalciuria at some time during the course of the disease [58]. Hypercalciuria is invariably present when patients develop hypercalcemia. In the 1950s, studies had already revealed similarities between hypercalcemia of sarcoidosis and the hypercalcemia of vitamin D toxicity, namely, increased intestinal absorption of calcium, hypercalciuria, and therapeutic efficacy of glucocorticoids [59,60]. The major distinguishing feature was in the amount of vitamin D associated with the hypercalcemia and/or hypercalciuria. Seasonal variation of the serum calcium level in sarcoidosis was correlated with availability of sunlight as a source of vitamin D [61]. In the late 1970s, two independent groups showed that the vitamin D-like principle that appeared to be responsible in sarcoidosis was, in fact, the active metabolite of vitamin D, $1,25(OH)_2D_3$ [56,62].

Ectopic production of $1,25(OH)_2D_3$ was confirmed by demonstrating high circulating concentrations of $1,25(OH)_2D_3$ in anephric patients with sarcoidosis on hemodialysis during hypercalcemic episodes [63,64]. This observation showed unequivocally that the kidney, usually the sole source of $1,25(OH)_2D_3$ in nonpregnant individuals, could not be the source of $1,25(OH)_2D_3$ in these patients. The serum calcium and $1,25(OH)_2D_3$ levels were positively correlated with indices of disease activity [65–67], namely, the extent of granuloma formation and the angiotensin-converting enzyme level. It was subsequently shown that the granulomatous tissue was, in fact, the site of $1,25(OH)_2D_3$ production. The lα-hydroxylase enzyme responsible for formation of $1,25(OH)_2D_3$ was present in lymph node homogenates [68]. Moreover, pulmonary alveolar macrophages [69] could be shown to catalyze the formation of an ^{3}H-labeled $25OHD_3$ metabolite. This metabolite was definitively identified as $1,25(OH)_2D_3$ by high-performance liquid chromatography (HPLC), by the chick intestinal receptor assay for $1,25(OH)_2D_3$, by UV spectroscopy, and by mass spectrometry [70]. The production of mRNA for 1α-hydroxyalse is markedly increased in alveolar macrophages isolated from hypercalcemic patients with sarcoid [71]. Importantly, control of the macrophage 1α-hydroxylase enzyme differs

from that of the renal 1α-hydroxylase. The renal 1α-hydroxylase is regulated at the level of transcription by calciotropic hormones, and is exquisitely autoregulated by $1,25(OH)_2D_3$ itself [72]. In contrast, the macrophage 1α-hydroxylase mRNA expression is potently stimulated by inflammatory agents, such as γ-interferon [73], and shows no feedback control in response to $1,25(OH)_2D_3$ [74]. Communication between signaling pathways of γ-interferon and the vitamin D receptor has recently been reported [75]. These mechanisms account for the uncontrolled synthesis of $1,25(OH)_2D_3$ and the characteristic finding of increased sensitivity to vitamin D in these patients [76], so that patients even without major increases in $1,25(OH)_2D_3$ can become hypercalemic. Conversely, abnormal $1,25(OH)_2D_3$ metabolism has been described in some patients with sarcoidosis who are normocalciuric and normocalcemic [77]. Another property of the macrophage 1α-hydroxylase enzyme is that it is inhibited in a dose-dependent fashion by dexamethasone and chloroquine that do not influence the renal 1α-hydroxylase enzyme that catalyzes synthesis of $1,25(OH)_2D_3$ [78]. These *in vitro* observations have direct clinical relevance.

There are several mechanisms by which calcium metabolism is disturbed in sarcoidosis [79]. First, $1,25(OH)_2D_3$ causes hypercalcemia, in part, by stimulating intestinal calcium absorption. A low calcium diet [80,81], alone or in association with cellulose phosphate [82], was found to normalize the calcium level in some patients with sarcoidosis. Second, $1,25(OH)_2D_3$ directly stimulates osteoclastic-mediated bone resorption; skeletal granulomas are not required for this effect [83–85]. The increased flux of calcium into the extracellular space by these gastrointestinal and skeletal mechanisms, aided by suppression of PTH [62–64], leads to hypercalciuria. Chronic hypercalciuria favors nephrocalcinosis and renal stone formation [86]. When the kidneys are unable to excrete the calcium presented to them, because of either declining renal function, enhanced bone resorption, a sudden influx of dietary calcium, dehydration, or any combination of these events, hypercalcemia ensues [87]. Granulomatous production of PTHrP may also play a role in abnormal calcium metabolism [88], where TNFα and interleukin-6, produced by macrophages, increase PTHrP gene expression. PTHrP was reported in one series to be present in 85% of biopsies of granulomatous tissue from patients with sarcoidosis [88].

b. Tuberculosis Longitudinal studies from the United States [89] and India [90] suggested that 16 to 28% of patients with tuberculosis develop hypercalcemia. However, in these early studies, vitamin D supplements were employed, increasing the risk and severity of hypercalcemia. A similar study from Greece [91] reported a figure as high as 48% when serum calcium was corrected to a normal albumin level. Other studies from the United Kingdom [92], Belgium [93], Hong Kong [94], and Malaysia [95] have shown a much lower prevalence of hypercalcemia, in the range of 0 to 2.3%. It is likely that hypercalcemia is not as common in tuberculosis as was previously thought [96]. This discrepancy might be attributable to regional differences in calcium and vitamin D intake, which can unmask hypercalcemia [97], along with increased sun exposure.

Reports of high circulating levels of $1,25(OH)_2D_3$ in three anephric patients with tuberculosis support an extrarenal source of the active vitamin D metabolite [98,99]. Positive correlation of the albumin-adjusted calcium level with the radiographic extent of the disease has been shown [94]. Hypercalcemia in tuberculosis may occur weeks to months after starting antituberculosis chemotherapy [89,90]. Thus, the hypercalcemia is not related to the presence of viable acid-fast bacilli, but rather to the granulomatous process and associated reactions. As with sarcoidosis, hypercalcemia in tuberculosis can be controlled by administration of glucocorticoids [100].

In patients with tuberculous pleuritis, the mean free $1,25(OH)_2D_3$ concentration in pleural fluid was selectively concentrated by 5.3-fold over that in serum [101]. Positive correlation between the concentrations of substrate ($25OHD_3$) and product [$1,25(OH)_2D_3$] in pleural fluid supported the idea that $1,25(OH)_2D_3$ was produced locally by activated inflammatory cells in or adjacent to the pleural space. The pleural fluid was found to have high concentrations of γ-interferon, a cytokine known to stimulate activated macrophages *in vitro* to synthesize $1,25(OH)_2D_3$ [102]. Cells obtained from bronchoalveolar lavage in patients with tuberculosis were also found to synthesize $1,25(OH)_2D_3$ *in vitro.* An important source of the active vitamin D metabolite appears to be the CD8+T lymphocytes at the granulomatous sites [103]. If one wonders etiologically about the production of $1,25(OH)_2D_3$ under these circumstances, the immunomodulatory functions of $1,25(OH)_2D_3$ acting as a beneficial local paracrine factor could be pertinent (see Chapter 79). Viewed in this context, hypercalcemia occurs when $1,25(OH)_2D_3$ is produced in such quantities that it gains entry into the circulation. Hypercalcemia in tuberculosis is usually mild and asymptomatic. Besides glucocorticoids, ketoconazole administration has been associated with a rapid decline in $1,25(OH)_2D_3$ and normalization of serum calcium levels [104]. Long-term antituberculosis therapy with isoniazid and rifampin can also be effective in treating the hypercalcemia by controlling the disease.

c. Other Granulomatous Diseases Besides the more detailed studies of hypercalcemia in tuberculosis, hypercalcemia has also been reported in other infectious diseases, including leprosy [105], coccidioidomycosis [106], histoplasmosis [107], candidiasis [108], cat-scratch disease [109], and pneumocystis carinii pneumonia [110]. Noninfectious associations, aside from sarcoidosis, have been reported with eosinophilic granuloma [111], berylliosis [112], silicone-induced granuloma [113], paraffin-induced granulomatosis [114], Wegener's granulomatosis [115], Langerhans' cell granulomatosis [116], Crohn's disease [117], and infantile fat necrosis [118]. The mechanism of increased production of the active vitamin D metabolite is believed to be shared by all of these granulomatous disorders. Spontaneous idiopathic excess production of calcitriol in the absence of granulomatous disease has also been reported in patients with elevated angiotensin converting enzyme levels [119], presumably with increased calcitriol production by macrophages. A possible role for $1,25(OH)_2D_3$ in these granulomatous disorders as noted above includes immunomodulatory features, which are discussed in Chapters 36 and 98.

2. LYMPHOMA

Hypercalcemia has been reported to occur in 5% [120] and 15% [121] of patients with Hodgkin's disease and non-Hodgkin's lymphoma (NHL), respectively. Up to 80% of patients with human T-cell leukemia virus type 1 (HTLV-l)-associated adult T-cell lymphoma/leukemia (ATLL) will develop hypercalcemia [122]. As is the case with other malignancies, hypercalcemia is a poor prognostic feature in lymphoma [123], adding substantially to morbidity and mortality. The humoral mediators of hypercalcemia in lymphoma are multiple and heterogeneous. However, evidence has shown $1,25(OH)_2D_3$ to be an important factor in many cases.

Hodgkin's disease is most consistently associated with $1,25(OH)_2D_3$ when hypercalcemia develops. Since the first report of hypercalcemia complicating Hodgkin's disease in 1956 [124], more than 60 cases have been described. In a retrospective review of the literature [125], 84% of patients had a peak serum calcium above 12 mg/dl, 74% of the patients had Ann Arbor stage III or IV disease, and 68% were symptomatic with night sweats, fever, and weight loss. Only 3 of 23 patients had radiological evidence of lytic bone lesions. In 17 hypercalcemic patients, all but one patient had an elevated $1,25(OH)_2D_3$ level. There is no evidence to implicate parathyroid hormone-related peptide (PTHrP) as a mediator of hypercalcemia in Hodgkin's disease. Two patients with Hodgkin's disease [126,127] were reported to have intermittent hypercalcemia during two consecutive summers or on vitamin D challenge. There was a close association between hypercalcemia and the abnormally raised $1,25(OH)_2D_3$ level, but serum $25OHD_3$ was within the normal range. These observations support the idea that the mechanism of the hypercalcemia in Hodgkin's disease is similar to that of the granulomatous diseases, namely, production by the lymphomatous tissue of $1,25(OH)_2D_3$.

A number of cases of $1,25(OH)_2D_3$-induced hypercalcemia in non-Hodgkin's lymphoma have been described. Most patients had bulky or advanced stage disease, but no clinically or radiographically evident bone lesions. In one case, the $1,25(OH)_2D_3$-mediated hypercalcemia was associated with transformation from a chronic lymphocytic leukemia to an aggressive high-grade non-Hodgkin's lymphoma [128]. Data supporting extrarenal synthesis of $1,25(OH)_2D_3$ are the presence of severe renal failure in a number of instances [129,130]; the demonstration of *in vitro* conversion of $25OHD_3$ to $1,25(OH)_2D_3$ by excised lymph node homogenates [131]; the prompt decline of $1,25(OH)_2D_3$ levels to normal after excision of an isolated splenic lymphoma [132] and a primary ovarian lymphoma [133]; and sensitivity to glucocorticoid suppression [130]. Five of ten patients with either AIDS or non-AIDS associated non-Hodgkin's lymphoma and hypercalcemia had frankly elevated serum $1,25(OH)_2D_3$ concentrations [134]. Other malignant lymphoproliferative diseases associated with $1,25(OH)_2D_3$–mediated hypercalcemia include lymphomatoid granulomatosis [135], dysgerminoma [136], and an inflammatory myofibroblastic tumor [137].

In a prospective study by Seymour *et al*. [138], a control group was composed of 16 patients with hypercalcemia and multiple myeloma. Using the mean serum $1,25(OH)_2D_3$ level of the control patients plus 3 standard deviations, the investigators defined the upper limit of expected serum $1,25(OH)_2D_3$ during hypercalcemia as 42 pg/ml, well below the upper limit of 76 pg/ml for the normocalcemic reference range. Thus, the typical hypercalcemic patient, if represented by this cohort of patients with multiple myeloma, shows a lower range of normal for $1,25(OH)_2D_3$ concentration. Of the 22 hypercalcemic patients with non-Hodgkin's lymphoma, 12 (55%) had elevated serum $1,25(OH)_2D_3$ levels. Moreover, the serum levels of corrected calcium and $1,25(OH)_2D_3$ were strongly correlated with one another. Even in the normocalcemic group with non-Hodgkin's lymphoma, 71% were hypercalciuric and 18% had elevated serum $1,25(OH)_2D_3$ levels.

The precise cell type responsible for the extrarenal synthesis of $1,25(OH)_2D_3$ in lymphoma remains to be established. There are two possibilities. One is the tumor-infiltrating reactive macrophage, recognized by

a "starry-sky" appearance [139] in intermediate and high-grade lymphomas, in which hypercalcemia is also most common. Alternatively, it may be that a particular clone of the malignant lymphoma cell synthesizes $1,25(OH)_2D_3$ [140]. Recent immunohistochemical analysis of the enzyme 1α-hydroxylase in a B-cell lymphoma associated with hypercalcemia and raised circulating levels of $1,25(OH)_2D_3$ suggests that the tumor itself is not a source of the steroid hormone [141]. Rather, macrophages adjacent to the tumor are likely to be the major site of ectopic $1,25(OH)_2D_3$ synthesis [141].

1,25-Dihydroxyvitamin D is only one cause of hypercalcemia in lymphoma. About half of the patients with non-Hodgkin's lymphoma and hypercalcemia have suppressed $1,25(OH)_2D_3$ levels. Additional circulating or local osteolytic factors are likely to be involved. Two of 22 patients in the study by Seymour *et al*. had elevated PTHrP levels. A few other cases of hypercalcemia in non-Hodgkin's lymphoma associated with elevated levels of PTHrP have been reported [142–144]. Cytokines such as interleukin-1, tumor necrosis factor-α (TNFα), and transforming growth factor (TGFβ) may also play a role in the pathogenesis of lymphoma-associated hypercalcemia.

Although HTLV-1-transformed lymphocytes were shown *in vitro* to possess the capacity to convert $25OHD_3$ to $1,25(OH)_2D_3$ [145], most studies have shown reduced $1,25(OH)_2D_3$ levels in hypercalcemia associated with HTLV-1-related adult T-cell leukemia/lymphoma [146,147]. PTHrP is most strongly implicated as the major mediator in this syndrome [148]. PTHrP messenger RNA has been demonstrated in HTLV-1-infected T cells [149] and tumor cells from adult T-cell lymphoma/leukemia (ATLL) patients with hypercalcemia [150]. Nevertheless, there are two well-documented instances of elevated $1,25(OH)_2D_3$ levels in ATLL [122,130]. In the first case, a PTHrP level was not available. In the second case, concomitant elevation of $1,25(OH)_2D_3$ and PTHrP was shown, suggesting the possibility of increased renal 1α-hydroxylase activity secondary to PTHrP. Alternatively, the tissue could be the site of both PTHrP and $1,25(OH)_2D_3$ formation. Most patients with hypercalcemia due to classic squamous cell carcinoma have elevated PTHrP levels and either suppressed or normal $1,25(OH)_2D_3$ levels.

IV. MECHANISMS OF VITAMIN D TOXICITY

A. General Mechanisms

Vitamin D toxicity may occur in patients due to any one of the three forms of vitamin D, namely, the vitamin D parent compound, 25OHD, or $1,25(OH)_2D$. Multiple factors may influence susceptibility to vitamin D toxicity and include the concentration of the vitamin D metabolite itself, vitamin D receptor (VDR) number, activity of lα-hydroxylase, the metabolic degradation pathway, and the capacity of the vitamin D–binding protein (DBP). Vitamin D_2 or D_3 toxicity is more difficult to manage than toxicity due to its metabolites 25OHD or $1,25(OH)_2D$. In part, this is due to the extensive lipid solubility of the parent compound in liver, muscle, and fat tissues and corresponding large storage capacity. As a result, the half-life of vitamin D ranges from 20 days to months. In contrast, the biological half-life of the less lipophilic compound 25OHD is shorter, approximately 15 days [151]. The biological half-life of the least lipophilic compound $1,25(OH)_2D$, is much shorter, approximately 15 hr [152]. In general, duration of toxicity is related to the half-life of the vitamin D compound. Thus, the hypercalcemia of parent vitamin D overdose can last for as long as 18 months, long after dosing is discontinued, because of its slow release from fat deposits. Over-dosage of 25OHD can persist for weeks also, but excessive $1,25(OH)_2D$ toxicity is more rapidly reversed because $1,25(OH)_2D$ is not stored in appreciable amounts in the body [66].

The toxicity of either parent vitamin D or 25OHD is due to 25OHD. In an investigation examining the concentrations of vitamin D_3 and its metabolites in the rat as influenced by various intakes of vitamin D_3 or 25OHD, Shepard and DeLuca found that large intakes of vitamin D_3, ranging from 0.65 to 6500 nmol/day, resulted in excessive concentrations of vitamin D_3 and $25OHD_3$ but not in $1,25(OH)_2D_3$ (Table II) [153]. Similarly, increased dosages of $25OHD_3$ ranging from 0.46 to 4600 nmol/day resulted in excessive amounts of $25OHD_3$, but not of vitamin D_3 or $1,25(OH)_2D_3$ (Table III). In the setting of toxicity due to overadministration of $1,25(OH)_2D_3$, the active metabolite itself is responsible for the hypercalcemia [154]. Unlike $1,25(OH)_2D$ whose production is tightly regulated in the kidney, the production of 25OHD is not tightly controlled by the liver. The high capacity for 25-hydroxylation of vitamin D in the liver as well as poor regulation at this site allows for massive amounts of 25OHD to be generated from large amounts of vitamin D. Thus, excessive concentrations of 25OHD are typically measured in vitamin D toxicity. As would be expected, PTH levels are suppressed in this form of hypercalcemia.

B. Role of Vitamin D Receptor (VDR) in Vitamin D Toxicity

Various investigations have helped to shed light on the interrelationship among vitamin D metabolites, the

TABLE II Plasma Concentrations of Vitamin D_3 and Metabolites in Rats Given Various Amounts of Vitamin D_3[a]

Amount (nmol/day)	Vitamin D_3 (ng/ml)	25OHD_3 (ng/ml)	Lactone (ng/ml)	24,25$(OH)_2D_3$ (ng/ml)	25,26$(OH)_2D_3$ (ng/ml)	1,25$(OH)_2D_3$ (pg/ml)	Plasma calcium (mg/100 ml)
0.65	11.3 ± 6.1	2.3 ± 1.9	< 0.06	0.56 ± 0.13	< 0.2	80 ± 60	9.0 ± 0.1
6.5	110 ± 43	14.7 ± 8.6	0.35 ± 0.12	3.98 ± 1.90	0.20 ± 0.36	77 ± 64	9.4 ± 0.4
65	368 ± 121	74.2 ± 14.5	10.3 ± 3.9	25.5 ± 5.2	7.60 ± 2.78	88 ± 9	9.7 ± 0.3
650	1339 ± 329[c]	643 ± 93[b]	64.5 ± 19.1[c]	73.5 ± 29.6[d]	16.4 ± 4.7[c]	51 ± 11[c]	12.4 ± 1.0[6]
6500	3108	1111	43.6	86.5	8.4	37	13.8

[a]Rats were orally dosed daily for 14 days with indicated amounts of vitamin D_3. Data are means of 5 rats ± SD. Reprinted with permission from Shepard RM, DeLuca HF 1980. Arch Biochem Biophys **202**:43–50.

[b]Differs from control group (0.65 nmol/day) and from group receiving 65 nmol/day at $p < 0.001$.

[c]Differs from group receiving 65 nmol/day at $p < 0.001$.

[d]Differs from control group (0.65 nmol/day) at $p < 0.001$ and from group receiving 65 nmol/day at $p < 0.010$.

VDR, and PTH in vitamin D toxicity. The biologically active form of vitamin D, 1,25$(OH)_2$D, as is typical of other steroid hormones, binds to a specific intracellular receptor protein (VDR) within its target tissues. The hormone-VDR complex then triggers subsequent transcriptional events by binding to DNA elements.

Regulation of cellular VDR numbers is believed to be an important mechanism by which cellular responsiveness to 1,25$(OH)_2$D is modulated, because the biological activity of 1,25$(OH)_2$D is proportional both to tissue VDR number and concentration of 1,25$(OH)_2$D (see Section II of this book for a detailed discussion). Increased VDR concentrations imply enhanced tissue responsiveness to 1,25-dihydroxyvitamin D, whereas decreased receptor numbers indicate reduced tissue responsiveness. Several investigations have suggested that exogenous 1,25$(OH)_2D_3$ can lead to homologous up-regulation of VDR *in vitro* and *in vivo,* in contrast to endogenous production of 1,25$(OH)_2D_3$. *In vitro* and *in vivo* administration of 1,25$(OH)_2D_3$ to rats has been shown to increase VDR content. *In vitro* exposure of human skin fibroblasts and osteosarcoma cells to 1,25$(OH)_2D_3$ has been shown to result in a three- to fivefold increase in VDR number [155]. Similarly, *in vivo* studies have shown increased VDR with exogenous administration of 1,25$(OH)_2D_3$. Costa and Feldman administered 1500 pmol/kg of 1,25$(OH)_2D_3$ daily to rats and found a 30% increase in intestinal VDR and a threefold increase in renal VDR concentration [156]. Reinhart *et al.* infused rats with 250 pmol/kg of 1,25$(OH)_2D_3$ daily for six days and noted a 22% increase in VDR levels in the intestine and a 37% increase in bone [157]. Goff and colleagues infused 36 ng of 1,25$(OH)_2D_3$ to rats over seven days and found

TABLE III Plasma Concentrations of Vitamin D_3 and Metabolites in Rats Given Various Amounts of 25OHD_3[a]

Amount (nmol/day)	Vitamin D_3 (ng/ml)	25OHD_3 (ng/ml)	Lactone (ng/ml)	24,25$(OH)_2D_3$ (ng/ml)	25,26$(OH)_2D_3$ (ng/ml)	1,25$(OH)_2D_3$ (pg/ml)	Plasma calcium (mg/100 ml)
0.46	< 0.5	6.2 ± 2.3	0.31 ± 0.05	2.29 ± 0.54	< 0.2	187 ± 72	9.8 ± 0.5
4.6	< 0.5	56.3 ± 11.4	3.02 ± 0.63	11.7 ± 3.3	< 0.2	192 ± 65	9.3 ± 0.5
46	< 0.5	199 ± 24	32.5 ± 8.5	57.3 ± 19.5	1.19 ± 0.49	82 ± 29	10.2 ± 0.4
460	< 0.5	436 ± 58	118 ± 26	170 ± 22	4.02 ± 1.02	33 ± 8	9.7 ± 0.4
4600	< 0.5	688 ± 145[b]	110 ± 38[c]	214 ± 117[d]	6.31 ± 1.79[e]	22 ± 1[f]	14.0 ± 0.5*

[a]Rats were orally dosed daily for 14 days with indicated amounts of 25OHD_3. Data are means of 5 rats ± SD. Reprinted with permission from Shepard RM, DeLuca HF 1980 Arch Biochem Biophys **202**:43–50.

[b]Differs from control group (0.46 nmol/day) at $p < 0.001$ and from group receiving 460 nmol/day at $p < 0.010$.

[c]Differs from control group (0.46 nmol/day) at $p < 0.001$.

[d]Differs from control group (0.46 nmol/day) at $p < 0.005$.

[e]Differs from group receiving 460 nmol/day at $p < 0.005$.

[f]Differs from control group (0.46 nmol/day) at $p < 0.001$ and from group receiving 460 nmol/day at $p < 0.050$.

*Differs from control group (0.46 nmol/day) and from group receiving 460 nmol/day at $p < 0.001$.

a 1.5-fold increase in duodenal VDR content and a three-fold increase in renal VDR content [158].

Goff *et al*. [158] also demonstrated that endogenously produced $1,25(OH)_2D_3$ has a different effect than exogenous administration of $1,25(OH)_2D_3$ on tissue VDR content. Rats fed a calcium-restricted diet resulting in "nutritional" hyperparathyroidism achieved a similar increase in endogenous $1,25(OH)_2D_3$ concentration as rats administered exogenous $1,25(OH)_2D_3$. However, calcium-restricted rats failed to up-regulate VDR content in the duodenum or kidney, presumably a consequence of the negative control of VDR by PTH [159]. This point has at least conceptual relevance in the case of vitamin D toxicity. Rather than down-regulation occurring during hypervitaminosis D, which is a more typical regulatory and protective event to limit tissue responsiveness, exposure of cells to exogenous $1,25(OH)_2D$ results in enhanced responsiveness by virtue of up-regulation. Such a mechanism would be of particular clinical relevance if the toxicity were due to overexposure of $1,25(OH)_2D$. Moreover, in this setting, the associated suppression of PTH would prevent the regulatory mechanism from being operative.

Evidence suggests that in parent vitamin D toxicity, target tissues are responding to high concentrations of 25OHD, not $1,25(OH)_2D$. Concentrations of $1,25(OH)_2D$ are typically only slightly increased, if at all. The hypercalcemia is due to the effects of pharmacologically high levels of 25OHD, even though in physiological settings, 25OHD has little potency. At high concentrations, 25OHD can compete for binding at VDR sites, and thereby produce biological effects similar to those of $1,25(OH)_2D$ on intestine and bone [160]. Beckman and colleagues [161] suggested, furthermore, that hypervitaminosis D, like excessive $1,25(OH)_2D$, is associated with homologous up-regulation of intestinal VDR. Their investigation demonstrated that supraphysiological amounts of vitamin D_2 or vitamin D_3 administered to rats at doses of 25,000 IU daily for six days resulted in increasing plasma 25OHD concentrations with significant up-regulation of intestinal VDR concentration and hypercalcemia. Plasma $1,25(OH)_2D$ levels were not altered substantially (see Table IV and Fig. 1).

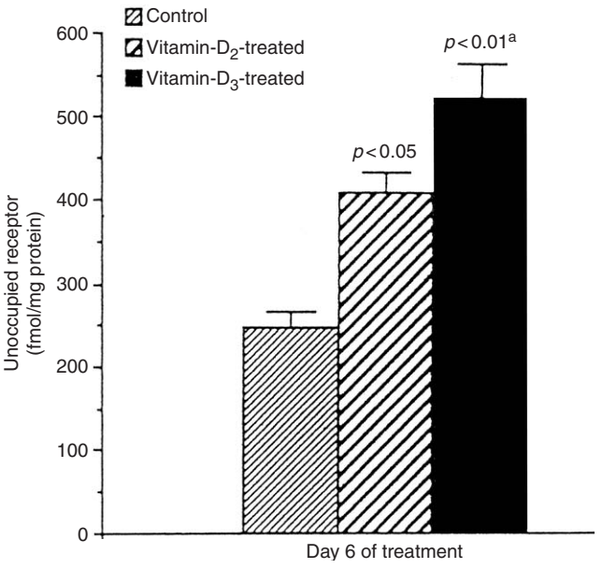

FIGURE 1 Intestinal VDR in rats treated six days with 25,000 IU/day of either vitamin D_2 or vitamin D_3 relative to the response in age-matched controls. [a]Response in vitamin D_3-treated rats significantly different from that in vitamin D_2-treated rats ($p < 0.05$). Reprinted with permission from Beckman MJ, *et al*. [161].

A comparison between hypervitaminosis D_3 and D_2 was also made [161]. No differences in 25OHD and plasma calcium concentrations were noted between either preparations. Concentrations of 25OHD in each case were markedly higher than the control group. The concentration of $1,25(OH)_2D$ was observed to be only slightly greater in the vitamin D_3-treated group than the vitamin D_2-treated group. Because the 25OHD

TABLE IV Changes in Body Weight, Plasma Calcium, and Plasma Vitamin D Metabolites in Rats Treated for Six Days with Either 25,000 IU/day of Vitamin D_2 or Vitamin D_3[a]

Group	Body weight (g)	Plasma calcium (mg/dl)	25OHD (ng/ml)	$1,25(OH)_2D$ (pg/ml)
Control	251 ± 5	9.5 ± 0.7	20 ± 2	112 ± 11
Vitamin D_2-treated	230 ± 17	11.8 ± 0.6[c]	466 ± 36[a]	123 ± 12
Vitamin D_3-treated	201 ± 18*	12.0 ± 0.9[c]	506 ± 67[b]	150 ± 8[c,d]

[a]Data represent means ± SE. Reprinted with permission from Beckman MJ *et al,*1990. Biochem Biophys Res Commun **169**:910–915.
[b]Significant difference at $p < 0.01$ of the treated groups relative to the control group.
[c]Significant difference at $p < 0.05$ of the treated group relative to the control group.
[d]Statistical significance between the D_2- and D_3-treated groups ($n = 6$).

concentrations were elevated twenty- to twenty-five-fold, whereas $1,25(OH)_2D$ showed only minimal increases, the biochemical and clinical changes associated with parent vitamin D toxicity were attributed to 25OHD. The data provided further support for the importance of 25OHD as the major toxic metabolite in vitamin D–associated hypercalcemia, as well as for the importance of increased intestinal VDR in the pathophysiological process that leads to enhanced effects of this metabolite.

C. Control of Renal 1α-Hydroxylase in Vitamin D Toxicity

Some investigators have suggested that toxic effects of excessive concentrations of 25OHD may result from PTH suppression and down-regulation of 1α-hydroxylase with increased concentrations of 25OHD. PTH and $1,25(OH)_2D$ have known reciprocal actions on 1α-hydroxylase and 24-hydroxylase activities. PTH stimulates 1α-hydroxylase activity and down-regulates 24-hydroxylase activity; $1,25(OH)_2D$, on the other hand, down-regulates 1α-hydroxylase activity and stimulates 24-hydroxylase activity.

Beckman and colleagues [162] studied the effects of an excess of vitamin D_3 and dietary calcium restriction on tissue 1α-hydroxylase and 24-hydroxylase activity in rats. Four groups of rats with different dietary calcium and vitamin D_3 concentrations were studied (normal calcium, NC; low calcium, LC; and the excess vitamin D groups with normal or low calcium, NCT and LCT). The data showed that in the setting of a calcium-restricted diet, a nutritional hyperparathyroidism ensued (Table V). Under conditions of excess vitamin D_3 at doses of 75,000 IU per week and on a calcium-restricted diet, elevations in PTH facilitated the elimination of $25OHD_3$ through its metabolism to $1,25(OH)_2D_3$ and or degradation to $24,25(OH)_2D_3$. The elevation in PTH was accompanied by increased activation of renal 1α-hydroxylase activity, lower concentrations of $25OHD_3$, increased activation of intestinal 24-hydroxylase activity, and lower renal VDR content compared to the normal calcium group (Table VI). In contrast, the normal calcium diet in the vitamin D_3 excess group contributed to the toxicity by virtue of suppressed PTH concentrations resulting in down-regulation of renal 1α-hydroxylase and decreased 24-hydroxylase activity, and, thus, higher $25OHD_3$ concentrations. On the other hand, dietary calcium restriction in the setting of vitamin D_3 excess seemed to be protective, providing less biological stimulation due to higher PTH concentrations with reduced VDR, increased activation of both 1α-hydroxylase and 24-hydroxylase activities, greater reductions in $25OHD_3$ concentrations, and lower concentrations of total calcium resulting in a less toxic state. So the low calcium diet protects, not only by contributing to less hypercalcemia, but also by facilitating metabolic pathways of vitamin D inactivation.

D. Inhibition of the Catabolic Pathway of 24-Hydroxylase

Others have proposed that inhibition of the enzymes that degrade the vitamin D metabolites may have a role in the pathogenesis of hypervitaminosis D. $1,25(OH)_2D$ is a known regulator of its own catabolism and an inhibitor of its synthesis. In the kidney, intestine and other targets $1,25(OH)_2D$ induces the enzyme 24-hydroxylase. This enzyme initiates a catabolic cascade that ultimately causes side chain oxidation, cleavage, and metabolic elimination of both $1,25(OH)_2D$ and 25OHD, and it accounts for 35–40% of the

TABLE V Changes in Plasma Calcium, Phosphorus, PTH, and $1,25(OH)_2D_3$ Concentrations in Response to Dietary Calcium Restriction and Vitamin D_3 Excess[a]

Treatment[b]	Calcium (mg/dl)	Phosphorus (mg/dl)	$25OHD_3$ (ng/ml)	PTH (pg/ml)	$1,25(OH)_2D_3$ (pg/ml)
NC	11.2 ± 0.1	9.3 ± 0.7	15.2 ± 1.7	48.0 ± 2.0	116.0 ± 7.0
NCT	14.6 ± 0.3[c]	9.5 ± 0.5	443 ± 43[c]	44.0 ± 3.0	48.0 ± 8.0
LC	9.1 ± 0.1[c]	8.9 ± 0.5	<1.0[c]	162.0 ± 10.0[c]	615.0 ± 110.0[c]
LCT	9.7 ± 0.4[d,e]	9.0 ± 0.7	244 ± 17[c,e]	154.0 ± 19.0[c,e]	99.0 ± 12.0

[a]Values are means ± SE (n = 6). Significant differences were measured by Tukey's multiple range test. Reprinted from Beckman MJ *et al.*, 1995. Arch Biochem Biophys **319**:535–539 with permission.

[b]NC, 1.0–1.2% Ca, normal D_3; NCT, 1.0–1.2% Ca, excess D_3; LC, 0.02% Ca, normal D_3; LCT, 0.02% Ca, excess D_3.

[c]Significant difference at $p < 0.001$ versus NC group.

[d]Significant difference at $p < 0.01$ versus NC group.

[e]Significant difference at $p < 0.001$ versus NCT group.

TABLE VI Changes in Renal VDR and Intestinal $25OHD_3$-Hydroxylases in Response to Dietary Calcium Restriction and Vitamin D_3 Excess[a]

Treatment	Renal VDR (fmol/mg protein)	Renal 1α-hydroxylase (pg/hr · mg tissue)	Renal 24-hydroxylase (pg/min · mg tissue)	Intestinal 24-hydroxylase (pg/min · mg tissue)
NC	200 ± 14	1.5 ± 0.2	6 ± 1	7 ± 1
NCT	541 ± 21[c]	1.1 ± 0.1	276 ± 15[c,e]	19 ± 7
LC	115 ± 8[c]	28.7 ± 5.6[b,c]	ND*	80 ± 16[d]
LCT	97 ± 6[c,e]	3.6 ± 0.4[d,e]	ND	174 ± 33[c,e]

[a]Values are means ± SE (n = 6–10). Groups NC, NCT, LC, and LCT are defined in Table V. Significant differences were measured by Tukey's multiple range test. Reprinted with permission from Beckman MJ 1995, *et al.* Arch Biochem Biophys **319**:535–539.

[b]ND, not detected.

[c]Significant difference at $p < 0.01$ versus NC group.

[d]Significant difference at $p < 0.05$ versus NC group.

[e]Significant difference at $p < 0.01$ versus NCT group.

catabolism of $1,25(OH)_2D$ [163]. The remainder of the metabolic degradation is due to other side chain oxidations and biliary clearance. Reinhart and Horst [164] initially proposed that blunting of the catabolic pathway of $1,25(OH)_2D_3$ with high concentrations of $24,25(OH)_2D_3$ in rat cells would competitively inhibit further inactivation of $1,25(OH)_2D_3$, resulting in an accumulation of $1,25(OH)_2D_3$ and toxicity.

Clinical investigations of the down-regulation of rat intestinal 24-hydroxylase and its inhibition by calcitonin may help to elucidate a role of this hormone in potentiating the toxicity of vitamin D. 24-Hydroxylation is important in the inactivation of both $1,25(OH)_2D_3$ and $25OHD_3$, and in the kidney is largely regulated inversely by 1α-hydroxylation [165]. In a study examining the effects of dietary calcium and vitamin D status on the regulation of intestinal 24-hydroxylase enzyme and mRNA expression, rats were fed normal or low calcium diets with variable amounts of vitamin D [166]. Half of the rats on the normal and low calcium diets were administered pharmacological doses of vitamin D_3 (25,000 IU three times weekly). Excess vitamin D_3 resulted in significant elevations in plasma $25OHD_3$ in both calcium groups (LCT and NCT), with a much larger increase noted in the normal calcium group (NCT). Hypercalcemia was most severe in the NCT group, whereas rats in the low calcium and vitamin D_3 excess group (LCT) had plasma calcium levels similar to the NC group (see Table VII). Because the NCT was accompanied by an increased calcitonin concentration compared to the LCT, the authors suggested that the increased calcitonin in the NCT group may have suppressed 24-hydroxylase activity, with resultant higher $25OHD_3$ and calcium concentrations [166]. This concept was further supported when rats, subjected to thyroparathyroidectomy (TPTX), which eliminated endogenous calcitonin (Table VIII), were found to have higher concentrations of 24-hydroxylase activity than the NCT group. Through inhibition of intestinal 24-hydroxylase activity, calcitonin could be associated

TABLE VII Changes in Plasma Calcium, 25OHD, $1,25(OH)_2D$, and Calcitonin Concentrations in Response to Dietary Calcium Restriction and Vitamin D_3 Excess[a]

Treatment	Calcium (mg/dl)	25OHD (ng/ml)	$1,25(OH)_2D$ (pg/ml)	Calcitonin (pg/ml)
NC	10.7 ± 0.2	108 ± 16	106 ± 17	21.2 ± 1.1
NCT	12.6 ± 0.3[b]	1812 ± 165*	96 ± 12	36.1 ± 2.5[b]
LC	9.7 ± 0.2[b]	47 ± 10[c]	459 ± 70[c]	15.9 ± 0.9
LCT	10.5 ± 0.2[c]	1130 ± 62[b,c]	188 ± 41	19.4 ± 2.3[c]

[a]Values are means ± SEM (n = 6). Groups NC, NCT, LC, and LCT are defined in Table V. Significant differences were measured by Tukey's multiple range test after analysis of variance. Reprinted from Beckman *et al.* [166] with permission.

[b]$p < 0.05$ versus NC group.

[c]$p < 0.05$ versus NCT group.

TABLE VIII Intestinal 24-Hydroxylase, Plasma Calcium, and $1,25(OH)_2D$ in Response to Vitamin D Excess and Thyroparathyroidectomy[a]

Treatment[b]	Calcium (mg/dl)	$1,25(OH)_2D$ (pg/ml)	24-Hydroxylase (pg/min • mg protein)
NCT	12.9 ± 0.4	86 ± 16	6.7 ± 0.5
NCT/TPTX	13.8 ± 0.3	78 ± 9	14.1 ± 0.2[c]
NCT/CT	9.7 ± 0.6[c]	80 ± 13	5.3 ± 0.2
NCT/TPTX/CT	10.3 ± 0.4[c]	82 ± 11	3.8 ± 0.2[c,d]

[a]Reprinted from Beckman et al. [166] with permission.

[b]NCT, Controls given excess vitamin D_3; NCT/TPTX, NCT animals that underwent TPTX; NCT/CT, NCT animals treated with 100 IU calcitonin 4 hr before death; NCT/TPTX/CT, NCT/TPTX animals treated with 100 IU calcitonin 4 hr before death.

[c]Different from NCT ($p < 0.001$).

[d]Different from NCT/TPTX ($p < 0.001$).

with reduced turnover and catabolism of $25OHD_3$, thereby potentiating its toxicity. Thus, increased expression of 24-hydroxylase activity in cases of pharmacological amounts of $25OHD_3$ may be an important mechanism to counteract vitamin D toxicity. A key role for 24-hydroxylase in preventing the development of vitamin D toxicosis was found in a recent animal study [167]. Growing dogs given 135-fold vitamin D_3 supplementation actually had a decrease in plasma $1,25(OH)_2D_3$ levels by 40% as compared to controls, despite an increase in $1,25(OH)_2D_3$ production. This was attributed to an upgraded catabolism of $1,25(OH)_2D_3$ by 24-hydroxylase, as evidenced by increased gene expression of renal and intestinal 24-hydroxylase, thus providing an efficient hormonal counteraction [167].

E. Vitamin D Binding–Protein and the Level of Free Metabolite in Vitamin D Toxicity

The vitamin D–binding protein (DBP) is a specific transport protein that binds large quantities of the circulating vitamin D metabolites (see Chapter 8). Similar to the situation for other steroid hormones, fat-soluble compounds, and thyroid hormones, only a small fraction of the metabolites circulate free in plasma. The binding affinity of the protein for the vitamin D metabolites is moderate and the capacity is great (only 5% of binding sites on DBP are normally occupied). In addition, the various metabolites have different binding affinities for the protein, in the following sequence: $25OHD > 24,25(OH)_2D > 1,25(OH)_2D$ [168]. Of note is the fact that the potent metabolite $1,25(OH)_2D$ has the least affinity for DBP, but the highest affinity for the intracellular VDR that triggers subsequent transcriptional events. Therefore, freeing bound $1,25(OH)_2D$ metabolite from DBP could promote its entry into various tissues and promote biological activity [169]. In states of vitamin D toxicity, the presence of elevated free $1,25(OH)_2D$ levels despite normal total $1,25(OH)_2D$ levels suggests that $1,25(OH)_2D$ is displaced from DBP by 25OHD, resulting in a rise of serum free calcitriol [170].

Evidence indicates that the biologically active form of the vitamin D steroid hormone is the free hormone that is accessible to cells [171]. Because of technical difficulties in measuring the free hormone, the determination of vitamin D status involves a measurement combining free vitamin D and DBP concentrations. In normal individuals, 85% of the total $1,25(OH)_2D$ is bound to DBP, 15% is bound to albumin, and 0.4% is free [172]. However, under conditions of altered or reduced albumin and DBP concentrations, as in liver or kidney disease, the free hormone may provide different information compared to the total measured concentration of vitamin D. Theoretically, total hormone concentration in such settings may erroneously suggest deficiency of vitamin D with needless institution of replacement therapy. Bikle and colleagues noted that subjects with liver disease have reduced DBP concentrations with low total $1,25(OH)_2D$ and 25OHD levels, whereas free forms are normal [173,174].

Similarly, in certain forms of renal disease, the concentrations of DBP and vitamin D metabolites are reduced, thus measurements of total hormone may provide an inaccurate reflection of vitamin D status. Koenig *et al.* [175] investigated free and total $1,25(OH)_2D$ concentrations in subjects with renal disease. Patients with nephrotic syndrome, with varying degrees of renal failure, and on chronic hemodialysis and peritoneal dialysis were studied. The serum concentrations of total and free $1,25(OH)_2D$ correlated well with one another in the patients with renal failure

and those undergoing hemodialysis. The concentrations of DBP and 25OHD, thus, were unaffected by renal function. The concentrations of total $1,25(OH)_2D$ accurately reflected free $1,25(OH)_2D$ in patients with varying degrees of renal failure when DBP levels remained normal. However, this did not hold true for the subjects with nephrotic syndrome or those on chronic peritoneal dialysis, who lost DBP and bound vitamin D metabolites into the urine or peritoneal fluid, respectively, with a rise in the percentage free $1,25(OH)_2D$ (also megalin may play a role as discussed in Chapter 10). Measurement of free metabolite in these particular patients may be important to avoid vitamin D toxicity when supplementation is instituted. Thus, in this context, the binding proteins of the vitamin D metabolites not only serve a transport function, but also may provide a buffering mechanism to protect against toxicity [176].

V. CLINICAL MANIFESTATIONS

The clinical manifestations of vitamin D toxicity resulting from hypercalcemia reflect the essential role of calcium in many tissues and targets, including bone, the cardiovascular system, nerves, and cellular enzymes. Initial signs and symptoms of hypervitaminosis D may be similar to other hypercalcemic states and include generalized weakness and fatigue. Central nervous system features may include confusion, difficulty in concentration, drowsiness, apathy, and coma [177]. Neuropsychiatric symptoms include depression and psychosis, which resolve following improvement of the hypercalcemia.

Hypercalcemia can affect the gastrointestinal tract and cause anorexia, nausea, vomiting, and constipation. It can induce hypergastrinemia, but only in men does it appear to be associated with peptic ulcer disease. There is no evidence that peptic ulcers are more common in any other form of hypercalcemia. Rarely, pancreatitis may be a presentation of either acute or chronic hypercalcemia.

In the heart, hypercalcemia may shorten the repolarization phase of conduction reducing the Q-T interval on the electrocardiogram (EKG). EKG changes in vitamin D toxicity have been mistaken for myocardial ischemia [178]. A more accurate EKG indication of the level of hypercalcemia is the Q-T interval corrected for rate. Bradyarrhythmias and first degree heart block have been described, but are rare. Hypercalcemia may potentiate the action of digitalis on the heart [179].

Kidney function is affected because high concentrations of calcium alter the action of vasopressin on the renal tubules. The net result is reduced urinary concentrating ability and a form of nephrogenic diabetes insipidus. This usually presents as polyuria, but rarely is the volume as high as that associated with central diabetes insipidus. Symptoms may include polydipsia, which is an expected consequence of polyuria. Hypercalciuria is one of the earliest signs of vitamin D toxicity and precedes the occurrence of hypercalcemia. The initial hypercalciuria may be ameliorated as renal failure progresses because of reduced calcium clearance. The pathophysiology of hypercalcemia can be rapidly worsened when dehydration develops. When reduced renal blood flow occurs, less calcium is presented to the renal glomerulus, and hypercalcemia can rapidly progress. Renal impairment from the hypercalcemia is reversible if of short duration. Chronic, uncontrolled hypercalcemia can lead to deposition of calcium phosphate salts in the kidney and permanent damage with eventual nephrocalcinosis. In an investigation of vitamin D–induced nephrocalcinosis, Scarpelli and colleagues [180] noted that cell damage, specifically in mitochondria, preceded intracellular calcium deposition. The hypercalcemia induced in rats by excessive vitamin D administration caused mitochondrial swelling, cell injury, and subsequent calcification.

Ectopic soft tissue calcification can be a particular problem in hypervitaminosis D. The tendency towards soft tissue calcification is compounded by the combination of hypercalcemia and hyperphosphatemia, often exceeding the solubility product of the two ions [181–183]. In rats exposed to excessive vitamin D, Hass and colleagues demonstrated that the pathological processes of vitamin D toxicity were related to dosage, length of time between doses, and duration of exposure [184]. For rats subjected to sublethal doses, generalized calcinosis was seen after only eight days, when a total of 300,000 units of ergosterol was administered. Pathologically, bones appeared more brittle than normal, with increased cortical bone resorption, increased numbers of osteoclasts, and reduced numbers of osteoblasts. Abnormal calcium deposits were noted in the aorta and its major branches, heart, kidney, muscle, and respiratory tract. The earliest evidence of hypervitaminosis D was in the proximal aorta. Muscle tissue was the least resistant to calcification, with the order of decreasing susceptibility being smooth muscle > cardiac muscle > skeletal muscle [185]. The liver, brain, and pituitary were not affected by high doses of vitamin D. Permanent dental changes have also been reported with hypervitaminosis D, including enamel hypoplasia and focal pulp calcification [186]. Bone mineral density can be decreased due to excessive bone resorption [182,187], changes which can be reversed when vitamin D levels return to normal [188].

VI. Diagnosis of Vitamin D Toxicity

With modern assays for calciotropic hormones, PTH, 25OHD, and 1,25$(OH)_2$D (see Chapter 58), one can readily differentiate vitamin D metabolite-mediated hypercalcemia from other causes of hypercalcemia. The circulating intact PTH level, preferably measured by the two-site immunoradiometric assay (IRMA) or immunochemiluminometric assay (ICMA), should be suppressed in virtually all hypercalcemic disorders with the exception of primary hyperparathyroidism, familial hypocalciuric hypercalcemia, administration of lithium or thiazides, and renal failure. Although patients with malignancy-associated hypercalcemia tend to have a higher serum calcium concentration than those with other causes of hypercalcemia, diminished glomerular filtration rate and subsequent reduction in renal calcium excretion can dramatically increase the serum calcium level in any hypercalcemic patient. In contrast to the low serum phosphorus level in patients with hypercalcemia due to PTH or PTHrP, the serum phosphorus level is at the upper limit of normal or frankly elevated in patients with vitamin D metabolite-mediated hypercalcemia. This is due to increased intestinal absorption and reduced renal clearance of phosphate. An elevated 25OHD concentration with normal 1,25$(OH)_2$D level is indicative of toxicity with exogenously administered vitamin D or 25OHD. The serum 1,25$(OH)_2$D level may be normally increased in patients with primary hyperparathyroidism due to the induction of renal 1α-hydroxylase by PTH. Abnormally high 1,25$(OH)_2$D levels, in the setting of suppressed PTH and hypercalcemia, indicate dysregulated production of 1,25$(OH)_2$D due to either granulomatous diseases, lymphoma, or toxicity with exogenous 1,25$(OH)_2$D or lα-OHD. In cases of hypercalcemia due to PTHrP or local osteolytic factors, the serum 1,25$(OH)_2$D concentration is usually suppressed. In patients with hypercalcemia due to toxicity with other vitamin D analogs such as dihydrotachysterol (DHT) [189] and calcipotriol, the active metabolites may not be recognized by the conventional competitive protein binding assays for 1,25$(OH)_2$D.

The diagnosis of vitamin D toxicity can be made on clinical grounds. Detailed clinical and drug history are of paramount importance in order to make an early diagnosis. Most patients who are suffering from vitamin D toxicity are taking vitamin D for osteoporosis, hypoparathyroidism, pseudohypoparathyroidism, hypophosphatemia, osteomalacia, or renal osteodystrophy in excessive dosages or at too frequent dosing intervals. Therefore, one should have a high index of suspicion in patients who are being treated with pharmacological dosages of vitamin D or its metabolites.

Patients with granulomatous diseases or lymphoma usually have widespread active disease when hypercalcemia develops. In such cases, the diagnosis is obvious at the time of presentation. However, exceptions do exist. In patients with unexplained hypercalcemia, if the 1,25$(OH)_2$D level is elevated and other more easily identifiable causes for this elevation such as primary hyperparathyroidism, pregnancy, and exogenous toxicity (by history) are excluded, measurement of angiotensin converting enzyme level and a systemic search for lymph node enlargement, pulmonary, renal, hepatosplenic, ocular, central nervous system, and bone marrow granulomas or lymphoma should be made.

VII. Treatment of Vitamin D Toxicity

Dietary calcium and vitamin D restriction and avoidance of exposure to sunlight and other ultraviolet light sources should be advised to patients at high risk to develop vitamin D metabolite-mediated hypercalcemia. Those at risk include patients with granulomatous diseases and lymphoma whose disease is widespread and active and patients who are already hypercalciuric. Daily dietary calcium intake should be minimized to approximately 400 mg or less in these patients. Any use of vitamin D supplements should be discontinued. The patient should be encouraged to use sunscreen [sun protection factor (SPF) >15] as much as possible when out of doors. The calcium level should be monitored closely in patients who have a previous history of hypercalcemia or hypercalciuria, or who have recently taken diets enriched in vitamin D and calcium, or who have a recent history of excessive sunlight exposure. A reduction in oxalate intake may also be advisable, so as to prevent an increase in oxalate absorption and hyperoxaluria, which may increase the risk of kidney stone formation, despite a reduction in urinary calcium excretion [190].

When hypercalcemia develops, the aforementioned preventive measures will help to ameliorate the severity of hypercalcemia. General measures in those who are symptomatic include hydration with normal saline and the judicious use of a loop diuretic, like furosemide. Specific inhibitors of bone resorption, such as bisphosphonates [182,187] and calcitonin, can be helpful. Recently, a 3-month-old infant with vitamin D intoxication due to oversupplementation (serum calcium 18.5 mg/dl and 25OHD 360 ng/ml) was treated with alendronate (5–10 mg/d) for 18 days with resolution of the hypercalcemia [22].

Glucocorticoids have proved to be particularly effective in vitamin D intoxication, granulomatous diseases, and lymphoma (see also Chapter 73). The precise

mechanism of action of glucocorticoids in calcium homeostasis is not known. Nonetheless, they are useful because they (1) directly inhibit gastrointestinal absorption of calcium by decreasing the synthesis of calcium-binding protein (calbindin-D) and decreasing active transcellular transport [191], (2) increase urinary excretion of calcium [192], and (3) may alter hepatic vitamin D metabolism to favor the production of inactive vitamin D metabolites, resulting in lower concentrations of 25OHD [193]. Evidence also suggests that they may increase the degradation of $1,25(OH)_2D$ at the receptor sites [194]. Glucocorticoids may also limit osteoclastic bone resorption [195]. Institution of glucocorticoid therapy results in prompt decline of the circulating $1,25(OH)_2D$ concentrations within 3 to 4 days [66]. Patients with nonhematological malignancies and those with primary hyperparathyroidism do not usually respond to glucocorticoids.

Aminoquinolones (chloroquine and hydroxychloroquine) are also capable of reducing the $1,25(OH)_2D$ and calcium concentrations in patients with sarcoidosis [196]. The theoretical advantage of aminoquinolones over glucocorticoids is that correction of the $1,25(OH)_2D$ should result in rapid recovery of at least some of the bone density lost to the disease [188]. In lymphoma cells, however, aminoquinolones do not have the same regulatory effects on the excess $1,25(OH)_2D$ as they do in granulomatous disease. In the presence of lymphoma, it is preferable to use steroid-containing antitumor regimens [198]. Owing to the limited experience with aminoquinolone drugs as antihypercalcemic agents and their potential side effects, they should be reserved for patients in whom steroid therapy is unsuccessful or specifically contraindicated. Ketoconazole, an antifungal agent, in high dosages can inhibit the mitochondrial cytochrome P450-linked 25OHD 1α-hydroxylase irrespective of whether it is renal [189] or extrarenal as in sarcoidosis [197] and tuberculosis [104].

VIII. EVIDENCE FOR BENEFITS OF HIGHER VITAMIN D LEVELS

A. Bone Health

Recent evidence indicates that the accepted threshold of vitamin D sufficiency might not be satisfactory, a shift in paradigm which could conceivably lead towards more zealous vitamin D replacement. Most of the daily vitamin D requirements in a healthy individual (3000–5000 IU) are met with cutaneously synthesized accumulations from solar sources during the preceding summer [199]. However, summer sun exposure is probably not sufficient. In 26 healthy men,

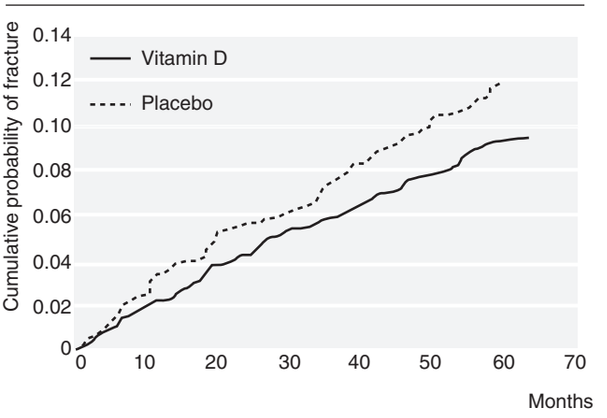

FIGURE 2 Cumulative probability of any first fracture in a randomized controlled trial according to treatment with vitamin D (n=1345) or placebo (n=1341), based on Cox regression; difference between two groups, $p=0.04$. Reproduced with permission from Trivedi, *et al*. [201].

25OHD levels went from 122 nmol/L in late summer to 74 nmol/L in late winter [200]. Furthermore, 25OHD levels that are well above the bottom end of the conventional reference range are probably, in fact, not optimal. Heaney and colleagues recently demonstrated that 25OHD needs to be at least 80 nmol/L (32.4 ng/ml) to maximize intestinal calcium absorption [14]. This finding was validated by the subsequent results of a large randomized controlled trial (n=2686), in which treatment with vitamin D in a dose sufficient to raise serum 25OHD from 53 to 74 nmol/L decreased fracture risk at hip, forearm, or spine by 33% ($p=0.02$) (see Fig. 2) [201]. Precisely how much more vitamin D supplementation might be necessary has not been determined, although some estimates can be made. It is known that an eight week course of additional vitamin D at 400 IU daily will raise 25OHD by 11 nmol/L [1] and that maintenance of a normal PTH in the absence of sun exposure requires 1000 IU of daily vitamin D [202]. It thus remains to be seen whether the recommended daily allowance (RDA) for vitamin D will be increased and whether this will enhance the potential for vitamin D toxicity (see also Chapters 61 and 62).

B. Cellular Health

Once thought to exert its effects solely on bone, kidney, and intestine, $1,25(OH)_2D$ and its synthetic analogs are increasingly recognized to possess a wider variety of noncalcemic roles, including antiproliferative, prodifferentiative, and immunomodulatory actions. It has now been ascertained that prostate, colon, skin, and

osteoblasts can express 1α-hydroxylase and synthesize $1,25(OH)_2D$ locally [24, 203]. An example of the clinical ramifications of the nonclassic actions of $1,25(OH)_2D$ is the efficacy of calcipotriene in treating psoriasis [4]. Another novel potential action of vitamin D might be to increase bone formation. A highly potent $1,25(OH)_2D$ analog (2MD) was recently shown to have an anabolic effect in the rodent skeleton [204] (Chapter 87). The noncalcemic actions of $1,25(OH)_2D$, like the calcemic ones, appear to be mediated through the VDR. One constraint in the use of vitamin D for cellular health has been the dose-limiting hypercalcemia, although this has been partially circumvented with the use of the newer synthetic vitamin D analogs. For example, paricalcitol, a less-calcemic vitamin D analog, has been found *in vitro* to inhibit proliferation of myeloid leukemia, myeloma, and colon cancer cells [27]. As with $1,25(OH)_2D$, the VDR controls most of the effects of the synthetic analogs on proliferation, apoptosis, differentiation, and angiogenesis [205].

The antiproliferative effects of $1,25(OH)_2D$ have been demonstrated directly in the prostate and colon and indirectly in the parathyroid. Human prostate cancer cells contain receptors for $1,25(OH)_2D$ and respond to vitamin D *in vitro* with increases in differentiation and apoptosis and decreases in proliferation, invasiveness, and metastases [26]. Epidemiologically, an association has been observed between decreased sun exposure or vitamin D deficiency and an increased risk of prostate cancer at an earlier age [26]. In a small clinical trial, 22 patients with prostate cancer recurrence were treated with calcitriol 0.5 μg/kg once weekly for 10 months with only transient hypercalcemia. This strategy of intermittent dosing apparently allows very high doses of calcitriol to be administered without hypercalcemic side-effects, although the primary efficacy endpoint of a 50% reduction in PSA was not achieved [206]. In the colon, similar *in vitro* evidence indicates that cultured transformed colon cancer cells can convert 25OHD to $1,25(OH)_2D$ [24]. mRNA for 1α-hydroxylase has been identified in normal colon tissue and in malignant and adjacent normal colon tissue [24]. As in prostate cancer, epidemiologic data suggest that the risk of dying from colorectal cancer is highest in areas with the least amount of sunlight (see Chapter 90). Finally, recent evidence suggests that local production of $1,25(OH)_2D$ regulates parathyroid cell growth and differentiation. The production of 1α-hydroxylase has been detected in parathyroid tissue, but at higher levels in adenomas and hyperplastic tissue. This implies that in addition to feedback control by circulating $1,25(OH)_2D$ levels, parathyroid cells may also be influenced by local 1α-hydroxylase activity with possible growth controlling effects [207].

$1,25(OH)_2D$ is known to exert a potent immunomodulatory effect on activated human lymphocytes *in vitro*. In fact, it has been proposed that $1,25(OH)_2D$ produced by macrophages in granulomatous disease exerts a paracrine immunoinhibitory effect on neighboring, activated lymphocytes to slow an otherwise overly exuberant immune response that may be detrimental to the host [208]. The physiological significance of this has been highlighted by the recent development of 1α-hydroxylase knockout mouse models [209,210], which present with multiple enlarged lymph nodes (see Chapter 67). An additional immunomodulatory action of vitamin D is inhibition of the autoimmune reaction targeted towards the β cells of the pancreas. In nonobese diabetic (NOD) mice, a murine model of human type I diabetes mellitus, $1,25(OH)_2D$ pretreatment decreased the incidence of type 1 diabetes [211] (see Chapter 99). More recently, a vitamin D–sufficient status alone was shown to confer partial protection against the development of type I diabetes mellitus in NOD mice [212]. These observations appear to have direct clinical relevance. The risk of type 1 diabetes mellitus was reduced by 80% in children treated with 2000 IU vitamin D daily after age 1 [25]. These noncalcemic actions of vitamin D thus have many potential pharmacologic applications; whether this will enhance the potential for vitamin D toxicity remains to be seen.

VIII. SUMMARY AND CONCLUSIONS

Vitamin D toxicity is not a common cause of hypercalcemia, but it can be life threatening if not identified quickly. The major causes of hypercalcemia are primary hyperparathyroidism and malignancy. If these two etiologies are excluded, vitamin D toxicity becomes an important diagnostic consideration. There are many forms of exogenous and endogenous vitamin D toxicity. Inadvertent excessive use of pharmaceutical preparations is the most common etiology of exogenous toxicity. Excessive amounts of the parent compound, vitamin D, can be most difficult to manage as compared to toxicity due to the metabolites 25OHD or $1,25(OH)_2D$. Extensive lipid solubility of vitamin D accounts for its extraordinary half-life and tendency for prolonged hypercalcemia. New clinical applications of $1,25(OH)_2D$ and its synthetic analogs have been accompanied by the increased potential for toxicity. Endogenous etiologies may result from ectopic production of $1,25(OH)_2D$ in granulomatous diseases, such as sarcoidosis and tuberculosis, or in lymphoma. Many different mechanisms have been proposed to account for vitamin D toxicity, including the vitamin D metabolite itself, VDR number, activity of 1α-hydroxylase,

inhibition of vitamin D metabolism, and the capacity of DBP. Mounting evidence that higher levels of vitamin D may have beneficial effects on bone and cellular health may predispose to enhanced administration of vitamin D in the future and thereby increased frequency of vitamin D toxicity.

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References

1. Barger-Lux MJ, Heaney RP, Dowell S, Chen TC, Holick MF 1998 Vitamin D and its major metabolites: serum levels after graded oral dosing in healthy men. Osteoporos Int **8**(3): 222–230.
2. Vieth R 1990 The mechanisms of vitamin D toxicity. Bone Miner **11**(3):267–272.
3. Jacobus CH, Holick MF, Shao Q, Chen TC, Holm IA, Kolodny JM, *et al.* 1992 Hypervitaminosis D associated with drinking milk. N Engl J Med **326**(18):1173–1177.
4. Holick MF 1998 Clinical efficacy of 1,25-dihydroxyvitamin D_3 and its analogs in the treatment of psoriasis. Retinoids **14**:12–17.
5. Koutkia P, Chen TC, Holick MF 2001 Vitamin D intoxication associated with an over-the-counter supplement. N Engl J Med **345**(1):66–67.
6. Lagman R, Walsh D 2003 Dangerous nutrition? Calcium, vitamin D, shark cartilage nutritional supplements and cancer-related hypercalcemia. Support Care Cancer **11**(4):232–235.
7. Haddad JG, Chyu KJ 1971 Competitive protein-binding radioassay for 25-hydroxycholecalciferol. J Clin Endocrinol Metab **33**(6):992–995.
8. Tangpricha V, Koutkia P, Rieke SM, Chen TC, Perez AA, Holick MF 2003 Fortification of orange juice with vitamin D: a novel approach for enhancing vitamin D nutritional health. Am J Clin Nutr **77**(6):1478–1483.
9. Vieth, R, Pinto TR, Reen BS, Wong MM 2002 Vitamin D poisoning by table sugar. Lancet **359**(9307):672.
10. Horst RL, Littledike ET, Riley JL, Napoli JL 1981 Quantitation of vitamin D and its metabolites and their plasma concentrations in five species of animals. Anal Biochem **116**(1):189–203.
11. Harrington DD, Page EH 1983 Acute vitamin D3 toxicosis in horses: case reports and experimental studies of the comparative toxicity of vitamins D_2 and D_3. J Am Vet Med Assoc **182**(12):1358–1369.
12. Hunt RD, Garcia FG, Walsh RJ 1972 A comparison of the toxicity of ergocalciferol and cholecalciferol in rhesus monkeys (Macaca mulatta). J Nutr **102**(8):975–986.
13. Trang HM, Cole DE, Rubin LA, Pierratos A, Siu S, Vieth R 1998 Evidence that vitamin D_3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D2. Am J Clin Nutr **68**(4):854–858.
14. Heaney RP, Dowell MS, Hale CA, Bendich A 2003 Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D. J Am Coll Nutr **22**(2):142–146.
15. Passeri G, Pini G, Troiano L, Vescovini R, Sansoni P, Passeri M, *et al.* 2003 Low vitamin D status, high bone turnover, and bone fractures in centenarians. J Clin Endocrinol Metab **88**(11):5109–5115.
16. Heaney RP, 2003 Vitamin D, nutritional deficiency, and the medical paradigm. J Clin Endocrinol Metab **88**(11): 5107–5108.
17. Parfitt AM, Gallagher JC, Heaney RP, Johnston CC, Neer R, Whedon GD 1982 Vitamin D and bone health in the elderly. Am J Clin Nutr **36**(5 Suppl):1014–1031.
18. Vieth R, Chan PC, MacFarlane GD 2001 Efficacy and safety of vitamin D_3 intake exceeding the lowest observed adverse effect level. Am J Clin Nutr **73**(2):288–294.
19. Administration, F.a.D, Over the Counter Report. 1979, Federal Register, p. 16164–16169.
20. Markestad T, Hesse V, Siebenhuner M, Jahreis G, Aksnes L, Plenert W, *et al.* 1987 Intermittent high-dose vitamin D prophylaxis during infancy: effect on vitamin D metabolites, calcium, and phosphorus. Am J Clin Nutr **46**(4):652–658.
21. Zeghoud F, Ben-Mekhbi H, Djeghri N, Garabedian M 1994 Vitamin D prophylaxis during infancy: comparison of the long-term effects of three intermittent doses (15, 5, or 2.5 mg) on 25-hydroxyvitamin D concentrations. Am J Clin Nutr **60**(3):393–396.
22. Bereket A, Erdogan T 2003 Oral bisphosphonate therapy for vitamin D intoxication of the infant. Pediatrics **111**(4 Pt 1): 899–901.
23. Tilyard MW, Spears GF, Thomson J, Dovey S 1992 Treatment of postmenopausal osteoporosis with calcitriol or calcium. N Engl J Med **326**(6):357–362.
24. Tangpricha V, Flanagan JN, Whitlatch LW, Tseng CC, Chen TC, Holt PR, *et al.* 2001 25-hydroxyvitamin D-1α-hydroxylase in normal and malignant colon tissue. Lancet **357**(9269): 1673–1674.
25. Hypponen E, Laara E, Reunanen A, Jarvelin MR, Virtanen SM 2001 Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. Lancet **358**(9292):1500–1503.
26. Chen TC, Holick MF 2003 Vitamin D and prostate cancer prevention and treatment. Trends Endocrinol Metab **14**(9): 423–430.
27. Kumagai T, O'Kelly J, Said JW, Koeffler HP 2003 Vitamin D2 analog 19-nor-1,25-dihydroxyvitamin D_2: antitumor activity against leukemia, myeloma, and colon cancer cells. J Natl Cancer Inst **95**(12):896–905.
28. Massry SG, Goldstein DA 1979 Is calcitriol [1,25(OH)2D3] harmful to renal function? JAMA **242**(17):1875–1876.
29. Aloia JF, Vaswani A, Yeh JK, Ellis K, Yasumura S, Cohn SH 1988 Calcitriol in the treatment of postmenopausal osteoporosis. Am J Med **84**(3 Pt 1):401–408.
30. Christiansen C, Rodbro P, Christensen MS, Hartnack B, Transbol I 1978 Deterioration of renal function during treatment of chronic renal failure with 1,25-dihydroxycholecalciferol. Lancet **2**(8092 Pt 1):700–703.
31. Slatopolsky E, Weerts C, Thielan J, Horst R, Harter H, Martin KJ 1984 Marked suppression of secondary hyperparathyroidism by intravenous administration of 1,25-dihydroxy-cholecalciferol in uremic patients. J Clin Invest **74**(6):2136–2143.
32. Caravaca F, Cubero JJ, Jimenez F, Lopez JM, Aparicio A, Cid MC, *et al.* 1995 Effect of the mode of calcitriol administration on PTH-ionized calcium relationship in uremic patients with secondary hyperparathyroidism. Nephrol Dial Transplant **10**(5):665–670.
33. Monier Faugere MC, Malluche HH 1994 Calcitriol pulse therapy in patients with end-stage renal failure. Curr Opin Nephrol Hypertens **3**(6):615–619.

34. Juergensen P, Santacroce S, Mooraki A, Cooper K, Finkelstein FO, Kliger AS 1994 Pulse oral calcitriol to treat hyperparathyroidism in 43 CAPD patients. Adv Perit Dial **10**:259–260.
35. Feinstein S, Algur N, Drukker A 1994 Oral pulse therapy with vitamin D_3 for control of secondary hyperparathyroidism. Pediatr Nephrol **8**(6):724–726.
36. Crocker JF, Muhtadie SF, Hamilton DC, Cole DE 1985 The comparative toxicity of vitamin D metabolites in the weanling mouse. Toxicol Appl Pharmacol **80**(1):119–126.
37. Brickman AS, Coburn JW, Friedman GR, Okamura WH, Massry SG, Norman AW 1976 Comparison of effects of 1 alpha-hydroxy-vitamin D_3 and 1,25-dihydroxy-vitamin D_3 in man. J Clin Invest **57**(6):1540–1547.
38. Kanis JA, Russell RG, Smith R 1977 Physiological and therapeutic differences between vitamin D, its metabolites, and analogs. Clin Endocrinol (Oxf) **7**(Suppl.):191s–201s.
39. Mortensen JT, Brinck P, Binderup L 1993 Toxicity of vitamin D analogs in rats fed diets with standard or low calcium contents. Pharmacol Toxicol **72**(2):124–127.
40. Gallagher JC, Bishop CW, Knutson JC, Mazess RB, DeLuca HF 1994 Effects of increasing doses of 1α-hydroxyvitamin D_2 on calcium homeostasis in postmenopausal osteopenic women. J Bone Miner Res **9**(5):607–614.
41. Sjoden, G, Smith C, Lindgren U, DeLuca HF 1985 1 alpha-hydroxyvitamin D2 is less toxic than 1α-hydroxyvitamin D_3 in the rat. Proc Soc Exp Biol Med **178**(3):432–436.
42. Pols HA, Birkenhager JC, van Leeuwen JP 1994 Vitamin D analogs: from molecule to clinical application. Clin Endocrinol (Oxf) **40**(3):285–292.
43. Bouillon R, Okamura WH, Norman AW 1995 Structure-function relationships in the vitamin D endocrine system. Endocr Rev **16**(2):200–257.
44. Slatopolsky, E, Finch J, Brown A 2003 New vitamin D analogs. Kidney Int **85**(Suppl.):83–87.
45. Salusky IB, Goodman WG 2002 Cardiovascular calcification in end-stage renal disease. Nephrol Dial Transplant **17**(2):336–339.
46. Teng M, Wolf M, Lowrie E, Ofsthun N, Lazarus JM, Thadhani R 2003 Survival of patients undergoing hemodialysis with paricalcitol or calcitriol therapy. N Engl J Med **349**(5):446–456.
47. Malluche HH, Monier Faugere MC, Koszewski NJ 2002 Use and indication of vitamin D and vitamin D analogs in patients with renal bone disease. Nephrol Dial Transplant 17 **10**(Suppl.):6–9.
48. Cannata Andia JB, 2000 Adynamic bone and chronic renal failure: an overview. Am J Med Sci **320**(2):81–84.
49. Brown AJ, Finch J, Grieff M, Ritter C, Kubodera N, Nishii Y, *et al.* 1993 The mechanism for the disparate actions of calcitriol and 22-oxacalcitriol in the intestine. Endocrinology **133**(3):1158–1164.
50. Hardman KA, Heath DA, Nelson HM 1993 Hypercalcaemia associated with calcipotriol (Dovonex) treatment. Bmj **306**(6882):896.
51. Bourke JF, Berth-Jones J, Mumford R, Iqbal SJ, Hutchinson PE 1994 High dose topical calcipotriol consistently reduces serum parathyroid hormone levels. Clin Endocrinol (Oxf) **41**(3): 295–297.
52. Dwyer C, Chapman RS 1991 Calcipotriol and hypercalcemia. Lancet **338**(8769):764–765.
53. Taylor AB, Stern PH, Bell NH 1982 Abnormal regulation of circulating 25-hydroxyvitamin D in the Williams syndrome. N Engl J Med **306**(16):972–975.
54. Ewart AK, Morris CA, Atkinson D, Jin W, Sternes K, Spallone P, *et al.* 1993 Hemizygosity at the elastin locus in a developmental disorder, Williams syndrome. Nat Genet **5**(1):11–16.
55. Omdahl, J, Holick M, Suda T, Tanaka Y, DeLuca HF 1971 Biological activity of 1,25-dihydroxycholecalciferol. Biochemistry **10**(15):2935–2940.
56. Papapoulos SE, Clemens TL, Fraher LJ, Lewin IG, Sandler LM, O'Riordan JL 1979 1,25-dihydroxycholecalciferol in the pathogenesis of the hypercalcemia of sarcoidosis. Lancet **1**(8117): 627–630.
57. Harrel G, Fisher S 1939 Blood chemical changes in Boeck's sarcoid with particular reference to protein, calcium, and phosphate values. J Clin Invest **18**:687–693.
58. Studdy PR, Bird R, Neville E, James DG 1980 Biochemical findings in sarcoidosis. J Clin Pathol **33**(6):528–533.
59. Anderson J, Harper C, Dent CE, Philpot GR 1954 Effect of cortisone on calcium metabolism in sarcoidosis with hypercalcemia; possibly antagonistic actions of cortisone and vitamin D. Lancet **267**(6841):720–724.
60. Albright F, Carroll EL Dempsey EF, Henneman PH 1956 The cause of hypercalcuria in sarcoid and its treatment with cortisone and sodium phytate. J Clin Invest **35**(11):1229–1242.
61. Taylor RL, Lynch HJ, Jr., and Wysor WG, Jr. 1963 Seasonal influence of sunlight on the hypercalcemia of sarcoidosis. Am J Med **34**:221–227.
62. Bell NH, Stern PH, Pantzer E, Sinha TK, DeLuca HF 1979 Evidence that increased circulating 1α,25-dihydroxyvitamin D is the probable cause for abnormal calcium metabolism in sarcoidosis. J Clin Invest **64**(1):218–225.
63. Barbour GL, Coburn JW, Slatopolsky E, Norman AW, Horst RL 1981 Hypercalcemia in an anephric patient with sarcoidosis: evidence for extrarenal generation of 1,25-dihydroxyvitamin D. N Engl J Med **305**(8):440–443.
64. Maesaka JK, Batuman V, Pablo NC, Shakamuri S 1982 Elevated 1,25-dihydroxyvitamin D levels: occurrence with sarcoidosis with end-stage renal disease. Arch Intern Med **142**(6):1206–1207.
65. Adams JS, Gacad MA, Anders A, Endres DB, Sharma OP 1986 Biochemical indicators of disordered vitamin D and calcium homeostasis in sarcoidosis. Sarcoidosis **3**(1):1–6.
66. Adams JS, 1989 Vitamin D metabolite-mediated hypercalcemia. Endocrinol Metab Clin North Am **18**(3):765–778.
67. Meyrier A, Valeyre D, Bouillon R, Paillard F, Battesti JP, Georges R 1985 Resorptive versus absorptive hypercalciuria in sarcoidosis: correlations with 25-hydroxy vitamin D_3 and 1,25-dihydroxy vitamin D_3 and parameters of disease activity. Q J Med **54**(215):269–281.
68. Mason RS, Frankel T, Chan YL, Lissner D, Posen S 1984 Vitamin D conversion by sarcoid lymph node homogenate. Ann Intern Med **100**(1):59–61.
69. Adams JS, Sharma OP, Gacad MA, Singer FR 1983 Metabolism of 25-hydroxyvitamin D_3 by cultured pulmonary alveolar macrophages in sarcoidosis. J Clin Invest **72**(5): 1856–1860.
70. Adams JS, Singer FR, Gacad MA, Sharma OP, Hayes MJ, Vouros P, *et al.* 1985 Isolation and structural identification of 1,25-dihydroxyvitamin D_3 produced by cultured alveolar macrophages in sarcoidosis. J Clin Endocrinol Metab **60**(5): 960–966.
71. Inui N, Murayama A, Sasaki S, Suda T, Chida K, Kato S, *et al.* 2001 Correlation between 25-hydroxyvitamin D_3 1α-hydroxylase gene expression in alveolar macrophages and the activity of sarcoidosis. Am J Med **110**(9):687–693.
72. Kong XF, Zhu XH, Pei YL, Jackson DM, Holick MF 1999 Molecular cloning, characterization, and promoter analysis of the human 25-hydroxyvitamin D_3–1α-hydroxylase gene. Proc Natl Acad Sci USA **96**(12):6988–6993.
73. Dusso AS, Kamimura S, Gallieni M, Zhong M, Negrea L, Shapiro S, *et al.* 1997 Gamma-interferon-induced resistance

to 1,25-(OH)2D3 in human monocytes and macrophages: a mechanism for the hypercalcemia of various granulomatoses. J Clin Endocrinol Metab **82**(7):2222–2232.
74. Monkawa T, Yoshida T, Hayashi M, Saruta T 2000 Identification of 25-hydroxyvitamin D_3 1α-hydroxylase gene expression in macrophages. Kidney Int **58**(2):559–568.
75. Vidal M, Ramana CV, Dusso AS 2002 Stat1-vitamin D receptor interactions antagonize 1,25-dihydroxyvitamin D transcriptional activity and enhance stat1-mediated transcription. Mol Cell Biol **22**(8):2777–2787.
76. Bell NH, Gill JR Jr. Bartter FC 1964 On the abnormal calcium absorption in sarcoidosis. Evidence for increased sensitivity to vitamin D. Am J Med **36**:500–513.
77. Basile JN, Liel Y, Shary J, Bell NH 1993 Increased calcium intake does not suppress circulating 1,25-dihydroxyvitamin D in normocalcemic patients with sarcoidosis. J Clin Invest **91**(4):1396–1398.
78. Reichel H, Koeffler HP, Barbers R, Norman AW 1987 Regulation of 1,25-dihydroxyvitamin D_3 production by cultured alveolar macrophages from normal human donors and from patients with pulmonary sarcoidosis. J Clin Endocrinol Metab **65**(6):1201–1209.
79. Fuss M, Pepersack T, Gillet C, Karmali R, Corvilain J 1992 Calcium and vitamin D metabolism in granulomatous diseases. Clin Rheumatol **11**(1):28–36.
80. Jackson WP, Dancaster C 1959 A consideration of the hypercalciuria in sarcoidosis, idiopathic hypercalciuria, and that produced by vitamin D; a new suggestion regarding calcium metabolism. J Clin Endocrinol Metab **19**(6):658–680.
81. Bell NH, Bartter FC 1967 Studies of 47-Ca metabolism in sarcoidosis: evidence for increased sensitivity of bone to vitamin D. Acta Endocrinol (Copenh) **54**(1):173–180.
82. Dwarakanathan A, Ryan WG 1987 Hypercalcemia of sarcoidosis treated with cellulose sodium phosphate. Bone Miner **2**(4):333–336.
83. Insogna KL, Dreyer BE, Mitnick M, Ellison AF, Broadus AE 1988 Enhanced production rate of 1,25-dihydroxyvitamin D in sarcoidosis. J Clin Endocrinol Metab **66**(1):72–75.
84. Fallon MD, Perry HM 3rd, Teitelbaum SL 1981 Skeletal sarcoidosis with osteopenia. Metab Bone Dis Relat Res **3**(3):171–4.
85. Alexandre C, Chappard D, Vergnon JM, Emonot A, Riffat G 1987 The bone in noncorticoid-treated sarcoidosis. A histomorphometric study. Rev Rhum Mal Osteoartic **54**(2):159–162.
86. Scholz DA, Keating FR, Jr. 1956 Renal insufficiency, renal calculi, and nephrocalcinosis in sarcoidosis; report of eight cases. Am J Med **21**(1):75–84.
87. Sandler LM, Winearls CG, Fraher LJ, Clemens TL, Smith R, JL O'Riordan 1984 Studies of the hypercalcaemia of sarcoidosis: effect of steroids and exogenous vitamin D_3 on the circulating concentrations of 1,25-dihydroxy vitamin D_3. Q J Med **53**(210):165–180.
88. Zeimer HJ, Greenaway TM, Slavin J, Hards DK, Zhou H, Doery JC, *et al.* 1998 Parathyroid-hormone-related protein in sarcoidosis. Am J Pathol **152**(1):17–21.
89. Abbasi AA, Chemplavil JK, Farah S, Muller BF, Arnstein AR 1979 Hypercalcemia in active pulmonary tuberculosis. Ann Intern Med **90**(3):324–328.
90. Sharma SC, 1981 Serum calcium in pulmonary tuberculosis. Postgrad Med J **57**(673):694–696.
91. Kitrou MP, Phytou Pallikari A, Tzannes SE, Virvidakis K, Mountokalakis TD 1982 Hypercalcemia in active pulmonary tuberculosis. Ann Intern Med **96**(2):255.
92. Davies PD, Brown RC, Woodhead JS 1985 Serum concentrations of vitamin D metabolites in untreated tuberculosis. Thorax **40**(3):187–190.
93. Fuss M, Karmali R, Pepersack T, Bergans A, Dierckx P, Prigogine T, *et al.* 1988 Are tuberculous patients at a great risk from hypercalcemia? Q J Med **69**(259):869–878.
94. Chan TY, Chan CH, Shek CC, Davies PD 1992 Hypercalcemia in active pulmonary tuberculosis and its occurrence in relation to the radiographic extent of disease. Southeast Asian J Trop Med Public Health **23**(4):702–704.
95. Tan TT, Lee BC, Khalid BA 1993 Low incidence of hypercalcaemia in tuberculosis in Malaysia. J Trop Med Hyg **96**(6):349–351.
96. Kelestimur F, Guven M, Ozesmi M, Pasaoglu H 1996 Does tuberculosis really cause hypercalcemia? J Endocrinol Invest **19**(10):678–681.
97. Chan TY 1997 Differences in vitamin D status and calcium intake: possible explanations for the regional variations in the prevalence of hypercalcemia in tuberculosis. Calcif Tissue Int **60**(1):91–93.
98. Felsenfeld AJ, Drezner MK, Llach F 1986 Hypercalcemia and elevated calcitriol in a maintenance dialysis patient with tuberculosis. Arch Intern Med **146**(10):1941–1945.
99. Peces R, Alvarez J 1987 Hypercalcemia and elevated 1,25(OH)$2D_3$ levels in a dialysis patient with disseminated tuberculosis. Nephron **46**(4):377–379.
100. Shai F, Baker RK, Addrizzo JR, Wallach S 1972 Hypercalcemia in mycobacterial infection. J Clin Endocrinol Metab **34**(2):251–256.
101. Barnes PF, Modlin RL, Bikle DD, Adams JS 1989 Transpleural gradient of 1,25-dihydroxyvitamin D in tuberculous pleuritis. J Clin Invest **83**(5):1527–1532.
102. Adams JS, Modlin RL, Diz MM, Barnes PF 1989 Potentiation of the macrophage 25-hydroxyvitamin D-1-hydroxylation reaction by human tuberculous pleural effusion fluid. J Clin Endocrinol Metab **69**(2):457–460.
103. Cadranel J, Garabedian M, Milleron B, Guillozo H, Akoun G, Hance AJ 1990 1,25(OH)2D2 production by T lymphocytes and alveolar macrophages recovered by lavage from normocalcemic patients with tuberculosis. J Clin Invest **85**(5):1588–1593.
104. Saggese G, Bertelloni S, Baroncelli GI, Di Nero G 1993 Ketoconazole decreases the serum-ionized calcium and 1,25-dihydroxyvitamin D levels in tuberculosis-associated hypercalcemia. Am J Dis Child **147**(3):270–273.
105. Ryzen E, Singer FR 1985 Hypercalcemia in leprosy. Arch Intern Med **145**(7):1305–6.
106. Lee JC, Catanzaro A, Parthemore JG, Roach B, Deftos LJ 1977 Hypercalcemia in disseminated coccidioidomycosis. N Engl J Med **297**(8):431–433.
107. Murray JJ, Heim CR 1985 Hypercalcemia in disseminated histoplasmosis. Aggravation by vitamin D. Am J Med **78**(5):881–884.
108. Kantarjian HM, Saad MF, Estey EH, Sellin RV, Samaan NA 1983 Hypercalcemia in disseminated candidiasis. Am J Med **74**(4):721–724.
109. Bosch X 1998 Hypercalcemia due to endogenous overproduction of active vitamin D in identical twins with cat-scratch disease. Jama **279**(7):532–534.
110. Ahmed B, Jaspan JB 1993 Case report: hypercalcemia in a patient with AIDS and pneumocystis carinii pneumonia. Am J Med Sci **306**(5):313–316.
111. Jurney TH 1984 Hypercalcemia in a patient with eosinophilic granuloma. Am J Med **76**(3):527–528.
112. Stoeckle JD, Hardy HL, Weber AL 1969 Chronic beryllium disease. Long-term follow-up of sixty cases and selective review of the literature. Am J Med **46**(4):545–561.
113. Kozeny GA, Barbato AL, Bansal VK, Vertuno LL, Hano JE 1984 Hypercalcemia associated with silicone-induced granulomas. N Engl J Med **311**(17):1103–1105.

114. Albitar S, Genin R, Fen-Chong M, Schohn D, Riviere JP, Serveaux MO, *et al.* 1997 Multisystem granulomatous injuries 28 years after paraffin injections. Nephrol Dial Transplant **12**(9):1974–1976.
115. Edelson GW, Talpos GB, Bone HG 3rd 1993, Hypercalcemia associated with Wegener's granulomatosis and hyperparathyroidism: etiology and management. Am J Nephrol **13**(4):275–277.
116. Al-Ali H, Yabis AA, Issa E, Salem Z, Tawil A, Khoury N, *et al.* 2002 Hypercalcemia in Langerhans' cell granulomatosis with elevated 1,25 dihydroxyvitamin D (calcitriol) level. Bone **30**(1):331–334.
117. Bosch X 1998 Hypercalcemia due to endogenous overproduction of 1,25-dihydroxyvitamin D in Crohn's disease. Gastroenterology **114**(5):1061–1065.
118. Cook JS, Stone MS, Hansen JR 1992 Hypercalcemia in association with subcutaneous fat necrosis of the newborn: studies of calcium-regulating hormones. Pediatrics **90**(1 Pt 1): 93–96.
119. Evron E, Goland S, von der Walde J, Schattner A, Sthoeger ZM 1997 Idiopathic calcitriol-induced hypercalcemia. A new disease entity? Arch Intern Med **157**(18):2142–2145.
120. Burt ME, Brennan MF 1980 Incidence of hypercalcemia and malignant neoplasm. Arch Surg **115**(6):704–7.
121. Vassilopoulou-Sellin R, Newman BM, Taylor SH, Guinee VF 1993 Incidence of hypercalcemia in patients with malignancy referred to a comprehensive cancer center. Cancer **71**(4):1309–1312.
122. Johnston SR, Hammond PJ 1992 Elevated serum parathyroid hormone-related protein and 1,25-dihydroxycholecalciferol in hypercalcemia associated with adult T-cell leukemia-lymphoma. Postgrad Med J **68**(803):753–755.
123. Major prognostic factors of patients with adult T-cell leukemia-lymphoma: a cooperative study. Lymphoma Study Group (1984–1987). Leuk Res **15**(2–3):81–90.
124. Gellhorn, A, Plimpton CH 1956 Hypercalcemia in malignant disease without evidence of bone destruction. Am J Med **21**(5):750–759.
125. Seymour JF, Gagel RF 1993 Calcitriol: the major humoral mediator of hypercalcemia in Hodgkin's disease and non-Hodgkin's lymphomas. Blood **82**(5):1383–1394.
126. Davies M, Hayes ME, Yin JA, Berry JL, Mawer EB 1994 Abnormal synthesis of 1,25-dihydroxyvitamin D in patients with malignant lymphoma. J Clin Endocrinol Metab **78**(5):1202–1207.
127. Karmali R, Barker S, Hewison M, Fraher L, Katz DR, O'Riordan J.L 1990 Intermittent hypercalcaemia and vitamin D sensitivity in Hodgkin's disease. Postgrad Med J **66**(779): 757–760.
128. Freeman NJ, Holik D 2003 Uncommon syndromes and treatment manifestations of malignancy: Case 3. Richter's syndrome heralded by refractory hypercalcemia. J Clin Oncol **21**(1):170–172.
129. Walker IR, 1974 Lymphoma with hypercalcemia. Can Med Assoc J **111**(9):928–930.
130. Breslau NA, McGuire JL, Zerwekh JE, Frenkel EP, Pak CY 1984 Hypercalcemia associated with increased serum calcitriol levels in three patients with lymphoma. Ann Intern Med **100**(1):1–6.
131. Mudde AH, van den Berg H, Boshuis PG, Breedveld FC, Markusse HM, Kluin PM, *et al.* 1987 Ectopic production of 1,25-dihydroxyvitamin D by B-cell lymphoma as a cause of hypercalcemia. Cancer **59**(9):1543–1546.
132. Rosenthal N, Insogna KL, Godsall JW, Smaldone L, Waldron JA, Stewart AF 1985 Elevations in circulating 1,25-dihydroxyvitamin D in three patients with lymphoma-associated hypercalcemia. J Clin Endocrinol Metab **60**(1): 29–33.
133. Rizvi AA, Bowman MA, Vaughters RB, 3rd, Isales C, Mulloy AL 2003 Primary ovarian lymphoma manifesting with severe hypercalcemia. Endocr Pract **9**(5):389–393.
134. Adams JS, Fernandez M, Gacad MA, Gill PS, Endres DB, Rasheed S, *et al.* 1989 Vitamin D metabolite-mediated hypercalcemia and hypercalciuria patients with AIDS- and non-AIDS-associated lymphoma. Blood **73**(1):235–239.
135. Scheinman SJ, Kelberman MW, Tatum AH, Zamkoff KW 1991 Hypercalcemia with excess serum 1,25 dihydroxyvitamin D in lymphomatoid granulomatosis/angiocentric lymphoma. Am J Med Sci **301**(3):178–181.
136. Grote TH, Hainsworth JD 1987 Hypercalcemia and elevated serum calcitriol in a patient with seminoma. Arch Intern Med **147**(12):2212–2213.
137. Ogose A, Kawashima H, Morita O, Hotta T, Umezu H, Endo N 2003 Increase in serum 1,25-dihydroxyvitamin D and hypercalcemia in a patient with inflammatory myofibroblastic tumor. J Clin Pathol **56**(4):310–312.
138. Seymour JF, Gagel RF, Hagemeister FB, Dimopoulos MA, Cabanillas F 1994 Calcitriol production in hypercalcemic and normocalcemic patients with non-Hodgkin's lymphoma. Ann Intern Med **121**(9):633–640.
139. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: summary and description of a working formulation for clinical usage. The Non-Hodgkin's Lymphoma Pathologic Classification Project. Cancer **49**(10): 2112–2135.
140. Cox M, Haddad JG 1994 Lymphoma, hypercalcemia, and the sunshine vitamin. Ann Intern Med **121**(9):709–712.
141. Hewison M, Kantorovich V, Liker HR AJ, Van Herle, Cohan P, Zehnder D, *et al.* 2003 Vitamin D-mediated hypercalcemia in lymphoma: evidence for hormone production by tumor-adjacent macrophages. J Bone Miner Res **18**(3): 579–582.
142. Kremer R, Shustik C, Tabak T, Papavasiliou V, Goltzman D 1996 Parathyroid hormone-related peptide in hematologic malignancies. Am J Med **100**(4):406–411.
143. Dodwell DJ, Abbas SK, Morton AR, Howell A 1991 Parathyroid hormone-related protein (50-69) and response to pamidronate therapy for tumor-induced hypercalcemia. Eur J Cancer **27**(12):1629–1633.
144. Wada S, Kitamura H, Matsuura Y, Katayama Y, Ohkawa H, Kugai N, *et al.* 1992 Parathyroid hormone-related protein as a cause of hypercalcemia in a B-cell type malignant lymphoma. Intern Med **31**(8):968–972.
145. Fetchick DA, Bertolini DR, Sarin PS, Weintraub ST, Mundy GR, Dunn JF 1986 Production of 1,25-dihydroxyvitamin D_3 by human T-cell lymphotrophic virus I-transformed lymphocytes. J Clin Invest **78**(2):592–596.
146. Dodd RC, Winkler CF, Williams ME, Bunn PA, Gray TK 1986 Calcitriol levels in hypercalcemic patients with adult T-cell lymphoma. Arch Intern Med **146**(10):1971–1972.
147. Fukumoto S, Matsumoto T, Ikeda K, Yamashita T, Watanabe T, Yamaguchi K, *et al.* 1988 Clinical evaluation of calcium metabolism in adult T-cell leukemia/lymphoma. Arch Intern Med **148**(4):921–925.
148. Senba M, Kawai K 1992 Hypercalcemia and production of parathyroid hormone-like protein in adult T-cell leukemia-lymphoma. Eur J Haematol **48**(5):278–279.
149. Motokura T, Fukumoto S, Takahashi S, Watanabe T, Matsumoto T, Igarashi T, *et al.* 1988 Expression of parathyroid hormone-related protein in a human T-cell lymphotrophic

virus type I-infected T-cell line. Biochem Biophys Res Commun **154**(3):1182–1188.
150. Moseley JM, Danks JA, Grill V, Lister TA, Horton MA 1991 Immunocytochemical demonstration of PTHrP protein in neoplastic tissue of HTLV-1 positive human adult T-cell leukemia/lymphoma: implications for the mechanism of hypercalcemia. Br J Cancer **64**(4):745–748.
151. Haddad JG Jr, Rojanasathit S 1976 Acute administration of 25-hydroxycholecalciferol in man. J Clin Endocrinol Metab **42**(2):284–290.
152. Kawakami, M, Imawari M, Goodman DS 1979 Quantitative studies of the interaction of cholecalciferol (vitamin D_3) and its metabolites with different genetic variants of the serum-binding protein for these sterols. Biochem J **179**(2):413–423.
153. Shepard R, DeLuca HF 1980 Plasma concentrations of vitamin D_3 and its metabolites in the rat as influenced by vitamin D_3 or 25-hydroxyvitamin D_3 intakes. Arch Biochem Biophys **202**:43–53.
154. Vieth R, McCarten K, Norwich KH 1990 Role of 25-hydroxy-vitamin D_3 dose in determining rat 1,25-dihydroxyvitamin D_3 production. Am J Physiol **258**(5 Pt 1): E780–9.
155. Reinhardt TA, Horst RL 1989 Ketoconazole inhibits self-induced metabolism of 1,25-dihydroxyvitamin D_3 and amplifies 1,25-dihydroxyvitamin D_3 receptor up-regulation in rat osteosarcoma cells. Arch Biochem Biophys **272**(2):459–465.
156. Costa EM, Feldman D 1986 Homologous up-regulation of the 1,25 (OH)2 vitamin D_3 receptor in rats. Biochem Biophys Res Commun **137**(2):742–747.
157. Reinhardt TA, Horst R, Engstrom G, Atkins K 1998 Ketoconazole potentiates 1,25(OH)2D-directed up-regulation of 1,25(OH)2D receptros in rat intestine and bone. Seventh Workshop on Vitamin D, In: Norman AW, *et al.* (eds) Vitamin D: molecular, cellular and clinical endocrinology, de Gruyter: Berlin, p. **233**.
158. Goff JP, Reinhardt TA, Beckman MJ, Horst RL 1990 Contrasting effects of exogenous 1,25-dihydroxyvitamin D [1,25-(OH)2D] versus endogenous 1,25-(OH)2D, induced by dietary calcium restriction, on vitamin D receptors. Endocrinology **126**(2):1031–1035.
159. Reinhardt TA, Horst R 1990 PTH down-regulates 1,25(OH)2D receptors and VDR mRNA *in vitro* and blocks homologous up-regulation of VDR *in vitro*. Endocrinology **127**:942–948.
160. Haussler MR, Cordy PE 1982 Metabolites and analogs of vitamin D. Which for what? JAMA **247**(6):841–844.
161. Beckman MJ, Horst RL, Reinhardt TA, Beitz DC 1990 Up-regulation of the intestinal 1,25-dihydroxyvitamin D receptor during hypervitaminosis D: a comparison between vitamin D_2 and vitamin D_3. Biochem Biophys Res Commun **169**(3):910–915.
162. Beckman MJ, Johnson JA, Goff JP, Reinhardt TA, Beitz DC, Horst RL 1995 The role of dietary calcium in the physiology of vitamin D toxicity: excess dietary vitamin D_3 blunts parathyroid hormone induction of kidney 1-hydroxylase. Arch Biochem Biophys **319**(2):535–539.
163. Haussler MR, 1986 Vitamin D receptors: nature and function. Annu Rev Nutr **6**:527–562.
164. Reinhardt TA, Horst RL 1989 Self-induction of 1,25-dihydroxyvitamin D_3 metabolism limits receptor occupancy and target tissue responsiveness. J Biol Chem **264**(27): 15917–15921.
165. Shigematsu, T, Horiuchi N, Ogura Y, Miyahara T, Suda T 1986 Human parathyroid hormone inhibits renal 24-hydroxylase activity of 25-hydroxyvitamin D_3 by a mechanism involving adenosine 3′,5′-monophosphate in rats. Endocrinology **118**(4):1583–1589.
166. Beckman MJ, Goff JP, Reinhardt TA, Beitz DC 1994 and Horst RL, *In vivo* regulation of rat intestinal 24-hydroxylase: potential new role of calcitonin. Endocrinology **135**(5): 1951–1955.
167. Tryfonidou MA, Oosterlaken-Dijksterhuis MA, Mol JA, van den Ingh T.S, van den Brom WE, Hazewinkel HA 2003 24-Hydroxylase: potential key regulator in hypervitaminosis D_3 in growing dogs. Am J Physiol Endocrinol Metab **284**(3): E505–13.
168. Mallon JP, Matuszewski D, Sheppard H 1980 Binding specificity of the rat serum vitamin D transport protein. J Steroid Biochem **13**(4):409–413.
169. Adams JS, 1984 Specific internalization of 1,25-dihydroxy-vitamin D_3 by cultured intestinal epithelial cells. J Steroid Biochem **20**(4A):857–862.
170. Pettifor JM, Bikle DD, Cavaleros M, Zachen D, Kamdar MC, Ross FP 1995 Serum levels of free 1,25-dihydroxyvitamin D in vitamin D toxicity. Ann Intern Med **122**(7):511–513.
171. Bouillon, RFA, Van Assche H, Van Baelen, Heyns W, De Moor P 1981 Influence of the vitamin D–binding protein on the serum concentration of 1,25-dihydroxyvitamin D_3. Significance of the free 1,25-dihydroxyvitamin D_3 concentration. J Clin Invest **67**(3):589–596.
172. Bikle DD, Siiteri PK, Ryzen E, Haddad JG 1985 Serum protein-binding of 1,25-dihydroxyvitamin D: a reevaluation by direct measurement of free metabolite levels. J Clin Endocrinol Metab **61**(5):969–975.
173. Bikle DD, Gee E, Halloran B, Haddad JG 1984 Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. J Clin Invest **74**(6):1966–1971.
174. Bikle DD, Halloran BP, Gee E, Ryzen E, Haddad JG 1986 Free 25-hydroxyvitamin D levels are normal in subjects with liver disease and reduced total 25-hydroxyvitamin D levels. J Clin Invest **78**(3):748–752.
175. Koenig KG, Lindberg JS, Zerwekh JE, Padalino PK, Cushner HM, Copley JB 1992 Free and total 1,25-dihydroxyvitamin D levels in subjects with renal disease. Kidney Int **41**(1):161–5.
176. Mendel CM, 1989 The free hormone hypothesis: a physiologically-based mathematical model. Endocr Rev **10**(3): 232–274.
177. Edelson GW, Kleerekoper M 1995 Hypercalcemic crisis. Med Clin North Am **79**(1):79–92.
178. Ashizawa, N, Arakawa S, Koide Y, Toda G, Seto S, Yano K 2003 Hypercalcemia due to vitamin D intoxication with clinical features mimicking acute myocardial infarction. Intern Med **42**(4):340–344.
179. Nussbaum SR, 1993 Pathophysiology and management of severe hypercalcemia. Endocrinol Metab Clin North Am **22**(2):343–362.
180. Scarpelli DG, Tremblay G, Pearse AG 1960 A comparative cytochemical and cytologic study of vitamin D–induced nephrocalcinosis. Am J Pathol **36**:331–353.
181. Shetty KR, Ajlouni K, Rosenfled PS, Hagen TC 1975 Protracted vitamin D intoxication. Arch Intern Med **135**(7): 986–988.
182. Rizzoli, R, Stoermann C, Ammann P, Bonjour JP 1994 Hypercalcemia and hyperosteolysis in vitamin D intoxication: effects of clodronate therapy. Bone **15**(2):193–198.
183. Hefti, E, Trechsel U, Fleisch H, Bonjour JP 1983 Nature of calcemic effect of 1,25-dihydroxyvitamin D3 in experimental hypoparathyroidism. Am J Physiol **244**(4): E313–E316.

184. Hass GM, Trueheart RE, Taylor CB, Stumpe M 1958 An experimental histologic study of hypervitaminosis D. Am J Pathol **34**(3):395–431.
185. Swierczynski, J, Nagel G, Zydowo MM 1987 Calcium content in some organs of rats treated with a toxic calciol dosis. Pharmacology **34**(1):57–60.
186. Giunta JL, Dental changes in hypervitaminosis D 1998 Oral Surg Oral Med Oral Pathol Oral Radiol Endod **85**(4):410–413.
187. Selby PL, Davies M, Marks JS, Mawer EB 1995 Vitamin D intoxication causes hypercalcemia by increased bone resorption which responds to pamidronate. Clin Endocrinol (Oxf) **43**(5):531–536.
188. Adams JS, Lee G 1997 Gains in bone mineral density with resolution of vitamin D intoxication. Ann Intern Med **127**(3):203–206.
189. Glass AR, Eil C 1988 Ketoconazole-induced reduction in serum 1,25-dihydroxyvitamin D and total serum calcium in hypercalcemic patients. J Clin Endocrinol Metab **66**(5): 934–938.
190. Kogan BA, Konnak JW, Lau K 1982 Marked hyperoxaluria in sarcoidosis during orthophosphate therapy. J Urol **127**(2): 339–340.
191. Feher JJ, Wasserman RH 1979 Intestinal calcium-binding protein and calcium absorption in cortisol-treated chicks: effects of vitamin D_3 and 1,25-dihydroxyvitamin D3. Endocrinology **104**(2):547–551.
192. Suzuki, Y, Ichikawa Y, Saito E, Homma M 1983 Importance of increased urinary calcium excretion in the development of secondary hyperparathyroidism of patients under glucocorticoid therapy. Metabolism **32**(2):151–156.
193. Lukert BP, Raisz LG 1990 Glucocorticoid-induced osteoporosis: pathogenesis and management. Ann Intern Med **112**(5):352–364.
194. Carre, M, Ayigbede O, Miravet L, Rasmussen H 1974 The effect of prednisolone upon the metabolism and action of 25-hydroxy- and 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA **71**(8):2996–3000.
195. Bilezikian JP, 1989 Etiologies and therapy of hypercalcemia. Endocrinol Metab Clin North Am **18**(2):389–414.
196. O'Leary TJ, Jones G, Yip A, Lohnes D, Cohanim M, Yendt ER 1986 The effects of chloroquine on serum 1,25-dihydroxyvitamin D and calcium metabolism in sarcoidosis. N Engl J Med **315**(12):727–730.
197. Adams JS, Sharma OP, Diz MM, Endres DB 1990 Ketoconazole decreases the serum 1,25-dihydroxyvitamin D and calcium concentration in sarcoidosis-associated hypercalcemia. J Clin Endocrinol Metab **70**(4):1090–5.
198. Adams JS, Kantorovich V 1999 Inability of short-term, low-dose hydroxychloroquine to resolve vitamin D–mediated hypercalcemia in patients with B-cell lymphoma. J Clin Endocrinol Metab **84**(2):799–801.
199. Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ 2003 Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. Am J Clin Nutr **77**(1):204–210.
200. Barger-Lux MJ, Heaney RP 2002 Effects of above average summer sun exposure on serum 25-hydroxyvitamin D and calcium absorption. J Clin Endocrinol Metab **87**(11):4952–4956.
201. Trivedi DP, Doll R, Khaw KT 2003 Effect of four monthly oral vitamin D3 (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: randomized double-blind controlled trial. BMJ **326**(7387):469.
202. Dawson-Hughes B, Dallal GE, Krall EA, Harris S, Sokoll LJ, Falconer G 1991 Effect of vitamin D supplementation on wintertime and overall bone loss in healthy postmenopausal women. Ann Intern Med **115**(7):505–512.
203. Schwartz GG, Whitlatch LW, Chen TC, Lokeshwar BL, Holick MF 1998 Human prostate cells synthesize 1,25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3. Cancer Epidemiol Biomarkers Prev **7**(5):391–395.
204. Shevde NK, Plum LA, Clagett-Dame M, Yamamoto H, Pike JW, DeLuca HF 2002 A potent analog of 1α,25-dihydroxyvitamin D3 selectively induces bone formation. Proc Natl Acad Sci USA **99**(21):13487–13491.
205. Guyton KZ, Kensler TW, Posner GH 2003 Vitamin D and vitamin D analogs as cancer chemopreventive agents. Nutr Rev **61**(7):227–238.
206. Beer TM, Lemmon D, Lowe BA, Henner WD 2003 High-dose weekly oral calcitriol in patients with a rising PSA after prostatectomy or radiation for prostate carcinoma. Cancer **97**(5):1217–1224.
207. Segersten, U, Correa P, Hewison M, Hellman P, Dralle H, Carling T, *et al.* 2002 25-hydroxyvitamin D_3-1α-hydroxylase expression in normal and pathological parathyroid glands. J Clin Endocrinol Metab **87**(6):2967–2972.
208. Lemire JM, 1995 Immunomodulatory actions of 1,25-dihydroxyvitamin D3. J Steroid Biochem Mol Biol **53**(1–6): 599–602.
209. Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, *et al.* 2001 Targeted ablation of the 25-hydroxyvitamin D 1α-hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. Proc Natl Acad Sci USA **98**(13):7498–7503.
210. Dardenne, O, Prud'homme J, Arabian A, Glorieux FH, St Arnaud R 2001 Targeted inactivation of the 25-hydroxyvitamin D_3-1α-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D–deficiency rickets. Endocrinology **142**(7):3135–3141.
211. Mathieu, C, Waer M, Laureys J, Rutgeerts O, Bouillon R 1994 Prevention of autoimmune diabetes in NOD mice by 1,25 dihydroxyvitamin D3. Diabetologia **37**(6):552–558.
212. Zella JB, McCary LC, DeLuca HF 2003 Oral administration of 1,25-dihydroxyvitamin D_3 completely protects NOD mice from insulin-dependent diabetes mellitus. Arch Biochem Biophys **417**(1):77–80.

CHAPTER 79

Extra-renal 1α-Hydroxylase Activity and Human Disease

MARTIN HEWISON Division of Medical Sciences, The University of Birmingham, Queen Elizabeth Medical Centre, Birmingham B15 2TH, UK

JOHN S. ADAMS Division of Endocrinology, Metabolism and Diabetes, Cedars-Sinai Medical Center, Los Angeles, USA

I. INTRODUCTION

The period of time following the publication of the first edition of this book has witnessed some remarkable advances in our understanding of the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase), including the cloning of the gene for this enzyme (CYP1α or CYP27b), the development of knockout animal models, and vastly improved tools for analysis of tissue-specific expression of CYP1α. In this chapter we have incorporated these new developments into the framework of the original chapter on the pathophysiology of dysregulated vitamin D metabolism associated with granuloma-forming and malignant lymphoproliferative disorders. We have placed the seminal observations of extra-renal 1α-hydroxylase activity in diseases, such as sarcoidosis, alongside the current studies that have highlighted a much wider tissue distribution of the enzyme, including epithelial cells. The fundamental structure of the chapter has been retained, but additional sections have been added. After this brief introduction (first section), the second section of the chapter will review the historical aspects of extra-renal synthesis of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) associated with inflammatory disease, including recent studies that have expanded the pathological relevance of extra-renal 1α-hydroxylase. The third section of the chapter will describe what we know about the mechanics and regulation of the vitamin D metabolizing enzymes present in inflammatory cells. The fourth section will recapitulate the potential immunological targets for active vitamin D metabolites and propose a model in which macrophage-derived vitamin D metabolites play a role in the modulation of the local immune responses. The fifth section will provide a comprehensive review of the various human diseases proposed to be associated with the overproduction of active vitamin D metabolites from an extra-renal source. The sixth and final section of this chapter will address the clinical aspects of disordered extra-renal 1α-hydroxylase; this will include a discussion of the diagnosis, treatment, and prevention of hypercalcemia and hypercalciuria in the patient with endogenous vitamin D intoxication.

II. VITAMIN D AND GRANULOMA-FORMING DISEASE: HISTORICAL PERSPECTIVE

A. Evidence of Endogenous Vitamin D Intoxication Associated with Sarcoidosis

A pathophysiological relationship between vitamin D and sarcoidosis was first recognized by Harrell and Fisher in 1939 [1]. Among the six hypercalcemic patients in their initial report, one was observed to experience a steep rise in the serum calcium concentration following ingestion of cod liver oil known to be enriched in vitamin D. Almost two decades passed before Henneman *et al*. [2] demonstrated in 1956 that the hypercalcemic syndrome of sarcoidosis, characterized

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by increased intestinal calcium absorption and bone resorption, was remarkably similar to that of exogenous vitamin D intoxication and was treatable by the administration of glucocorticoids. This is summarized in Section IV of this chapter and is documented in greater detail in Chapters 5, 7, and 93–96. In 1963, Taylor and coworkers [3] performed the first, large-scale seasonal evaluation of serum calcium levels in patients with sarcoidosis. They found that there was a significant increase in the mean serum calcium concentration in 345 patients with sarcoidosis from winter to summer, but no such change in over 12,000 control subjects. This was the first evidence that there was an association between enhanced vitamin D synthesis, known to occur principally during the summer months, and the blood level of calcium in patients with sarcoidosis. This observation was prospectively confirmed by Dent [4], who was able to increase the serum calcium concentration in patients with active sarcoidosis upon exposure to whole body ultraviolet radiation. The Dent study also helped validate the earlier work of Hendrix [5] who achieved resolution of hypercalcemia and hypercalciuria in two patients with sarcoidosis by institution of vitamin D–deficient diets and environmental sunlight deprivation.

B. Evidence for the Extra-renal Overproduction of an Active Vitamin D Metabolite

The above mentioned studies led Bell *et al.* [6] to propose in 1964 that development of a clinical abnormality in calcium balance in patients with active sarcoidosis resulted from an increase in target organ responsiveness to vitamin D. This view persisted for more than a decade. However, after the discovery of $1,25(OH)_2D$ as the active vitamin D hormone [7–9] and the development of sensitive and specific assays for the hormone in blood [10–12], investigators were quick to determine that the hypercalcemia of sarcoidosis was the result of an increase in the circulating concentrations of a vitamin D metabolite that interacted with the vitamin D receptor (VDR) [13–16]. The fact that a vitamin D hormone was made outside the kidney in hypercalcemic patients with sarcoidosis was first discovered by Barbour and colleagues in 1981 [17]. These investigators reported high concentrations of a vitamin D metabolite detected as $1,25(OH)_2D$ in the circulation of a hypercalcemic, anephric patient with active sarcoidosis. Two years later, Adams *et al.* [18] determined the macrophage to be the extra-renal source of the active vitamin D metabolite. Unequivocal structural characterization of the metabolite as $1,25(OH)_2D$ was obtained by these same investigators in 1985 [19].

C. Cloning of the CYP1α Gene Provides New Perspectives for Extra-renal Synthesis of $1,25(OH)_2D$

The original studies describing synthesis of $1,25(OH)_2D$ by activated macrophages and the potential consequences of this with respect to inflammatory diseases such as sarcoidosis has stimulated a much broader appreciation of extra-renal activation of vitamin D. This is summarized in section IV of this chapter and is documented in greater detail in Chapters 7, 93, 94, and 95. Further investigation of macrophage 1α-hydroxylase activity also highlighted several crucial differences between the activity of the enzyme in these cells when compared to its classical renal counterpart. For example, the macrophage 1α-hydroxylase is not subject to the exquisite autoregulation characteristic of its kidney counterpart, raising the possibility that renal and extra-renal synthesis of $1,25(OH)_2D$ is catalyzed by distinct enzymes. This and other mechanistic features of extra-renal 1α-hydroxylase are discussed in section III of this chapter. The most significant contributing factor to our current understanding of extra-renal $1,25(OH)_2D$ production has been the cloning of the gene for 1α-hydroxylase (CYP1α). After initial isolation of the mouse gene (Cyp1α) from renal tissue [20], it is notable that the human homolog (CYP1α) was cloned from keratinocytes, a well-established extra-renal site for $1,25(OH)_2D$ production [21]. That this gene was identical to that in the kidney strongly supported the notion of a single but differentially regulated 1α-hydroxylase protein in renal and extra-renal tissues. Further support for this postulate was provided by Mawer and colleagues who showed that macrophages from patients harboring mutations in the CYP1α gene had impaired levels of $1,25(OH)_2D$ production similar to that observed in the renal enzyme [22]. The availability of sequence information has also facilitated the development of specific antisera and probes for 1α-hydroxylase. This has further emphasized the widespread tissue distribution of 1α-hydroxylase, but has also helped to confirm the identity between the renal and extra-renal enzymes [23,24]. Advances in our understanding of extra-renal 1α-hydroxylase have also led to its implication in diseases beyond the original observation of abnormal synthesis of $1,25(OH)_2D$ in some patients with sarcoidosis. A key development has been the expression and function of 1α-hydroxylase in breast, prostate, and colon cancer, and this is discussed in greater detail in Chapters 93–95. In the remainder of this chapter, we will focus on the established link between extra-renal 1α-hydroxylase and granulomatous diseases.

III. PATHOPHYSIOLOGY OF DISORDERED CALCIUM BALANCE IN SARCOIDOSIS: A MODEL FOR THE EXTRA-RENAL PRODUCTION OF AN ACTIVE VITAMIN D METABOLITE IN HUMAN DISEASE

A. Clinical Evidence for Dysregulated Overproduction of the Vitamin D Hormone

As has been described in detail in earlier chapters, the synthesis of $1,25(OH)_2D$ by the renal 1α-hydroxylase is normally strictly regulated with levels of the hormone product being some 1000-fold less plentiful in the circulation than that of the principal substrate for the enzyme, 25-hydroxyvitamin D (25OHD). Hormone synthesis in the kidney is stimulated by an increase in the serum parathyroid hormone (PTH) concentration, a decrease in the serum phosphate concentration, and a decrease in the activity of the competing vitamin D 24-hydroxylase (24-hydroxylase). Synthesis of $1,25(OH)_2D$ is inhibited by a decrease in the circulating PTH levels, increased serum phosphate, and increased 24-hydroxylase activity. There are now at least four clear lines of clinical evidence to indicate that endogenous $1,25(OH)_2D$ production in hypercalcemic/hypercalciuric patients with sarcoidosis is dysregulated and not bound by the same set of endocrine factors known to regulate $1,25(OH)_2D$ synthesis in the kidney [25].

First, hypercalcemic patients with sarcoidosis possess a frankly high or inappropriately elevated serum $1,25(OH)_2D$ concentration, although their serum PTH level is suppressed and their serum phosphate concentration is relatively elevated [26,27]. If $1,25(OH)_2D$ synthesis were under the regulation of PTH, phosphate, and $1,25(OH)_2D$ itself, then $1,25(OH)_2D$ concentrations in such patients should be low. Second, the serum $1,25(OH)_2D$ concentration in patients with active sarcoidosis is very sensitive to an increase in available substrate [28], while the serum $1,25(OH)_2D$ level in normal individuals is not influenced by small or even moderate increments in the circulating 25OHD concentration. Clinically, this aspect of dysregulation is manifest by the long-recognized association of the appearance of hypercalciuria and/or hypercalciuria in sarcoidosis patients in the summer months or following holidays to geographic locations at lower latitudes than those at which the patient normally resides [28,29]. This link between an increase in cutaneous vitamin D synthesis and the development of a clinical abnormality in calcium balance can be replicated by the oral administration of vitamin D [15,27,30] to such patients. It can also be substantiated on a biochemical basis by demonstration of a positive correlation between the serum 25OHD and $1,25(OH)_2D$ concentrations in patients with active sarcoidosis, but not in normal human subjects [31].

Third, the rate of endogenous $1,25(OH)_2D$ production, which is significantly increased in patients with sarcoidosis [32], is unusually sensitive to inhibition by factors (i.e. drugs) that do not influence the renal 1α-hydroxylase at the same doses. Anti-inflammatory concentrations of glucocorticoids have long been recognized as effective combatants of sarcoidosis-associated hypercalcemia and have also been shown to dramatically lower elevated $1,25(OH)_2D$ levels [30,31,33]. On the other hand, administration of the same glucocorticoid doses to patients without sarcoidosis is not associated with a clinically relevant reduction in the serum $1,25(OH)_2D$ or calcium concentration. Chloroquine and its hydroxylated analog, hydroxychloroquine, are other examples of pharmaceutical agents that appear to act preferentially on the extra-renal vitamin D-1α-hydroxylase reaction, which is active in patients with sarcoidosis [34–36]. Fourth, the serum calcium and $1,25(OH)_2D$ concentrations are positively correlated to indices of disease activity in patients with sarcoidosis [37–39]; patients with widespread disease and high angiotensin-converting enzyme (ACE) activity are more likely to be hypercalciuric or frankly hypercalcemic.

B. Correlates *In Vitro* for Dysregulated $1,25(OH)_2D$ Production *In Vivo*

Investigators have now generated a substantial body of experimental data from cells, including inflammatory cells harvested directly from patients with sarcoidosis, to indicate that the dysregulated vitamin D hormone synthesis in sarcoidosis is probably not due to expression of a 1α-hydroxylase that is different from the renal enzyme, but rather to expression of the authentic 1α-hydroxylase in a macrophage, not a kidney cell [22]. In fact, each of the above mentioned pieces of clinical evidence for dysregulated vitamin D hormone production in this disease can be borne out *in vitro* in cells from patients with this disease [40].

1. Subcellular Localization, Substrate Selectivity, and Kinetics of the Macrophage Vitamin D-1-hydroxylase

As is the case with the 1α-hydroxylase of renal origin, the macrophage enzyme is a mitochondrial mixed function oxidase with detectable cytochrome P450 activity [41] (see Fig. 1). Like the renal 1α-hydroxylase reconstituted from mitochondrial extracts, the presence

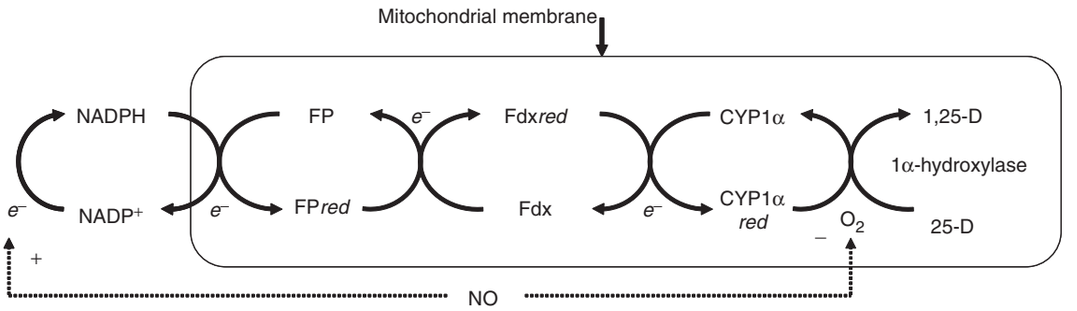

FIGURE 1 Mitochondrial electron transport associated with the vitamin D-1α-hydroxylase reaction: a model for interaction with nitric oxide (NO). NADPH supplies the electron transport chain of accessory proteins associated with 1α-hydroxylase, consisting of a flavoprotein reductase (FP), a ferredoxin (Fdx), and the 1α-hydroxylase cytochrome P450 (CYP1α). A stimulatory effect on the enzyme may also be mediated by relatively low intracellular NO levels. An electron *(e⁻)* generated from NO is donated to oxidized NADP, thus forming NADPH. On the other hand, the inhibitory effect on the enzyme which occurs at relatively high NO levels in the cell results from competition with O_2 binding to the P450 heme group, inhibiting the enzyme.

of a flavoprotein, ferredoxin reductase, an electron source, and molecular oxygen (O_2) are all required for electron transfer to the cytochrome P450 and for the insertion of an oxygen atom in the substrate [41]. Also, like the renal 1α-hydroxylase, we now know the macrophage 1α-hydroxylase is inhibited by the napthoquinones, molecules which compete with reductase for donated electrons, and by the imidazoles, compounds which compete with the enzyme for receipt of O_2 [42]. Similar to the 1α-hydroxylase isolated from the mitochondria of proximal renal tubular epithelial cells, the macrophage enzyme requires a secosterol (vitamin D sterol molecule with an open B-ring) as substrate [43]. Also similar to the renal 1α-hydroxylase, the macrophage enzyme has a particular affinity for secosterols bearing a carbon-25 hydroxy group as is encountered in the two preferred substrates for this enzyme, 25OHD and 24,25-dihydroxyvitamin D (24,25$(OH)_2$D) [43,44]; the calculated Km (affinity) of the 1α-hydroxylase in pulmonary alveolar macrophages derived directly from patients with active sarcoidosis is in the range of 50–100 nM for these two substrates [43,44]. The availability of cDNA sequences for 1α-hydroxylase expression studies has shed more light on the catalytic properties of the enzyme (20,21,45,46) but, as yet, has failed to provide a clear mechanism for the differential regulation of 1,25$(OH)_2$D production in renal and extra-renal tissues. Some of the potential explanations for this are discussed in the following sections.

2. MACROPHAGES LACK RESPONSIVENESS TO PTH, CALCIUM, PHOSPHATE

In vivo there appear to be three major regulators of the renal 1α-hydroxylase—the serum concentration of calcium, parathyroid hormone, and phosphate [47] (left panel, Fig. 2). Hypocalcemia enhances the activity of the renal 1α-hydroxylase, but much of this stimulatory effect may be indirectly mediated through parathyroid hormone (PTH). Any decrease in the serum calcium concentration below normal is a stimulus for increased secretion of PTH [48] which, in turn, is a direct stimulator of the renal 1α-hydroxylase [49]. Recent promoter-reporter analyses have shown that both PTH and calcitonin stimulate transactivation of 1α-hydroxylase [50,51], although other studies have suggested that PTH can also effect changes in 1,25$(OH)_2$D production via transient alteration in the phosphorylation status of the ferredoxin, which contributes electrons to 1α-hydroxylase [52]. A change in the serum phosphate concentration is the other major regulator of renal 1,25$(OH)_2$D production; in adult humans, dietary phosphorus restriction causes an increase in circulating concentrations of 1,25$(OH)_2$D to 80% above control values, an increase not due to accelerated metabolic clearance of this hormone [53]. Dietary phosphorous supplementation will have the opposite effect. Although the mechanism by which a drop in the serum phosphate level will increase renal 1,25$(OH)_2$D production remains uncertain [54], there is no doubt that there exists a concerted, cooperative attempt of the calcium-phosphorous-PTH axis in man to regulate the conversion of 25OHD to 1,25$(OH)_2$D in the kidney. For example, a drop in the serum calcium concentration will be immediately registered by the parathyroid cell calcium receptor, which will release its inhibition on PTH production and secretion. An increase in the circulating PTH will directly stimulate the renal 1α-hydroxylase, while a PTH-mediated phosphaturic response and a subsequent decrement in

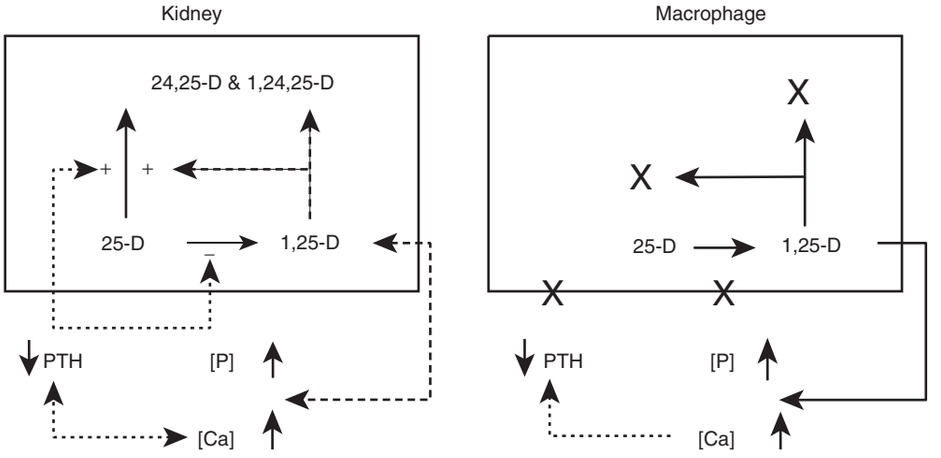

FIGURE 2 Model distinguishing the regulation of 1α-hydroxylase in the proximal renal tubular epithelial cell of the kidney (**left panel**) and in the granuloma-forming disease-activated macrophage (**right panel**). In the kidney, the enzymatic conversion of substrate 25-hydroxyvitamin D (25-D) to product 1,25-dihydroxyvitamin D (1,25-D) is subject to negative feedback control with down-regulation of enzyme activity under the influence of 1) a calcium-mediated decrease in the circulating parathyroid (PTH); 2) a 1,25-D-mediated increase in the serum phosphate [P] level; and 3) a 1,25-D–mediated increase in vitamin D 24-hyroxylase activity (24,25-dihydroxyvitamin D (24,25-D) and 1,24,25-trihydroxyvitamin D (1,24,25-D) production). The macrophage lacks responsiveness to changes in the extracellular PTH and [P] and harbors little or no detectable vitamin D-24-hydroxylase (lack of regulation designated **X**).

the serum phosphate level will indirectly promote $1,25(OH)_2D$ production.

The macrophage 1α-hyroxylase, on the other hand, is immune to the stimulatory effects of PTH and phosphate [42,55] (right panel, Fig. 2). The macrophage plasma membrane is not enriched with PTH receptors [56], and there is no evidence that any PTH receptors which are resident in the macrophage membrane are responsive to PTH or PTHrP in terms of stimulating the protein kinase signaling pathways that are associated with stimulation of the renal 1α-hydroxylase. Similarly, the macrophage enzyme appears to be uninfluenced by changes in the extracellular phosphate concentration [42]. Moreover, exposure of activated macrophages expressing 1α-hydroxylase to a calcium ionophore stimulates the hydroxylation reaction [57], while increasing the extracellular calcium concentration has the opposite, inhibitory effect on the renal 1α-hydroxylase [58]. These observations appear to confirm the fact that the three most important extracellular signaling systems for the renal 1α-hydroxylase are not heeded by the macrophage enzyme and provide an explanation for why $1,25(OH)_2D$ production by the macrophage in diseases like sarcoidosis is not subject to negative feedback control as reflected by a drop in the serum PTH concentration and an increase in the circulating calcium and phosphate level. Furthermore, with the possible exception of insulin-like growth factor-1 (IGF-1) [59], there is no evidence that the macrophage 1α-hydroxylation reaction is influenced by any of the other endocrine factors, including estrogen, prolactin, and growth hormone, purported to increase the renal production of $1,25(OH)_2D$ [60–63]. By contrast, macrophage 1α-hydroxylase activity is potently inhibited by anti-inflammatory agents, such as glucocorticoids, which have little or no effect on the renal enzyme. *In vivo*, this is likely to be due in part to the effects of glucocorticoids on macrophage differentiation and apoptosis. However, studies *in vitro* suggest that there is also direct inhibition of macrophage 1α-hydroxylase activity by glucocorticoids [40].

3. Macrophages Lack $1,25(OH)_2D$-directed 24-hydroxylase Activity

The other major contributor to the circulating $1,25(OH)_2D$ level is the activity of 24-hydroxylase. Like the 1α-hydroxylase, 24-hydroxylase is a heme-binding mitochondrial enzyme requiring NADPH, molecular oxygen, and magnesium ions [52,64]. The cDNA and gene sequences for human, rat, and chicken 24-hydroxylase, now referred to as CYP24/Cyp24, were cloned several years prior to CYP1α [65–67].

As depicted in Fig. 2 (left panel), expression of CYP24 is stimulated in kidney cells by $1,25(OH)_2D$, especially if the protein kinase C (PKC) pathway is also up-regulated [68–70]. PTH appears to exert an opposite, inhibitory effect on CYP24 gene transcription and $24,25(OH)_2D$ synthesis [71]. There is dual impact of this mitochondrial, cytochrome P450-linked enzyme system on vitamin D and calcium balance in adult animals, including man. Because it is coexpressed in the kidney along with the 1α-hydroxylase, the first point of impact is on regulation of substrate 25OHD available to the 1α-hydroxylase. Like the 1α-hydroxylase, the 24-hydroxylase exhibits a preference for 25-hydroxylated secosterol substrates [72]. Although its affinity for 25OHD is reported to be somewhat less than that of renal 1α-hydroxylase, its capacity for substrate is substantially greater [44]. Hence, when up-regulated under the influence of circulating or locally-produced $1,25(OH)_2D$ or diminished serum PTH levels, the 24-hydroxylase has the capacity to compete with 1α-hydroxylase for substrate 25OHD. Under physiological conditions, this state of competitive substrate deprivation for the 1α-hydroxylase will persist until the serum calcium and PTH concentration are normalized. The second point of impact of the vitamin D-24-hydroxylase on the circulating $1,25(OH)_2D$ concentration is at the level of catabolism of $1,25(OH)_2D$ itself. Although both 25OHD and $1,25(OH)_2D$ are metabolized by 24-hydroxylase [73], current data strongly suggest that the latter is the preferred substrate [64]. Considering the fact that the 24-hydroxylase is the initial step in the conversion of $1,25(OH)_2D$ to non-biologically-active, water-soluble, excretable metabolites of the hormone, up-regulation of this enzyme will contribute to the lowering of $1,25(OH)_2D$ hormone levels. In contrast to precursor monocytic cells, the macrophage lacks detectable 24-hydroxylase activity (Fig. 2, right panel) [43]. Therefore, unlike the renal tubular epithelial cells and indeed other epithelial cells [74], macrophages do not possess the capability of shunting substrate 25OHD or the 1α-hydroxylase product $1,25(OH)_2D$ down the catabolic 24-hydroxylase pathway. The net result is dysregulated overproduction of $1,25(OH)_2D$ by the macrophage, escape of the hormone into the general circulation, and the eventual development of hypercalcemia.

4. MACROPHAGE 1α-HYDROXYLASE EXHIBITS RESPONSIVENESS TO IMMUNE CELL REGULATORS

The lack of negative feedback control on the 1α-hydroxylase expressed in the macrophage as just described can account for the failure to appropriately inhibit $1,25(OH)_2D$ synthesis in inflammatory diseases like sarcoidosis. However, this does not adequately explain the fact that $1,25(OH)_2D$ production rates are increased well above normal in patients with these diseases at a time when the renal 1α-hydroxylase is inhibited. This observation suggests that there must be an alternative, "nonclassical" set of factors that stimulate the synthesis of $1,25(OH)_2D$ by the macrophage but not by the kidney.

Clinical observations from a number of investigative groups around the world indicate that sarcoidosis patients with diffuse, infiltrative pulmonary disease are at greater risk to develop dysregulated vitamin D metabolism. Cultured pulmonary alveolar macrophages (PAM) from such patients were more likely to synthesize more $1,25(OH)_2D$ *in vitro* on a per cell basis than PAM from a host with less intense or no alveolitis [75,76]. These results led to the conclusion that the specific activity of the 1α-hydroxylase reaction in macrophages from patients with active pulmonary sarcoidosis was regulated by endogenously-synthesized factors, which also modulated the intensity of the host immune response. Of the various bioactive cytokines concentrated in the alveolar space of patients with active sarcoidosis [77,78], IFNγ was found to be the principal cytokine stimulator of the sarcoid macrophage 1α-hydroxylation reaction [77]; by itself at maximally effective concentrations *in vitro*, IFNγ increased basal hydroxylase activity over four-fold. However, it is now clear that other immunomodulators are also able to stimulate macrophage 1α-hydroxylase, including other cytokines such as tumor necrosis factor α (TNFα) [43,79] and interleukin-2 (IL-2) [55], as well as pathogen-associated peptides such as bacterial lipopolysaccharide (LPS) [42,55]. As all of these factors have been implicated in the maturation of macrophage responsiveness within the innate immune system, it seems likely that up-regulated 1α-hydroxylase activity is a common feature of activated macrophages. However, two key questions remain: first, is there a specific mechanism involved in up-regulation of macrophage 1α-hydroxylase activity; second, why is macrophage 1α-hydroxylase activity pathologically elevated in patients with inflammatory diseases, such as sarcoidosis? These issues are addressed in the following sections.

a. Cytokines Despite the advent of gene sequence information for CYP1α and in particular promoter analyses for classical 1α-hydroxylase regulators such as PTH, our current understanding of the molecular mechanisms involved in regulating macrophage 1α-hydroxylase activity is still poor. It seems likely that several pathways are involved—for example, IFNγ signals via Janus Kinase 1 (JAK1) and JAK2 with subsequent phosphorylation of signal transducers and activators of transcription alpha (STAT alpha) and subsequent transregulation of target genes via cis-acting

promoter elements [80]. However, the JAK/STAT pathway is essential for the effects of many cytokines and growth factors, including some members of the interleukin family (e.g. IL-2 and IL-6) [81]. The JAK/STAT system may also interact with other signaling pathways, including p38 mitogen-activated protein kinase (MAP kinase) and nuclear factor-κB (NF-κB) [82,83]. The net effect of this extensive cross-talk means that a variety of factors, including the cytokines outlined above together with appropriate growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), may be able to stimulate 1α-hydroxylase in macrophages. Disappointingly, the availability of CYP1α gene sequence information and promoter-reporter constructs has shed no further light on the nuclear transactivation factors that mediate cytokine-induced synthesis of $1,25(OH)_2D$. Nevertheless, the CYP1α promoter region includes putative AP-1 and NF-κB binding sites, which are potential targets for cytokine-regulation of 1α-hydroxylase [50,51].

Alternatively signaling via cytokines such as IFNγ may lead to the activation of other, calcium-dependent pathways in the macrophage, specifically the protein kinase C (PKC) [84] and phospholipase A2 (PLA2) pathways [85,86]. Because the macrophage 1α-hydroxylase was not influenced by attempts to directly stimulate or inhibit PKC, attention has focused on the PLA2 pathway and the endogenous arachidonic acid metabolic cascade as the signal transduction pathway of most influence over the macrophage enzyme. Further dissection of the intracellular arachidonate metabolic pathway in this cell demonstrated that signal transduction through the 5-lipoxygenase pathway, specifically with the generation of leukotriene C4 (LTC4), was most critical to an increase in $1,25(OH)_2D$ synthesis [87]. These studies were extended to investigate another compound with potential actions in the PLA2-arachidonic acid pathway, the 4-amino quinoline derivative chloroquine. $1,25(OH)_2D$ synthesis by macrophages was completely inhibited by exposure to 10^{-6} M chloroquine *in vitro* [36]. Furthermore, this effect is independent of chloroquine's apparent ability to alter the pH of intracellular organelles. When given orally to a hypercalcemic patient with sarcoidosis, chloroquine [34,36] or its analog hydroxychloroquine [35] can effectively reduce the serum $1,25(OH)_2D$ and calcium concentration within a matter of 36 hours.

b. Lipopolysaccharide (LPS) LPS is a bioactive lipid extractable from the cell wall of infectious microorganisms, including the mycobacterium. On macrophages, LPS interacts with a complex that includes the cell surface CD14 receptor and toll-like receptor 4 (TLR4), together with the accessory proteins MD-2 and MyD88 [88]. TLR4 is one of ten TLR proteins, similar to the TNF receptor family, that function as pathogen-recognition receptors and which signal via NF-κB and p38 MAP kinase [89]. TLR4/CD14 is strongly expressed on cells from the immune system, including macrophages and dendritic cells (DCs), and LPS is a potent inducer of 1α-hydroxylase in human monocyte/macrophage-like cells [42,55] (Fig. 3). TLRs are also expressed by epithelial cells at "barrier" sites, including the skin, lungs, gastrointestinal tract, and distal nephron. Here, as with macrophages, TLR expression is able to support LPS inducibility of $1,25(OH)_2D$ production. In recent studies, Hewison and co-workers demonstrated the presence of CD14 and TLR4 on cortical collecting duct HCD cells, but not proximal tubule HKC-8 cells [90]. As a consequence, HCD cells showed potent induction of 1α-hydroxylase activity in response to both PTH and LPS, while HKC-8 cells responded to PTH alone. Thus, it seems likely that TLR expression and signaling acts as a pivotal mechanism in regulating extra-renal 1α-hydroxylase activity. In fact, the most reproducibly effective stimulation of the macrophage 1α-hydroxylase

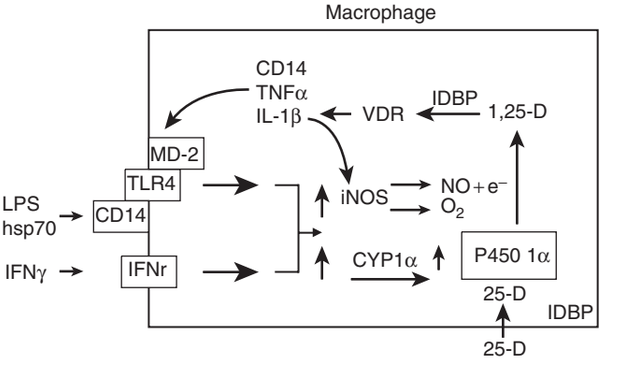

FIGURE 3 Proposed mechanism for the amplification of macrophage 1α-hydroxylase. Macrophage stimulatory agents lipopolysaccharide (LPS), interferon-γ (IFNγ), and heat-shock proteins (hsps) with their respective cell-surface receptor molecules (LPS and hsps: CD14, toll-like receptor 4 (TLR4), MD-2 complex. IFNγ: IFN receptor (IFNr). This leads to: 1) up-regulation of CYP1α mRNA expression and increased levels of 1α-hydroxylase protein (P4501α); 2) up-regulation of iNOS expression. Nitric oxide (NO) is synthesized from an extracellular source of molecular oxygen (O_2) and L-arginine (L-arg). NO can serve as an electron-donating source for enzymatic conversion of 25-hydroxyvitamin D (25-D) to 1,25-dihydroxyvitamin D (1,25-D). 3) increased conversion of 25-D to 1,25-D. The transport of 25-D to mitochondrial 1α-hydroxylase and 1,25-D to nucleus is facilitated by hsp70-like intracellular vitamin D–binding proteins (IDBP). NO and 1,25-D act in an intracrine fashion to up-regulate expression of the cytokines interleukin-1β (IL-1ß), tumor necrosis factor α (TNFα), and the LPS receptor molecules; TNF and CD14 promote intracrine stimulation of NO and 1,25-D synthesis.

in vitro is achieved by coexposure of macrophages to IFNγ and LPS [55]. LPS and IFNγ commonly activate different signal transduction pathways, but as outlined previously, there is potential for cross-talk between these pathways, which may have a significant impact on transactivation of CYP1α. Notably, IFNγ and LPS are also the two most effective stimulators of nitric oxide (NO) synthesis in macrophages, and this led Adams and co-workers to hypothesize that production of NO and $1,25(OH)_2D$ in macrophage-like cells may be functionally linked [91–93].

c. Nitric Oxide (NO) The generation of NO in the macrophage is under the control of the enzyme inducible nitric oxide synthase (iNOS) [94]. In contrast to the more stringently regulated, constitutively expressed isoforms of the enzyme (cNOS) that are localized to endothelial cells and neurons, are regulated by calcium, and are capable of producing only modest amounts of NO, the calcium-independent iNOS remains tonically active when "induced" and is capable of generating large quantities of NO in and around the cell [95]. It is therefore interesting that two of the major stimulators of the human macrophage 1α-hydroxylase, IFNγ and LPS, are also key transcriptional regulators of the iNOS gene [96,97], which is itself a cytochrome P450-linked oxidase [98]. These observations coupled with the fact that NO has established inhibitory effects on other cytochrome P450s [99,100] suggested a possible link with the enzymes involved in vitamin D metabolism. Data indicate that NO may act as an alternative to NADPH as a source of unpaired electrons for the 1α-hydroxylase reaction in macrophages [91,92]. However, as the amount of NO generated inside the macrophage continues to increase, there is a reflex decrease in $1,25(OH)_2D$ production [93], suggesting that there is a built-in limit on the ability of the cell to produce active vitamin D. This inhibitory effect of NO on the macrophage 1α-hydroxylase is almost certainly due to competition of NO with oxygen for binding to the heme center of the enzyme. A similar effect has been very recently demonstrated for a number of heme-containing enzymes [99,100], including those involved in steroid hormone metabolism [101].

d. Other Potential Regulators of the Macrophage 1α-hydroxylase Another potential autoregulator of macrophage $1,25(OH)_2D$ synthesis is the stress-induced heat shock-70 (hsp70) family of proteins [102,103]. These proteins are ubiquitously distributed in the nuclear, cytoplasmic, mitochondrial, and endoplasm reticular compartments of eukaryotic cells. Hsp70s were first recognized as heat-shock-responsive ATP-binding proteins with ATPase activity [104]. Stress-induced proteins have a well-established link with immune responses and inflammatory disease [104,105]. This stems first from the fact that peptides bound or linked to HSPs can elicit potent antigen-specific immunity [106]. In addition, proteins such as hsp70 are able to stimulate cells of the innate immune system directly, and thus act as "danger"-signaling molecules in a similar fashion to LPS [107]. These observations are supported by studies which have shown that TLRs act as exogenous and enodogenous signal transduction pathways for hsp responses [108,109]. A specific link between hsp/TLR signaling and vitamin D metabolism has yet to be studied, but it seems likely that hsps will act as important regulators of the macrophage 1α-hydroxylase. This may be due in part to TLR/NF-κB/p38 MAP kinase-mediated transcriptional regulation as detailed in section 4.b. However, hsps are also characterized functionally by their ability to bind and release hydrophobic segments of an unfolded polypeptide chain in an ATP-hydrolytic reaction cycle [103]. This so-called "chaperone" function of hsp70 is critical for a number of intracellular protein-protein interactions [105], and in the targeting and translocation of molecules across the endoplasmic and mitochondrial membrane [105,110–112].

In macrophage-like cells, hsp70 expression is known to be induced by both physical (i.e. heat) and cytokine (i.e. IFN) stimuli, but its expression is also dramatically enhanced by $1,25(OH)_2D$ [113]. Furthermore, in a series of studies we have shown that proteins from the hsp70 family have a high capacity for intracellular binding of 25-hydroxylated vitamin D metabolites [114]. These hsp70 homologs now termed *intracellular vitamin D–binding proteins* (IDBPs) were originally identified as vitamin D–resistant cells from New World primates [115]. Initially, these proteins were thought to be the functional basis for the end-organ resistance to $1,25(OH)_2D$ in New World primates. However, subsequent data have shown that the IDBPs are in fact potent activators of $1,25(OH)_2D$–induced gene transactivation [116]. Rather, the underlying cause of vitamin D resistance in New World primates has now been shown to be due to constitutive overexpression of a vitamin D response element-binding protein (VDRE-BP) from the heterogeneous nuclear ribonucleoprotein in the A family (see Chapter 21), with the IDBPs acting as a putative compensatory mechanism by acting as intracellular chaperones for $1,25(OH)_2D$ [117]. Crucially, cells overexpressing IDBPs also showed increased synthesis of $1,25(OH)_2D$, indicating that their chaperone activity was not restricted to increased VDR-mediated transactivation [116]. Thus, it is possible that up-regulated IDBP expression in disease-activated macrophages is another key factor in the increased synthesis of $1,25(OH)_2D$ by these cells. Because of its

capacity to bind 25OHD as well as $1,25(OH)_2D$, hsp70/IDBP may serve to concentrate, on a relatively low-affinity, high-capacity binding protein, substrate for the 1α-hydroxylase from the general circulation. In fact, by virtue of their organelle-targeting sequences, hsp70s or related molecules may be critical in the directed translocation of 25OHD to the inner mitochondrial membrane where the 1α-hydroxylase actually resides. In this respect, IDBPs may provide a crucial link between the intracellular organelles and endocytic proteins, such as megalin, which are known to act as an interface with serum-bound steroid hormones [118]. Megalin is strongly expressed in the proximal renal tubules where it plays a pivotal role in transporting 25OHD [119], but its role in directing macrophage metabolism of vitamin D remains unclear. The actions of megalin are discussed in greater detail in Chapter 10.

IV. LOCAL IMMUNOREGULATORY EFFECTS OF ACTIVE VITAMIN D METABOLITES

A. Intracrine/Autocrine Action on the Monocyte/Macrophage

The immunomodulatory properties of $1,25(OH)_2D$ and synthetic analogs of vitamin D are discussed extensively in Chapter 36, which also outlines potential therapeutic applications for auto-immune disease and host-graft rejection. However, a key question remains as to the normal physiological function of locally synthesized $1,25(OH)_2D$. Furthermore, what is the purpose of $1,25(OH)_2D$ production in patients with granuloma-forming disease like sarcoidosis? Is local synthesis of the hormone by macrophages beneficial or detrimental to the host? These are important questions that investigators in a number of centers around the world have been addressing since the 1980s when it became known that the activated, circulating monocytes and tissue macrophages expressed the VDR [120,121]. Expression of the receptor for the active vitamin D hormone indicated that the macrophage could actually be a target for the $1,25(OH)_2D$ that the cell itself was making. Indeed, investigators have suggested that $1,25(OH)_2D$ has the potential to interact with the monocyte/macrophage in either an intracrine or autocrine mode [122,123] (left panel, Fig. 4). For example, incubation with a VDR-saturating concentration of $1,25(OH)_2D$ increases IL-1ß expression by eight-fold and decreases by 1000-fold the concentration of stimulator lipopolysaccharide (LPS) required to achieve maximal IL-1ß gene expression [124]. This extraordinary priming effect of the $1,25(OH)_2D$ for LPS stimulation of the IL-1 gene can also be observed for another monokine gene product, TNF [125] and is due to $1,25(OH)_2D$–mediated induction of the gene for CD14, which acts as an accessory receptor with TLR4.

The multiplicity and complexity of actions of $1,25(OH)_2D$ on the macrophage are detailed in Chapter 36. Suffice it to say that it is now widely accepted that the actions of hormone are directed toward stimulation of macrophage function. For example, $1,25(OH)_2D$ is known to enhance giant cell formation [126], monokine production [127–129], and cytotoxic function [130,131]. Conversely, $1,25(OH)_2D$ acts as a

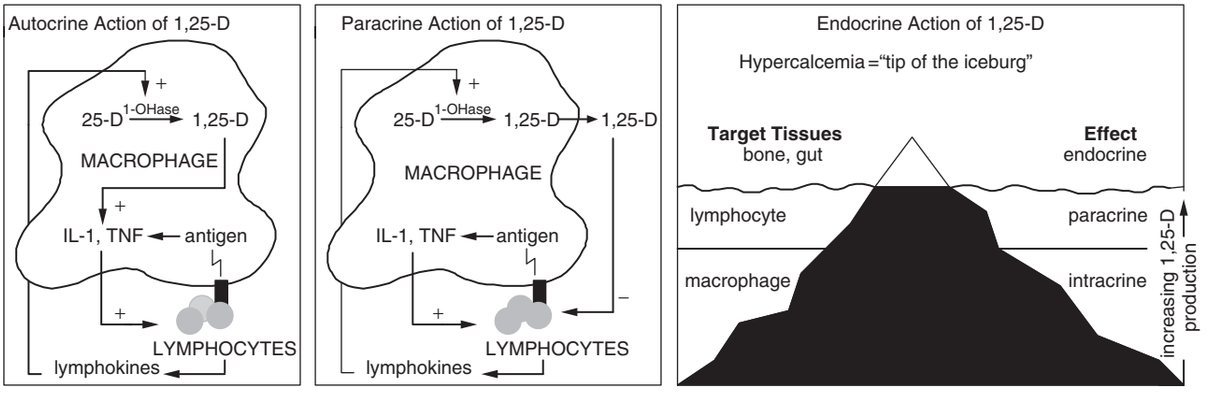

FIGURE 4 Schemes for local production and action of macrophage-derived 1,25-dihydroxyvitamin D (1,25-D). In an autocrine or intracrine mode (**left panel**), 1,25-D promotes antigen handling and monokine production. In a paracrine mode (**middle panel**), 1,25-D acts in a negative feedback fashion to "brake" what may turn out to be an overexuberant lymphocyte response to presented antigen and local monokines. If the immune response and 1,25-D production are persistent, then the hormone can escape the local inflammatory microenvironment and act in an endocrine mode (**right panel**) to alter host calcium balance.

potent suppressor of antigen presentation by both macrophages [132] and other professional antigen presenting cells (APCs), such as dendritic cells (DCs) [133]. The latter has attracted considerable recent attention because of the link between vitamin D responsiveness in DCs and macrophages. Specifically, the ability of $1,25(OH)_2D$ and vitamin D analogs to inhibit DC function appears to be dependent on the suppression of DC differentiation, thereby maintaining DCs in an immature, immune tolerant state [134]. Importantly, studies *in vitro* indicate that this is accompanied by a reinduction of the macrophage marker CD14 [135,136], suggesting that the suppressive effects of $1,25(OH)_2D$ on DCs are counterbalanced by enhanced macrophage development/function. In this way, $1,25(OH)_2D$ may play an important role in modulating the balance between innate (macrophage/phagocytic) and acquired (DC/APC) immune responses. The significance of this has been further underlined through recent studies by us and others who have shown that monocyte-derived DCs express the same 1α-hydroxylase as renal cells and macrophages and are able to synthesize significant levels of $1,25(OH)_2D$ [137,138]. As a result, DC differentiation and function was potently suppressed by 25OHD, as well as $1,25(OH)_2D$, suggesting that this may be the pivotal mechanism linking vitamin D status with normal immune function. This process would also be consistent with the overproduction of $1,25(OH)_2D$ observed in diseases such as sarcoidosis. Specifically, the enhanced *localized* synthesis of $1,25(OH)_2D$ by DCs following an immune challenge would further stimulate tissue macrophage population. This, in turn, may lead to even higher levels of $1,25(OH)_2D$ production and in some cases potential spill-over into *systemic* levels of the hormone.

These functional consequences of hormone action indicate that elaboration of $1,25(OH)_2D$ by activated macrophages and DCs in human diseases like sarcoidosis is important in modulation of the local cellular immune response to the granuloma-causing antigen, promoting antigen processing, containment, and destruction. The system is designed for maximal efficiency in that the hormone interacts with the VDR in the same cell in which the hormone is made. Thus, under normal circumstances, relatively high intracellular and local concentrations of hormone can be achieved to modulate macrophage/DC action without having a generalized, endocrine effect on the host (see Fig. 4).

B. Paracrine Action on Lymphocyte

As outlined above, vitamin D is a potent autocrine/intracrine regulator of both innate and acquired immune reponses. The effects of $1,25(OH)_2D$ on acquired, lymphocyte-directed immune reponses are due, at least in part, to indirect actions via the suppression of antigen presentation by macrophages and DCs. However, there is also a breadth of evidence for direct effects of $1,25(OH)_2D$ on lymphocytes [139–142]. Specifically, if lymphokine stimulation of macrophage $1,25(OH)_2D$ synthesis were persistent because of difficulty in macrophage-mediated elimination of the offending antigen, then one might conceive of a situation in which the lipid soluble vitamin D hormone escapes the confines of the macrophage (middle panel, Fig. 4). Once outside of the macrophage, $1,25(OH)_2D$ would be free to interact in a paracrine fashion with antigen-activated T- and B-lymphocytes in the local inflammatory microenvironment. The many reported actions of active vitamin D metabolites on cells of the T-lymphocyte (T-cell) lineage are also described in Chapter 36. In general and in contrast to stimulatory effects of the hormone on monocyte-macrophage cells, $1,25(OH)_2D$ and most of the nonhypercalcemic analogs of vitamin D will inhibit T-cell responsiveness to mitogen or antigen challenge. Interaction of the VDR with its cognate ligand in activated lymphocytes and natural killer (NK) cells inhibits cellular proliferation [143,144], generally decreases lymphokine production [143,145], and inhibits T-cell-directed B-cell immunoglobulin synthesis [146] and delayed-type hypersensitivity reactions [147]. More recent studies have shown that $1,25(OH)_2D$ and vitamin D analogs also influence the nature of T-cell responses by promoting specific T-cell subgroups. Initial studies indicated that $1,25(OH)_2D$ preferentially enhanced a shift from potentially damaging cellular-based T-cell responses involving type 1 helper T-cells (Th1) to a more benign humoral-based immunity via Th2 cells [148–150]. However, more recent work has highlighted an exciting alternative role for $1,25(OH)_2D$ as a potent stimulator of suppressor T-cells also termed T-regulatory cells [151–153]. In view of the importance of T-regulatory cells in directing immune tolerance, the implications for the effects of $1,25(OH)_2D$ on these cells are considerable both in terms of its therapeutic potential and as a basis for a clearer role for vitamin D in normal immune responses. These and other issues are discussed in much greater detail in Chapter 36.

In summary it is now clear that immunomodulatory actions of either exogenously added or locally synthesized $1,25(OH)_2D$ can be broadly divided into either autocrine/intracrine *activation* of macrophage function or paracrine *suppression* of lymphocyte function. We have postulated that this apparent paradox in immunoactions of the hormone, to dampen lymphocyte activity

while stimulating monocyte/macrophage function, is designed to maximize the ability of the host to combat and contain the granuloma-causing antigen, while controlling the potentially self-destructive lymphocytic response to that offending antigen. In other words, in order to prevent "overstimulation" of lymphocytes by monokines elaborated at the site of inflammation, some hormone produced by the macrophage will escape the confines of the cell in which it was made, will interact with neighboring, VDR-expressing, activated lymphocytes, and will tend to restrain what might be an otherwise overzealous, self-destructive T-cell and B-cell response to the offending antigen. As depicted in the right panel of Fig. 4, only at times of heightened immunoreactivity (i.e. extraordinary disease activity) does monokine production escape the confines of the site of inflammation and spill over into the general circulation causing elevated $1,25(OH)_2D$ concentrations. This model would indicate that the endocrine actions of a locally produced vitamin D metabolite that escapes the inflammatory microenvironment is the exception rather than the rule. It also suggests that $1,25(OH)_2D$ is by design an immunomodulatory cytokine in these lymphoproliferative diseases and not a hormone meant to modulate calcium homeostasis in the host.

The overall importance of vitamin D as part of the normal immunomodulatory machinery is still open to some debate. Studies *in vivo* using animals [154–157] and humans [158,159] have highlighted significant immune abnormalities associated with vitamin D deficiency. Likewise, vitamin D supplementation has been shown to have positive effects on immune responses [160]. The extent to which this is mediated via macrophage 1α-hydroxylase and the contribution of this mechanism to the effects of UV exposure on diseases such as TB [161] are still under scrutiny and are discussed in much greater detail in Chapter 36. However, further light has been shed on this issue following the recent development of VDR [162] and 1α-hydroxylase [163] knockout mice. Although initial analysis of the VDR knockout mouse revealed minimal changes in the immune function [164], subsequent studies have documented dysregulated T-helper cell function in these animals [165]. In a similar fashion both VDR and 1α-hydroxylase knockout mice have characteristic lymph nodes dysplasia that is consistent with abnormal DC function [134,163]. These observations have underlined the potential importance of vitamin D metabolism and signaling as a regulator of immune responses and in future studies it will be interesting to assess the way these animals respond to specific inflammatory diseases.

C. Accumulation of $1,25(OH)_2D$ at Sites of Inflammation

1. Granuloma-forming Diseases

Although much effort has been expended to ascertain the immunomodulatory potential of active vitamin D metabolites in human disease, considerably less is known about the immunoactions of these endogenously synthesized molecules *in vivo* in man and animals. If $1,25(OH)_2D$ is truly a naturally occurring "cytokine," then one should be able to document accumulation of the metabolite at sites of inflammation and show that the inflammatory cells at this site are under the influence of the locally-produced vitamin D metabolite. This was first accomplished by Barnes *et al.* [166], not in sarcoidosis, but in the infectious granuloma-forming disease, tuberculosis. They determined that the pleural space in nonhypercalcemic/calciuric patients infected with mycobacterium tuberculosis was one such site of $1,25(OH)_2D$ accumulation. They detected a steep gradient for free, biologically-active $1,25(OH)_2D$ across the visceral pleura in patients' tuberculous effusions (but not in patients with nontuberculous effusions), showed that PPD-reacting T-cell clones from these patients expressed the VDR, and determined that the stimulated proliferation of these T-cell clones was susceptible to $1,25(OH)_2D$–mediated inhibition. They also showed that the pleural fluid of these patients contained an IFN-like peptide that stimulated the synthesis of $1,25(OH)_2D$ by heterologous sarcoid macrophages [167]. Collectively, these data supported the idea put forward by Rook and colleagues [168] that there exists in the pleural microenvironment of patients with active pulmonary tuberculosis a system whereby: 1) mycobacterium-activated macrophages are stimulated to make $1,25(OH)_2D$; 2) this synthetic reaction is supported by proliferating and lymphokine- (i.e. IFNγ) producing lymphocytes in the local site of inflammation; and 3) the local accumulation of lymphokines, in turn, acts to further augment the local production of $1,25(OH)_2D$ by the macrophage (see Fig. 4). Investigators [169] have viewed this sort of positive feedback effect of IFNγ on macrophage $1,25(OH)_2D$ production *in vivo* as an efficient mechanism for dealing with antigens, like myco-bacteria, the "sarcoid antigen," or certain viruses that are difficult for the host to irradicate.

2. Other Inflammatory Disease States

Mawer and colleagues [170,171] have demonstrated substrate-dependent accumulation of $1,25(OH)_2D$ in the synovial fluid of patients with "inflammatory arthritis," including subjects with rheumatoid arthritis. These investigators speculate that the local increase in $1,25(OH)_2D$ synthesis may contribute to periarticular

bone loss in such individuals. A positive tissue-to-serum gradient for $1,25(OH)_2D$ has also been suggested in peritoneal dialysis patients, particularly when afflicted with peritonitis [164,165]; peritoneal macrophages from such patients have been shown to metabolize 25OHD to $1,25(OH)_2D$ *in vitro* [172,173].

V. HUMAN DISEASES ASSOCIATED WITH THE EXTRA-RENAL OVERPRODUCTION OF ACTIVE VITAMIN D METABOLITES (see Table I)

A. Granuloma-Forming Diseases

1. SARCOIDOSIS

Sarcoidosis is the human disease most commonly complicated by endogenous vitamin D intoxication [1–6,14–19,174–177]. In the most expansive studies published on the topic, roughly 10% of patients with sarcoidosis will develop hypercalcemia [177], and up to 50% will suffer from hypercalciuria [177] at some time during the course of their disease. In their retrospective, worldwide review of serum calcium concentrations in 3,676 patients with sarcoidosis, James *et al.* [177] recorded an 11% incidence of hypercalcemia (serum calcium ≥ 10.5 mg/dL). Studdy *et al.* [176] studied 547 patients with biopsy-proven sarcoidosis in Great Britain and found hypercalcemia to be 38% more frequent in men than women and more common among Caucasians than individuals of West Indian descent. Although not systematically studied, the frequency of hypercalcemia among patients with sarcoidosis tends to be consistently higher in North America than in Northern Europe [178]. This is perhaps due to the lower latitude and more direct sunlight exposure in the United States.

Although fractional intestinal calcium absorption may be increased under the influence of $1,25\text{-}(OH)_2D$ and fractional urinary calcium excretion may be decreased in patients with renal insufficiency [38], the principal source of calcium which accumulates in the circulation in this disease is the skeleton. This fact is perhaps most strongly confirmed by the observations of Rizatto *et al.* [179] who documented in serial fashion a significant decrease in bone mineral density in a group of patients with chronic active sarcoidosis in whom anti-inflammatory agents, including glucocorticoids, were not used in management compared to age- and sex-matched control subjects. This fact is confirmed by the long-standing observations that hypercalcemia persists in patients with active sarcoidosis in absence of ingested calcium and may be contributed to by increased bone resorption [33]. The proximal cause of bone loss is increased osteoclast-mediated bone resorption [180] and does not require the presence of extensive granulomata in the bone [181]. These observations suggest that an osteoclast activating factor (OAF) exists in this disease. One such bonafide OAF is of course already known, namely $1,25(OH)_2D$.

TABLE I Human Disease Associated with 1,25-Dihydroxyvitamin D-mediated Hypercalcemia/Hypercalciuria

Granuloma-forming diseases	
Infectious	
Tuberculosis	[182–191]
Leprosy	[192]
Candidiasis	[193]
Crytococcosis	[194]
Histoplasmosis	[195]
Coccidioidomycosis	[196]
AIDS-related pneumocystis	[197]
Noninfectious	
Sarcoidosis	[14–19]
Silicone-induced granulomatosis	[198]
Eosinophilic granuloma	[199]
Wegener's granulomatosis	[200]
Langerhans cell histiocytosis	[201]
Berylliosis	[202]
Infantile fat necrosis	[203]
Crohn's disease	[204,205]
Malignant lymphoproliferative disease	
Hodgkin's disease	[209–211, 213]
Non-Hodgkin's lymphoma	[212, 214–216]
Dysgerminoma/seminoma	[217]

2. TUBERCULOSIS

Of the other human granuloma-forming diseases reported to be associated with vitamin D metabolite-mediated hypercalcemia, tuberculosis is the most commonly reported aside from sarcoidosis. Hypercalcemia has been recognized as a complication of infection with mycobacterium tuberculosis for over eight decades [182]. That this disturbance in calcium balance is caused by the extra-renal overproduction of an active vitamin D metabolite was confirmed by investigators in the mid 1980s [183,184]. As is the case with sarcoidosis, the circulating vitamin D metabolite causing hypercalcemia 1) appears to be $1,25(OH)_2D$ [185,186]; 2) is synthesized by disease-activated macrophages [187,188], 3) is abnormally responsive to small changes in the serum concentration of substrate 25OHD [189], and 4) is reducible under the influence of glucocorticoid *in vivo* [190,191]. The prevalence of hypercalcemia

in patients may be as high as 26% [182] and may be even higher, particularly in the era of AIDS, because of frequent association of hyopalbuminemia (i.e. from malnutrition) in patients with tuberculosis. The source of $1,25(OH)_2D$ in this disease is, as it is in all of the other granuloma-forming diseases, extra-renal [183] most likely arising from the macrophage [187].

3. OTHER INFECTIOUS DISEASES

Hypercalciuria or overt hypercalcemia has also been observed in a number of infectious diseases, most characterized by widespread granuloma formation and macrophage proliferation in infected tissue. Included among these diseases are leprosy [192], disseminated candidiasis [193], crytococcosis [194], histoplasmosis [195], and coccidioidomycosis [196]. Hypercalcemia in most of these conditions has been documented to be associated with inappropriately elevated serum concentrations of $1,25(OH)_2D$. The true prevalence and incidence of hypercalcemia and hypercalciuria in patients with these diseases in unknown. However, it is likely that this complication of dysregulated vitamin D metabolism and action associated with these diseases will increase in frequency as the number of immunocompromised patients, especially those with AIDS, increases worldwide. For example, hypercalcemia in association with elevated circulating levels of $1,25(OH)_2D$ has been reported in an AIDS patient with pneumocystis [197]; both serum calcium and $1,25(OH)_2D$ concentrations dropped in this patient with successful treatment of his opportunistic infection.

4. NONINFECTIOUS GRANULOMA-FORMING DISEASES

The syndrome of extra-renal overproduction of $1,25(OH)_2D$ has also been documented in adult patients with widespread silicone-induced granulomata [198], eosinophilic granuloma [199], Wegener's granulomatosis [200], and Langerhans cell histiocytosis [201]. Although the active vitamin D metabolite was not measured, dysregulated calcium balance in the granuloma-forming pulmonary disease berylliosis is also attributed to the extra-renal production of $1,25(OH)_2D$ [202]. In addition, $1,25(OH)_2D$–mediated hypercalcemia has been observed in newborn infants suffering from massive subcutaneous fat necrosis [203]; this is a transient disorder associated with birth trauma and characterized histopathologically by the proliferation of "foreign body-type" giant cells around cholesterol-shaped crystals in necrotizing, subcutaneous adipose tissue. Finally, there are also reports of elevated serum levels of $1,25(OH)_2D$ and associated hypercalcemia in patients with inflammatory bowel disease [204]. The possible impact of extra-renal 1α-hydroxylase in this clinical situation is an exciting new development, in part because of the prevalence of Crohn's disease particularly in developed countries [205], but also because of several recent reports which have documented expression of 1α-hydroxylase along the gastrointestinal tract [24,206–208].

B. Malignant Lymphoproliferative Disorders

By the 1980s, data was accumulating to suggest that a vitamin D–mediated disturbance in calcium metabolism was not confined to patients with granuloma-forming diseases and could also be observed in patients with lymphoproliferative neoplasms [209–212]. More recent reports [213,214] indicate that the extra-renal overproduction of $1,25(OH)_2D$ is the most common cause of hypercalciuria and hypercalcemia in patients with non-Hodgkin and Hodgkin lymphoma, especially in patients with B-cell neoplasms, whether or not the tumor is associated with AIDS in the patient [212]. In fact, in the Seymour study [206] 71% of normocalcemic patients with non-Hodgkin lymphoma had hypercalciuria (fractional urinary calcium excretion >0.15 mg/dL glomerular filtrate) and most of these had serum $1,25(OH)_2D$ levels that were above the mid range of normal or frankly elevated. As is the situation with hypercalciuric/calcemic patients with sarcoidosis or other granuloma-forming disease and elevated circulating $1,25(OH)_2D$ levels, the serum concentrations of PTH are suppressed and PTHrP normal (i.e. not elevated) in lymphoma patients, indicative of the state of dysregulated overproduction of the active vitamin D hormone. Results of clinical studies of hypercalcemic patients with lymphoma pre and post successful anti-tumor therapy [212–215] are most compatible with either the tumor being an immediate source of an active vitamin D metabolite or the source of a soluble factor (i.e. peptide), which stimulates the production of $1,25(OH)_2D$ in the kidney or in other inflammatory cells. Recent data from our group suggest that the latter is the case. Specifically, we carried out extensive analysis of a patient with hypercalcemia and raised circulating levels of $1,25(OH)_2D$ associated with a splenic B-cell lymphoma [216]. The abnormalities in serum $1,25(OH)_2D$ and calcium were corrected following resection of the spleen, and subsequent immunonhistochemical analysis of this tissue revealed increased expression of 1α-hydroxylase in macrophages adjacent to the tumor, but not in the tumor itself. This raises further potentially important questions: 1) what is the nature of the tumor-derived factor that is able to stimulate macrophage 1α-hydroxylase?

2) Is macrophage-derived $1,25(OH)_2D$ a contributing factor to the hypercalcemia associated with other types of tumors? In seeking to answer the latter question, current studies within our group have focused on the expression and function of macrophage 1α-hydroxylase in dysgerminomas, gonadal tumors that are associated with granulomata and which have shown previously to be linked to hypercalcemia [217].

C. Non-Granuloma-forming Conditions

As outlined in section II.C, cloning of the gene for 1α-hydroxylase (CYP1α) has enabled a much more comprehensive appraisal of the tissue distribution of this enzyme than was previously available. Indeed, the human cDNA for CYP1α was cloned from keratinocytes, a well-established extra-renal source of $1,25(OH)_2D$ [218,219]. Studies have defined both the renal and extra-renal tissues that express 1α-hydroxylase [23,24]. Specific sites of interest include skin [24,218,219], prostate [220], placenta [24,221,222] (one of the first tissues to show extra-renal synthesis of $1,25(OH)_2D$ [223]), parathyroids [224], vasculature [225,226], gut [24,206–208], brain [24], and pancreas [24]. The precise function of 1α-hydroxylase expression at these sites is currently the focus of considerable attention. In tissues such as the prostate [220,227], colon, [206–208], and parathyroids [224,228], the enzyme has been postulated to fulfill an antitumor function by increasing local concentrations of antiproliferative $1,25(OH)_2D$, and this is discussed in greater detail in Chapters 93–96. However, in many extra-renal tissues particularly the pancreas, gut, vasculature, and brain, it is probable that 1α-hydroxylase will fulfill a function which is more akin to the macrophage, namely as a generator of immunomodulatory $1,25(OH)_2D$. For example, the most potent activator of 1α-hydroxylase in cultured human endothelial cells [225], as well as epithelial cells from the distal nephron [90], is LPS, with both cell types, like macrophages, showing strong expression of CD14/TLR4. Thus, the function of extra-renal 1α-hydroxylase may be far more diverse than originally thought: putative roles in preclampsia [221], implantation [222], and vascular disease [225] have been proposed in addition to its link with common cancers [206–208,220,227]. Nevertheless, at the present moment in time the most well-documented pathological paradigm for extra-renal 1α-hydroxylase remains the overproduction of $1,25(OH)_2D$ associated with granulomatous disease. The clinical management of this is therefore discussed in greater detail in the following sections.

VI. DIAGNOSIS, PREVENTION, AND TREATMENT OF THE PATIENT WITH ENDOGENOUS VITAMIN D INTOXICATION

A. Diagnosis

The diagnosis of so-called "endogenous" vitamin D intoxication is made when the following three criteria are met. First is the presence of hypercalciuria and/or hypercalcemia in a patient with an inappropriately elevated serum $1,25(OH)_2D$ (i.e. the serum $1,25(OH)_2D$ concentration is not suppressed below 20 pg/ml). Second is the presence in the serum of an appropriately suppressed PTH level if the patient's free (ionized) serum calcium concentration is high; this is evidence that the calcium sensing receptor in the plasma membrane of the host's parathyroid cell is normally operative. This distinguishes the patient with primary hyperparathyroidism and elevated $1,25(OH)_2D$ levels, in whom the calcium-sensing receptor signal transduction pathway to control PTH synthesis and release is disrupted, and from the individual with endogenous vitamin D intoxication and elevated $1,25(OH)_2D$ levels. The other major exception here is the patient with absorptive hypercalciuria who possesses, as a primary or secondary abnormality, an inappropriately elevated circulating $1,25(OH)_2D$ concentration [229]. Third is the exclusion of exogenous vitamin D intoxication arising from the oral or parental administration of an active vitamin D metabolite or the substrate for endogenous synthesis of an active vitamin D metabolite. The most common cause of exogenous vitamin D intoxication occurs with the ingestion or injection of large doses of vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol), and can usually be detected by measuring a frankly elevated serum 25OHD level; most, if not all, currently available serum assays for 25OHD do not distinguish $25OHD_2$ from $25OHD_3$ [230]. Exogenous vitamin D intoxication may occur in patients taking too much 1-α-OHD, dihydrotachysterol (DHT), or $1,25(OH)_2D$ itself; the two former compounds undergo 25-hydroxylation in the host hepatocyte. In these instances, the $1,25(OH)_2D$, not the 25OHD, concentration will be elevated, making the distinction from endogenous vitamin D intoxication from the extra-renal overproduction of $1,25(OH)_2D$ impossible on strictly biochemical grounds. In these situations, a complete knowledge of the medications to which the patient has access is critical for making the correct diagnosis. Examples of such patients would be those receiving relatively large amounts of vitamin D, a vitamin D metabolite, or a vitamin D analog (i.e. patients with hypoparathyroidism, renal failure, and

psoriasis, respectively). Most of the newer vitamin D analogs currently in clinical use [231] will not be measured efficiently in the serum $1,25(OH)_2D$ assay, so awareness of the use of these kinds of topically- and orally-administered agents is of particular importance to the diagnosing clinician.

B. Early Detection and Prevention of Hypercalciuria/Hypercalcemia

1. Identifying Patients at Risk

Considering the fact that the means of specifically inhibiting the production of active metabolites of vitamin D or of blocking the response of cells to active vitamin D derivatives is not yet available, the best way to treat vitamin D–mediated abnormalities in calcium balance is to prevent their occurrence. The first step is to identify patients at risk. This encompasses primarily patients with granuloma-forming disease as well as patients with malignant lymphoproliferative disorders, especially B-cell and Hodgkin's lymphoma. Disordered calcium balance in these groups of patients results from the endogenous and dysregulated overproduction of $1,25(OH)_2D$ by inflammatory cells. Production of the offending vitamin D metabolite is, in turn, directly related to the amount of substrate 25OHD available to the macrophage 1α-hydroxylase (see Fig. 2) as well as to the severity and activity of the underlying disease. In terms of sarcoidosis, for example, patients at risk would be those with: 1) widespread, active disease; 2) a previous history of hypercalciuria or hypercalcemia; 3) a diet enriched in vitamin D and/or calcium; 4) a recent history of sunlight exposure or treatment with vitamin D; and 5) an intercurrent condition, or medicinal treatment of an intercurrent condition, that increases bone resorption or decreases the glomerular filtration rate.

2. Screening Patients at Risk

Since hypercalciuria almost always precedes the development of overt hypercalcemia in this set of disorders, patients at risk should be checked for the presence of occult hypercalciuria. This is best accomplished by a fasting two-hour urine collection for calcium and creatinine. If the calcium:creatinine ratio (gm:gm) is not abnormally high (<0.16), then a 24-hour urine collection for the fractional calcium excretion rate is necessary to establish hypercalciuria; presumably due to increased bone resorption, many patients with vitamin D–mediated hypercalciuria will be hypercalciuric even in the absence of recent food (calcium) ingestion. If screening is to be done only on an annual basis, then the late summer or early autumn when 25OHD levels are usually at their peak is the best time [76].

3. Prevention of Hypercalcemia

Prevention of an overt disorder in calcium balance is obviously preferable to dealing with hypercalcemia. This is especially true for patients 1) in whom one episode of hypercalciuria or hypercalcemia has already been documented and 2) are taking supplemental vitamin D and calcium preparations for another medical indication (i.e. osteoporosis). Prevention is best achieved by monitoring the serum calcium and urinary calcium excretion rate on a regular basis. If either is high, then the serum concentration of 25OHD and $1,25(OH)_2D$ level should be obtained; the former should be evaluated to rule out the existence or coexistence of exogenous vitamin D intoxication. For patients determined to be at risk from the appropriate monitoring analyses, measures to prevent worsening hypercalciuria anf frank hypercalcemia should be instituted. These measures should include: 1) the use of UVB-absorbing sunscreens on exposed body parts when they anticipate being out-of-doors for periods in excess of 20–30 minutes; 2) caution against ingestion of vitamin and food supplements containing ≥400 IU vitamin D; 3) education on the vitamin D and calcium content of foods, vitamin supplements, and medicinal agents like antacids; 4) caution against the regular ingestion of elemental calcium in excess of 1000 mg daily; and 5) education regarding the earliest signs of hypercalciuria (i.e. nocturia).

C. Treatment of Hypercalciuria and Hypercalcemia

Because they reside on the same pathophysiological spectrum, the treatment aim of normalization of the urinary calcium excretion rate is the same whether the patient is hypercalciuric or frankly hypercalcemic. There are three general therapeutic goals. First is reduction in the serum concentration of the offending vitamin D metabolite or derivative. In patients with exogenous vitamin D intoxication, this is usually accomplished by cessation or a reduction in the dose of the vitamin D preparation being used by that patient. Remembering that 25OHD has a serum half-life of months, vitamin D–intoxicated patients may require as much as a year off therapy. In patients suffering from endogenous intoxication with $1,25(OH)_2D$ made by inflammatory cells, reduction in the serum $1,25(OH)_2D$ level can be most reliably achieved by treatment with anti-inflammatory doses of glucocorticoid (adult dose of 40 mg prednisone or equivalent per day). At these doses, steroid therapy should have little effect on the

renal 1α-hydroxylase, so there is little concern for inducing hypocalcemia with glucocorticoid administration. In patients with extra-renal production of the hormone, steroid therapy should result in a drop in the serum $1,25(OH)_2D$ concentration within a matter of 3–4 days, followed shortly thereafter by a decrease in the filtered load of calcium and urinary calcium excretion rate provided that the patient's glomerular filtration rate is maintained.

In patients who fail glucocorticoids or in whom glucocorticoids are contraindicated, treatment with the 4-aminoquinoline class of drugs like chloroquine (250 mg twice daily) or hydroxychloroquine (up to 400 mg daily) may be effective [34–36]. A less desirable therapeutic alternative is the cytochrome P450 inhibitor ketoconazole [232]; it will effectively reduce the serum $1,25(OH)_2D$ concentration [233–235], but the therapeutic margin of safety is narrow; doses of the drug that inhibit the macrophage P450 system are very close to those that will also inhibit endogenous glucocorticoid and sex steroid production. The second goal of therapy is to limit the actions of the vitamin D derivative at its target tissues, the gut and bone. A reduction in intestinal calcium absorption is best accomplished by elimination of as much calcium as possible from the diet. Such conservative measures are rarely effective in patients with active, widespread disease, so glucocorticoid administration may also be required to block vitamin D–mediated calcium absorption and bone resorption [236]. Although not well studied, other skeletal antiresorptive agents, like calcitonin and the bisphosphonates, do not appear to be particularly effective in blocking active vitamin D metabolite-mediated bone resorption. The third goal of therapy is to enhance urinary calcium excretion to a point where the filtered load of calcium is insufficient to cause either hypercalcemia or hypercalciuria. This can be achieved by maintenance of the vascular volume (glomerular filtration rate) and urinary flow rate, and if needed, by the use of a "loop" diuretic, like furosemide, to inhibit calcium reabsorption from the urine. The effects of successfully reducing the serum $1,25(OH)_2D$ concentration and managing hypercalcemia/hypercalciuria on the patient's skeleton long-term are not known. There is preliminary evidence that successful treatment of exogenous vitamin D intoxication may result in a transient increase in bone mineral density [237].

References

1. Harrell GT, Fisher S 1939 Blood chemical changes in Boeck's sarcoid. J Clin Invest **18**:687–693.
2. Henneman PH, Dempsey EF, Carrol EJ, Albright F 1956 The causes of hypercalcemia in sarcoid and its treatment with cortisone. J Clin Invest **35**:1229–1242.
3. Taylor RL, Lynch HJ Jr, Wysor WG 1963 Seasonal influence of sunlight on the hypercalcemia of sarcoidosis. Am J Med **35**:67–89.
4. Dent CE, Flynn FV, Nabarro JDN 1953 Hypercalcemia and impairment of renal function in generalized sarcoidosis. Br Med J **2**:808–810.
5. Hendrix JZ 1963 The remission of hypercalcemia and hypercalciuria in systemic sarcoidosis by vitamin D depletion. Clin Res **11**:220–225.
6. Bell NH, Gill JR Jr, Barter FC 1964 Abnormal calcium absorption in sarcoidosis: evidence for increased sensitivity to vitamin D. Am J Med **36**:500–513.
7. Holick MF, Schnoes THK, DeLuca HF, Suda T, Cousins RJ 1992 Isolation and identification of 1,25-dihydroxycholecalciferol. A metabolite of vitamin D active in intestine. J NIH Res **4**:88–96.
8. Bell NH 1985 Vitamin D endocrine system. J Clin Invest **76**:1–6.
9. Hughes MF, Baylink DJ, Jones PG, Haussler MR 1976 Radioligand receptor assay for 25-hydroxyvitamin D_2/D_3 and 1α,25-dihydroxyvitamin D_2/D_3. J Clin Invest **58**:61–70.
10. Clemens TL, Hendy GN, Graham RF 1978 A radioimmunoassay for 1,25-dihydroxycholecalciferol. Clin Sci Mol Med **54**:329–332.
11. Gray TK, McAdoo T 1979 Radioimmunoassay for 1,25-dihydroxyvitamin D_3. In: Norman AW, Schaefer K, von-Herrath D (eds) Vitamin D Basic Research and Its Clinical Application. Berlin: de Gruyter, pp. 763–767.
12. Bouillon R, DeMoor P, Baggiolini EG, Uskokovic MR 1980 A radioimmunoassay for 1,25-dihydroxycholecalciferol. Clin Chem **26**:562–567.
13. Holick MF 1990 The use and interpretation of assays for vitamin D and its metabolites. J Nutr **120**:1464–1469.
14. Bell NH, Stern PH, Pantzer E, Sinha TK, DeLuca 1999 Evidence that increased circulating 1,25-dihydroxyvitamin D is the probable cause for abnormal calcium metabolism in sarcoidosis. J Clin Invest **64**:218–225.
15. Papapoulos SE, Clemens TL, Fraher LJ, Lewin IG, Sandler LM, O'Riordan JL 1979 1,25-Dihydroxycholecalciferol in the pathogenesis of the hypercalcemia of sarcoid. Lancet **1**: 627–630.
16. Stern PH, Olazabal J, Bell NH 1980 Evidence for abnormal regulation of circulating 1,25-dihydroxyvitamin D in patients with sarcoidosis. J Clin Invest **66**:852–855.
17. Barbour GL, Coburn JW, Slatopolsky E, Norman AW, Horst RL 1981 Hypercalcemia in an anephric patient with sarcoidosis. N Engl J Med **305**:440–443.
18. Adams JS, Sharma OP, Gacad MA, Singer FR 1983 Metabolism of 25-hydroxyvitamin D_3 by cultured alveolar macrophages in sarcoidosis. J Clin Invest **72**:1856–1860.
19. Adams JS, Singer FR, Gacad MA, Sharma OP, Hayes MJ, Vouros P, Holick MF 1985 Isolation and structural identification of 1,25-dihydroxyvitamin D_3 produced by cultured alveolar macrophages in sarcoidosis. J Clin Endocrinol Metab **60**: 960–966.
20. Takeyama K, Kitanaka S, Sato T, Kobori M, Yanagisawa J, Kato S 1997 25-Hydroxyvitamin D_3 1α-hydroxylase and vitamin D synthesis. Science **277**:1827–1830.
21. Fu GK, Lin D, Zhang MY, Bikle DD, Shackleton CH, Miller WL, Portale AA 1997 Cloning of human 25-hydroxyvitamin D-1α-hydroxylase and mutations causing vitamin D–dependent rickets type 1. Mol Endocrinol **11**:1961–1970.

22. Smith SJ, Rucka AK, Berry JL, Davies M, Mylchreest S, Paterson CR, Heath DA, Tassabehji M, Read AP, Mee AP, Mawer EB 1999 Novel mutations in the 1α-hydroxylase (P450c1) gene in three families with pseudovitamin D–deficiency rickets resulting in loss of functional enzyme activity in blood-derived macrophages. J Bone Miner Res **14**:730–739.
23. Zehnder D, Bland R, Walker EA, Bradwell AR, Howie AJ, Hewison M, Stewart PM Expression of 25-hydroxyvitamin D_3–1α-hydroxylase in the human kidney. J Am Soc Nephrol **10**:2465–2473.
24. Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM, Hewison M 2001 Extra-renal expression of 25-hydroxyvitamin D_3-1α-hydroxylase. J Clin Endo Metab **86**:888–894.
25. Bell NH 1991 Endocrine complications of sarcoidosis. Endo Metab Clin North Am **20**:645–654.
26. Basile JN, Leil Y, Shary J, Bell NH 1993 Increased calcium intake does not suppress circulating 1,25-dihydroxyvitamin D in normocalcemic patients with sarcoidosis. J Clin Invest **91**: 1396–1398.
27. Sandler LM, Winearls CG, Fraher LJ, Clemens TL, Smith R, O'Riordan JLH 1984 Studies of the hypercalcemia of sarcoidosis. Quart J Med **53**:165–180.
28. Papapoulos SE, Clemens TL, Fraher LJ 1979 Dihydroxycholecalciferol in the pathogenesis of the hypercalcemia of sarcoid. Lancet **1**:627–630.
29. Cronin CC, Dinneen SF, O'Mahony MS, Bredin CP, O'Sullivan DJ 1990 Precipitation of hypercalcemia in sarcoidosis by foreign sun holidays: report of four cases. Postgrad Med J **66**:307–309.
30. Insogna KL, Dreyer BE, Mitnich M, Ellison AF, Broadus A 1988 Enhanced production of 1,25-dihydroxyvitamin D in sarcoidosis. J Clin Endocrinol Metab **66**:72–75.
31. Stern PH, De Olazabal J, Bell NH 1980 Evidence for abnormal regulation of circulating 1α,25-dihydroxyvitamin D in patients with sarcoidosis and normal calcium metabolism. J Clin Invest **66**:852–855.
32. Shulman LE, Schoenrich E, Harvey A 1952 The effects of adrenocorticotropic hormone (ACTH) and cortisone on sarcoidosis. Bull John Hopkins Hosp **91**:371–415.
33. Anderson J, Dent CE, Harper C, Philpot GR 1954 Effect of cortisone on calcium metabolism in sarcoidosis with hypercalcemia. Lancet **2**:720–724.
34. O'Leary TJ, Jones G, Yip A, Lohnes D, Cohanim M, Yendt ER 1986 The effects of chloroquine on serum 1,25-dihydroxyvitamin D and calcium metabolism in sarcoidosis. N Engl J Med **315**:727–730.
35. Barre PE, Gascon-Barre M, Meakins JL, Goltzman D 1987 Hydroxychloroquine treatment of hypercalcemia in a patient with sarcoidosis. Am J Med **82**:1259–1262.
36. Adams JS, Diz MM, Sharma OP 1989 Effective reduction in the serum 1,25-dihydroxyvitamin D and calcium concentration in sarcoidosis-associated hypercalcemia with short-course chloroquine therapy. Ann Int Med **111**:437–438.
37. Singer FR, Adams JS 1986 Abnormal calcium homeostasis in sarcoidosis. N Engl J Med **315**:755–756.
38. Meyrier A, Valeyre D, Bouillon R, Paillard F, Battesti JP, Georges R 1985 Resorptive versus absorptive hypercalciuria in sarcoidosis. Quart J Med **54**:269–281.
39. Adams JS, Gacad MA, Anders A, Endres DB, Sharma DP 1986 Biochemical indicators of disordered vitamin D and calcium homeostasis in sarcoidosis. Sarcoidosis **3**:1–6.
40. Adams JS, Gacad MA 1985 Characterization of 1α-hydroxylation of vitamin D_3 sterols by cultured macrophages from patients with sarcoidosis. J Exp Med **161**:755–765.
41. Shany S, Adams JS 1993 Subcellular localization of the 25-hydroxyvitamin D_3-1-hydroxylase and partial purification from the chick myelomonocytic cell line HD-11. J Bone Min Res **8**:269–276.
42. Adams JS, Ren S-Y, Arbelle JE, Horiuchi N, Gray RW, Clemens TL, Shany S 1994 Regulated production and intracrine action of 1,25-dihydroxyvitamin D_3 in chick myelomonocytic cell line HD-11. Endocrinology **134**:2567–2573.
43. Reichel H, Koeffler HP, Norman AW 1987 Synthesis *in vitro* of 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 by interferon-gamma-stimulated normal human bone marrow and alveolar macrophages. J Biol Chem **262**:10931–10937.
44. Fraser DR 1980 Regulation of the metabolism of vitamin D. Physiol Rev **60**:551–613.
45. Nakamura Y, Eto TA, Taniguchi T, Miyamoto K, Nagatomo J, Shiotsuki H, Sueta H, Higashi S, Okuda KI, Setoguchi T 1997 Purification and characterization of 25-hydroxyvitamin D_3 1α-hydroxylase from rat kidney mitochondria. FEBS Lett **419**:45–48.
46. Sakaki T, Sawada N, Nonaka Y, Ohyama Y, Inouye K 1999 Metabolic studies using recombinant *Escherichia coli* cells producing rat mitochondrial CYP24. CYP24 can convert 1α,25-dihydroxyvitamin D_3 to calcitroic acid. Eur J Biochem **262**:43–48.
47. Reichel H, Koeffler HP, Norman AW 1989 The role of the vitamin D endocrine system in health and disease. N Engl J Med **320**:980–991.
48. Taylor R 1994 A new receptor for calcium ions. J NIH Res **6**:25–27.
49. Garabedian M, Holick MF, DeLuca HF, Boyle IT 1972 Control of 25-hydroxy-cholecalciferol metabolism by parathyroid glands. Proc Natl Acad Sci **69**:1973–1976.
50. Murayama A, Takeyama K, Kitanaka S, Kodera Y, Hosoya T, Kato S 1998 The promoter of the human 25-hydroxyvitamin D_3 1alpha-hydroxylase gene confers positive and negative responsiveness to PTH, calcitonin, and 1α,25$(OH)_2D_3$. Biochem Biophys Res Commun **249**:11–16.
51. Kong XF, Zhu XH, Pei YL, Jackson DM, Holick MF 1999 Molecular cloning, characterization, and promoter analysis of the human 25-hydroxyvitamin D_3-1α-hydroxylase gene. Proc Natl Acad Sci USA **96**:6988–6993.
52. Henry HL 1992 Vitamin D hydroxylases. J Cell Biochem **49**:4–9.
53. Portale AA, Halloran BP, Murphy MM, Morris RC, Jr. 1986 Oral intake of phosphorus can determine the serum concentration of 1,25-dihydroxyvitamin D by determining its production rate in humans. J Clin Invest **77**:7–12.
54. Tenenhouse HS, Martel J, Gauthier C, Zhang MY, Portale AA 2001 Renal expression of the sodium/phosphate cotransporter gene, Npt2, is not required for regulation of renal 1 alpha-hydroxylase by phosphate. Endocrinology **142**:1124–1129.
55. Reichel H, Koeffler HP, Barbers R, Norman AW 1987 Regulation of 1,25-dihydroxyvitamin D_3 production by cultured alveolar macrophages from normal human donors and patients with pulmonary sarcoidosis. J Clin Endocrinol Metab **65**:1201–1209.
56. Hakeda Y, Hiura K, Sato T, Okazaki R, Matsumoto T, Ogata E, Ishitani R, Kumegawa M 1989 Existence of parathyroid hormone-binding sites on murine hemopoietic blast cells. Biochem Biophys Res Commun **163**:1481–1486.
57. Yuan JY, Freemont AJ, Mawer EB, Hayes ME 1992 Regulation of 1α,25-dihydroxyvitamin D_3 synthesis in macrophages from arthritic joints by phorbol ester, dibutyryl-cAMP and calcium ionophore (A23187). FEBS Lett **311**: 71–74.

58. Bland R, Walker EA, Hughes SV, Stewart PM, Hewison M 1999 Constitutive expression of 25-hydroxyvitamin D_3-1α-hydroxylase in a transformed human proximal tubule cell line: evidence for direct regulation of vitamin D metabolism by calcium. Endocrinology **140**:2027–2034.
59. Nesbitt T, Drezner MK 1993 Insulin-like growth factor-1 regulation of renal 25-hydroxyvitamin D-1α-hydroxylase activity. Endocrinology **132**:133–138.
60. Henry HH 1981 25(OH)D_3 metabolism in kidney cell cultures: lack of a direct effect of estradiol. Am J Physiol **240**:E119–E124.
61. Adams ND, Garthwaite TL, Gray RW 1979 The interrelationship among prolactin, 1,25-dihydroxyvitamin D, and parathyroid hormone in humans. J Clin Endocrinol Metab **49**:628–630.
62. Kumar R, Merimee TJ, Silva P, Epstein FH 1979 The effect of chronic excess or deficiency of growth hormone on plasma 1,25-dihydroxyvitamin D levels in man. In: Norman AW, Schaefer K, von Herrath D (eds.) Vitamin D, Basic Research and Its Clinical Application. Berlin: de Gruyter pp.1005–1009.
63. Brixen K, Nielsen HK, Bouillon R, Flyvbjerg A, Mosekilde L 1992 Effects of short-term growth hormone treatment on PTH, calcitriol, thyroid hormones, insulin, and glucagon. Acta Endocrinol **127**:331–336.
64. Omdahl JL, Bobrovnikova EA, Choe S, Dwivedi PP, May BK 2001 Overview of regulatory cytochrome P450 enzymes of the vitamin D pathway. Steroids **66**:381–389.
65. Ohyama Y, Noshiro M, Okuda K 1991 Cloning and expression of cDNA encoding 25-hydroxyvitamin D_3 24-hydroxylase. FEBS Letter **278**:195–198.
66. Chen K, Goto H, DeLuca HF 1992 Isolation and expression of human 1,25-dihydroxyvitamin D_3 24-hydroxylase cDNA. J Bone Mineral Res **7**:S148.
67. Chen KS, Prahl JM, DeLuca HF 1993 Isolation and expression of human 1,25-dihydroxyvitamin D_3-24-hydroxylase. Proc Natl Acad Sci **90**:4543–4547.
68. Ismail R, Elaroussi MA, DeLuca HF 1993 Regulation of chicken kidney vitamin D_3 24-hydroxylase mRNA by 1,25-dihydoxyvitamin D_3 and parathyroid hormone. J Bone Min Res **8**:S208.
69. Uchida M, Shinki T, Ohyama Y, Noshiro M, Okda K, Suda T 1993 Protein kinase C up-regulates 1α,25-dihydroxyvitamin D_3 induced expression of the 24-hydroxylase gene. J Bone Min Res **8**:S171.
70. Chen ML, Boltz MA, Armbrecht HJ 1993 Effects of 1,25-dihydroxyvitamin D_3 and phorbol ester on 25-hydroxyvitamin D_3-24-hydroxylase cytochrome P-450 messenger ribonucleic acid levels in primary cultures of rat renal cells. Endocrinology **132**:1782–1788.
71. Zierold C, Mings JA, DeLuca HF 2003 Regulation of 25-hydroxyvitamin D_3-24-hydroxylase mRNA by 1,25-dihydroxyvitamin D_3 and parathyroid hormone. J Cell Biochem **88**:234–237.
72. Henry HL 1979 Regulation of the hydroxylation of 25-hydroxyvitamin D_3 *in vivo* and in primary cultures of chick kidney cells. J Biol Chem **254**:2722–2729.
73. Inouye K, Sakaki T 2001 Enzymatic studies on the key enzymes of vitamin D metabolism; 1α-hydroxylase (CYP27B1) and 24-hydroxylase (CYP24). Biotechnol Annu Rev **7**:179–194.
74. Xie Z, Munson SJ, Huang N, Portale AA, Miller WL, Bikle DD 2002 The mechanism of 1,25-dihydroxyvitamin D_3 autoregulation in keratinocytes. J Biol Chem **277**:36987–36990.
75. Adams JS, Gacad MA, Singer FR, Sharma OP 1986 Production of 1,25-dihydroxyvitamin D_3 by pulmonary alveolar macrophages from patients with sarcoidosis. NY Acad Sci **465**:587–594.
76. Adams JS 1992 Hypercalcemia and hypercalciuria. Sem Resp Med **13**:402–410.
77. Adams JS, Modlin RL, Diz MM, Barnes PF 1989 Potentiation of the macrophage 25-hydroxyvitamin D-1α-hydroxylation reaction by human tuberculous pleural effusion fluid. J Clin Endocrinol Metab **69**:457–460.
78. Hunninghake GW 1986 Role of alveolar macrophage and lung T-cell derived mediators in pulmonary sarcoidosis. Ann NY Acad Sci **465**:82–90.
79. Pryke AM, Duggan C, White CP, Posen S, Mason RS 1990 Tumor necrosis factor-alpha induces vitamin D-1-hydroxylase activity in normal human alveolar macrophages. J Cell Physiol **142**:652–656.
80. Platanias LC, Fish EN 1999 Signaling pathways activated by interferons. Exp Hematol **27**:1583–1592.
81. Imada K, Leonard WJ 2000 The Jak-STAT pathway. Mol Immunol **37**:1–11.
82. Ishihara K, Hirano T 2002 Molecular basis of the cell specificity of cytokine action. Biochim Biophys Acta **1592**:281–296.
83. Xaus J, Comalada M, Valledor AF, Cardo M, Herrero C, Soler C, Lloberas J, Celada A 2001 Molecular mechanisms involved in macrophage survival, proliferation, activation, or apoptosis. Immunobiology **204**:543–550.
84. Celada A, Schreiber RD 1996 Role of protein kinase C and intracellular calcium mobilization in the induction of macrophage tumoricidal activity by interferon-gamma. J Immunol **137**:2373.
85. Wightman PD, Humes JL, Davies P, Bonney RJ 1981 Identification of two phospholipase A2 activities in resident mouse peritoneal macrophages. Biochem J **195**:427.
86. Wightman PD, Dahlgren M, Bonney RS 1982 Protein kinase activation of phospholipase A2 in sonicates of mouse peritoneal macrophages. J Biol Chem **257**:6650.
87. Adams JS, Gacad MA, Diz MM, Nadler JL 1990 A role for endogenous arachidonate metabolites in the regulated expression of the 25-hydroxyvitamin D-1-hydroxylation reaction in cultured alveolar macrophages from patients with sarcoidosis. J Clin Endocrinol Metab **70**:595–600.
88. Sabroe I, Jones EC, Usher LR, Whyte MK, Dower SK 2002 Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. J Immunol **168**:4701–4710.
89. Barton GM, Medzhitov R 2002 Toll-like receptors and their ligands. Curr Top Microbiol Immunol **270**:81–92.
90. Bland R, Zehnder D, Hughes SV, Ronco PM, Stewart PM, Hewison M 2001 Regulation of vitamin D-1α-hydroxylase in a human cortical collecting duct cell line. Kidney International 60:1277–1286.
91. Adams JS, Ren SY, Arbelle JE, Clemens TL, Shany S 1994 A role for nitric oxide in the regulated expression of the 25-hydroxyvitamin D-1-hydroxylation reaction in the chick myelomonocytic cell line HD-11. Endocrinology **134**:499–502.
92. Adams JS, Ren S-Y, Arbelle J, Shany S, Gacad MA 1995 Coordinate regulation of nitric oxide and 1,25-dihydroxyvitamin D production in the avian myelomonocytic cell line HD-11. Endocrinology **136**:2262–2269.
93. Adams JS, Ren S-Y 1996 Autoregulation of 1,25-dihydroxyvitamin D synthesis in macrophage mitochondria by nitric oxide. Endocrinology **137**:4514–4517.
94. Marletta MA 1994 Nitric oxide synthase: aspects concerning structure and catalysis. Cell **78**:927–930.
95. Nathan C, Xie Q-W 1994 Nitric oxide synthases: Roles, tolls, and controls. Cell **78**:915–918.
96. Salkowski CA, Detore G, McNally R, van Rooijen N, Vogel SN 1997 Regulation of inducible nitric oxide synthase messenger RNA expression and nitric oxide production by lipopolysaccharide *in vivo*: the roles of macrophages, endogenous IFN-gamma, and TNF receptor-1-mediated signaling. J Immunol **158**:905–912.

97. Alley EW, Murphy WJ, Russell SW 1995 A classical enhancer element responsive to both lipopolysaccharide and interferon-gamma augments induction of the iNOS gene in mouse macrophages. Gene **158**:247–251.
98. White KA, Marletta MA 1992 Nitric oxide synthase is a cytochrome P-450 type hemoprotein. Biochemistry **31**: 6627–6631.
99. Khatsenko OG, Gross SS, Rifkind AB, Vane JR 1993 Nitric oxide is a mediator of the decrease in cytochrome P450-dependent metabolism caused by immunostimulants. Proc Natl Acad Sci **90**:11147–11151.
100. Stadler J, Trockfeld J, Schmalix WA, Brill T, Siewert JR, Greim H, Doehmer J 1994 Inhibition of cytochromes P450-1A by nitric oxide. Proc Natl Acad Sci **91**:3559–3563.
101. Van Voorhis BJ, Dunn MS, Snyder GD, Weiner CP 1994 Nitric oxide: An autocine regulator of human granulosa-luteal cell steroidogenesis. Endocrinology **135**:1799–1806.
102. Hartl FU 1996 Molecular chaperones in cellular protein folding. Nature **381**:571–580.
103. Rutherford SL, Zuker CS 1994 Protein folding and the regulation of signaling pathways. Cell **79**:1129–1132.
104. Minowada G, Weich WJ 1995 Clinical implications of the stress response. J Clin Invest **95**:3–12.
105. Moseley P 2000 Stress proteins and the immune response. Immunopharmacol **48**:299–302.
106. More S, Breloer M, Fleischer B, von Bonin A 1999 Activation of cytotoxic T-cells *in vitro* by recombinant gp96 fusion proteins irrespective of the "fused" antigenic peptide sequence. Immunol Lett **69**:275–282.
107. Wallin RP, Lundqvist A, More SH, von Bonin A, Kiessling R, Ljunggren HG 2002 Heat-shock proteins as activators of the innate immune system. Trends Immunol **23**:130–135.
108. Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H 2002 HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J Biol Chem **277**:15107–15112.
109. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK 2002 Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem **277**:15028–15034.
110. Berthold J, Bauer MF, Schneider H-C, Klaus C, Dietmeier K, Neupert W, Brunner M 1995 The MIM complex mediates preprotein translocation across the mitochondrial inner membrane and couples it to the mt-Hsp70/ATP driving system. Cell **81**:1085–1093.
111. Brodsky JL 1996 Post-translational protein translocation: not all hsc70s are created equal. Trends Biochem Sci **21**:122–126.
112. Voos W, Martin H, Krimmer T, Pfanner N 1999 Mechanisms of protein translocation into mitochondria. Biochim Biophys Acta **1422**:235–254.
113. Polla BS, Healy AM, Wojno WC, Krane SM 1987 Hormone 1α,25-dihydroxyvitamin D_3 modulates heat shock response in monocytes. Am J Physiol **252**:C640–C649.
114. Gacad MA, Adams JS 1998 Proteins in the heat shock-70 family specifically bind 25-hydroxyvitamin D_3 and 17-beta-estradiol. J Clin Endocrinol Metab **83**:1264–1267.
115. Gacad MA, Chen H, Arbelle JE, LeBon T, Adams JS 1997 Functional characterization and purification of an intracellular vitamin D–binding protein in vitamin D–resistant new world primate cells. Amino acid sequence homology with proteins in the hsp-70 family. J Biol Chem **272**:8433–8440.
116. Wu S, Ren S, Chen H, Chun RF, Gacad MA, Adams JS 2000 Intracellular vitamin D–binding proteins: novel facilitators of vitamin D–directed transactivation. Mol Endocrinol **14**: 1387–1397.
117. Adams JS, Chen H, Chun RF, Nguyen L, Wu S, Ren SY, Barsony J, Gacad MA 2003 Novel regulators of vitamin D action and metabolism: Lessons learned at the Los Angeles zoo. J Cell Biochem **88**:308–314.
118. Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, Melsen F, Christensen EI, Willnow TE 1999 An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D_3. Cell **96**:507–515.
119. Willnow TE, Nykjaer A 2002 Pathways for kidney-specific uptake of the steroid hormone 25-hydroxyvitamin D_3. Curr Opin Lipidol **13**:255–260.
120. Provvedini DM, Tsoukas CD, Deftos LJ, Manolagas SC 1983 1,25-dihydroxyvitamin D_3 receptors in human leukocytes. Science **221**:1181–1182.
121. Bhalla AK, Amento EP, Clemens TL, Holick MF, Krane SM 1983 Specific high-affinity receptors for 1,25-dihydroxyvitamin D_3 in human peripheral blood mononuclear cells. J Clin Endocrinol Metab **57**:1308–1310.
122. Rigby W 1988 The immunobiology of vitamin D. Immunol Today **9**:54–58.
123. Hewison M 1992 Vitamin D and the immune system. J Endocrinology **132**:173–175.
124. Fagan DL, Prehn JL, Jordan SC, Adams JS 1991 The human myelomonocytic cell line U937 as a model for studying alterations in monokine gene expression by 1,25-dihydroxyvitamin D. Mol Endocrinol **5**:179–186.
125. Prehn JL, Fagan DL, Jordan SC, Adams JS 1992 Potentiation of lipopolysaccharide-induced tumor necrosis factor-alpha expression by 1,25-dihydroxyvitamin D_3. Blood **80**:2811–2816.
126. Bar-Shavit Z, Teitelbaum SL, Reitsma P, Hall A, Pegg LE, Trial J, Kahn AJ 1983 Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA **80**:5907–5910.
127. Bhalla AK, Amento EP, Krane SM 1986 Differential effects of 1,25-dihydroxyvitamin D_3 on human lymphocytes and monocyte/macrophages: inhibition of interleukin-2 and augmentation of interleukin-1 production. Cell Immunol **98**: 311–322.
128. Ucla C, Roux-Lombard P, Dayer J-M, Mach B 1990 IFNγ drastically modifies the regulation of IL-1 genes by endotoxin in U937 cells. J Clin Invest **85**:185–191.
129. Morel PA, Manolagas SC, Provvedini DM, Wegmann DR, Chiller JM 1986 Interferon-gamma-induced IA expression in WEHI-3 cells is enhanced by the presence of 1,25-dihydroxyvitamin D_3. J Immunol **136**:2181–2186.
130. Rook GAW, Steele J, Fraher L, Barker S, Karmali R, O'Riordan J, Stanford J 1986 Vitamin D_3, gamma-interferon, and control of proliferation of mycobacterium tuberculosis by human monocytes. Immunology **57**:159–163.
131. Cohen MS, Mesler DE, Snipes RG, Gray TK 1986 1,25-dihydroxyvitamin D_3 activates secretion of hydrogen peroxide by human monocytes. J Immunol **136**:1049–1053.
132. Rigby WF, Waugh M, Graziano RF 1990 Regulation of human monocyte HLA-DR and CD4 antigen expression and antigen presentation by 1,25-dihydroxyvitamin D_3. Blood **76**:189–197.
133. Penna G, Adorini L 2000 1α,25-dihydroxyvitamin D_3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T-cell activation. J Immunol **164**:2405–2411.
134. Griffin MD, Lutz W, Phan VA, Bachman LA, McKean DJ, Kumar R 2001 Dendritic cell modulation by 1α,25-dihydroxyvitamin D_3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity *in vitro* and *in vivo*. Proc Natl Acad Sci USA **98**:6800–6805.

135. Piemonti L, Monti P, Sironi M, Fraticelli P, Leone BE, Dal Cin E, Allavena P, Di Carlo V 2000 Vitamin D_3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. J Immunol **164**:4443–4451.
136. Berer A, Stockl J, Majdic O, Wagner T, Kollars M, Lechner K, Geissler K, Oehler L 2000 1,25-Dihydroxyvitamin D_3 inhibits dendritic cell differentiation and maturation *in vitro*. Exp Hematol **28**:575–583.
137. Hewison M, Freeman L, Hughes SV, Evans KN, Bland R, Eliopoulos AG, Kilby MD, Moss PA, Chakraverty R 2003 Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. J Immunol **170**: 5382–5890.
138. Fritsche J, Mondal K, Ehrnsperger A, Andreesen R, Kreutz M 2003 Regulation of 25-hydroxyvitamin D_3-1α-hydroxylase and production of 1α,25-dihydroxyvitamin D_3 by human dendritic cells. Blood.
139. Deluca HF, Cantorna MT 2001 Vitamin D: its role and uses in immunology. FASEB J **15**:2579–2585.
140. Lemire JM 1992 Immunomodulatory role of 1,25-dihydroxyvitamin D_3. J Cell Biochem **49**:26–31.
141. Lemire JM, Adams JS, Kermani-Arab V, Bakke AC, Sakai R, Jordan SC 1985 1,25-dihydroxyvitamin D suppresses human T helper-inducer lymphocyte activity *in vitro*. J Immunol **134**:219–224.
142. Tsoukas CD, Provvedini DM, Manolagas SC 1984 1,25-dihydroxyvitamin D_3, a novel immunoregulatory hormone. Science **224**:1438–1440.
143. Nun JD, Katz DR, Barker S, Fraher LJ, Hewison M, Hendy GN, O'Riordan JL 1986 Regulation of human tonsillar T-cell proliferation by the active metabolite of vitamin D_3. Immunol **59**:479–484.
144. Rigby WF, Noelle RJ, Krause K, Fanger MW 1985 The effects of 1,25-dihydroxyvitamin D_3 on human T lymphocyte activation and proliferation. J Immunol **135**:2279–2286.
145. Rigby WFC, Denome S, Fanger MW 1987 Regulation of lymphokine production and human T lymphocyte activation by 1,25-$(OH)_2D_3$. J Clin Invest **79**:1659–1664.
146. Lemire JM, Adams JS, Sakai R, Jordan SC 1984 1,25-dihydroxyvitamin D suppresses proliferation and immunoglobulin production by normal human peripheral blood mononuclear cells. J Clin Invest **74**:857–861.
147. Lemire JM, Adams JS 1992 1,25-dihydroxyvitamin D inhibits delayed-type hypersensitivity mediated by T-cell clones inducing experimental auto-immune encephalomyelitis. J Bone Min Res **7**:171–178.
148. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A 2001 1α,25-dihydroxyvitamin D_3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. J Immunol **167**:4974–4980.
149. Lemire JM, Archer DC, Beck L, Spiegelberg HL 1995 Immunosuppressive actions of 1,25-dihydroxyvitamin D_3: preferential inhibition of Th1 functions. J Nutr **125**(6 Suppl): 1704S–1708S.
150. Rook GA, Hernandez-Pando R, Lightman SL 1994 Hormones, peripherally activated prohormones, and regulation of the Th1/Th2 balance. Immunol Today **15**:301–303.
151. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, de Waal-Malefyt R, Coffman RL, Hawrylowicz CM, O'Garra A 2002 *In vitro* generation of interleukin 10-producing regulatory CD4(+) T-cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J Exp Med **195**:603–616.
152. Gregori S, Casorati M, Amuchastegui S, Smiroldo S, Davalli AM, Adorini L 2001 Regulatory T-cells induced by 1α,25-dihydroxyvitamin D_3 and mycophenolate mofetil treatment mediate transplantation tolerance. J Immunol **167**: 1945–1953.
153. Adorini L 2003 Tolerogenic dendritic cells induced by vitamin D receptor ligands enhance regulatory T-cells inhibiting autoimmune diabetes. Ann N Y Acad Sci **987**:258–252.
154. Aslam SM, Garlich JD, Qureshi MA 1998 Vitamin D deficiency alters the immune responses of broiler chicks. Poult Sci **77**:842–849.
155. Yang S, Smith C, Prahl JM, Luo X, DeLuca HF 1993 Vitamin D deficiency suppresses cell-mediated immunity *in vivo*. Arch Biochem Biophys **303**:98–106.
156. McMurray DN, Bartow RA, Mintzer CL, Hernandez-Frontera E 1990 Micronutrient status and immune function in tuberculosis. Ann N Y Acad Sci **587**:59–69.
157. Wientroub S, Winter CC, Wahl SM, Wahl LM 1989 Effect of vitamin D deficiency on macrophage and lymphocyte function in the rat. Calcif Tissue Int **44**:125–130.
158. Mariani E, Ravaglia G, Forti P, Meneghetti A, Tarozzi A, Maioli F, Boschi F, Pratelli L, Pizzoferrato A, Piras F, Facchini A 1999 Vitamin D, thyroid hormones, and muscle mass influence natural killer (NK) innate immunity in healthy nonagenarians and centenarians. Clin Exp Immunol **116**:19–27.
159. Yener E, Coker C, Cura A, Keskinoglu A, Mir S 1995 Lymphocyte subpopulations in children with vitamin D–deficient rickets. Acta Paediatr Jpn **37**:500–502.
160. The EURODIAB Substudy 2 Study Group 1999 Vitamin D supplement in early childhood and risk for Type I (insulin-dependent) diabetes mellitus. Diabetologia **42**:51–54.
161. Jeevan A, Kripke ML 1989 Effect of a single exposure to ultraviolet radiation on *Mycobacterium bovis* bacillus Calmette-Guerin infection in mice. J Immunol **143**: 2837–2843.
162. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S 1997 Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia, and growth retardation after weaning. Nat Genet **16**:391–396.
163. Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, Goltzman D 2001 Targeted ablation of the 25-hydroxyvitamin D 1α-hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. Proc Natl Acad Sci U S A **98**:7498–7503.
164. Mathieu C, Van Etten E, Gysemans C, Decallonne B, Kato S, Laureys J, Depovere J, Valckx D, Verstuyf A, Bouillon R 2001 *In vitro* and *in vivo* analysis of the immune system of vitamin D receptor knockout mice. J Bone Miner Res **16**:2057–2065.
165. O'Kelly J, Hisatake J, Hisatake Y, Bishop J, Norman A, Koeffler HP 2002 Normal myelopoiesis but abnormal T lymphocyte responses in vitamin D receptor knockout mice. J Clin Invest **109**:1091–1099.
166. Barnes PF, Modlin RL, Bikle DD, Adams JS 1989 Transpleural gradient of 1,25-dihydroxyvitamin D in tuberculous pleuritis. J Clin Invest **83**:1527–1532.
167. Adams JS, Modlin RL, Diz MM, Barnes PF 1989 Potentiation of the macrophage 25-hydroxyvitamin D-1-hydroxylation reaction by human tuberculous pleural effusion fluid. J Clin Endocrinol Metab **69**:457–460.
168. Rook GAW, Taverne J, Leveton C, Steele J 1987 The role of gamma-interferon, vitamin D_3 metabolites, and tumor necrosis factor in the pathogenesis of tuberculosis. Immunology **62**:229–234.

169. Rook GAW 1988 The role of vitamin D in tuberculosis. Am Rev Respir Dis **138**:768–770.
170. Mawer EB, Hayes ME, Still PE, Davies M, Lumb GA, Palit J, Holt PJL 1991 Evidence for nonrenal synthesis of 1,25-dihydroxyvitamin D in patients with inflammatory arthritis. J Bone Min Res **6**:733–739.
171. Hayes ME, Bayley D, Still P, Palit J, Denton J, Freemont AJ, Cooper RG, Mawer EB 1992 Differential metabolism of 25-hydroxyvitamin D_3 by cultured synovial fluid macrophages and fibroblast-like cells from patients with arthritis. Ann Rheum Dis **51**:220–226.
172. Hayes ME, O'Donoghue DJ, Ballardie FW, Mawer EB 1987 Peritonitis induces the synthesis of 1α,25-dihydroxyvitamin D_3 in macrophages from CAPD patients. FEBS **220**:307–310.
173. Shany S, Rapoport J, Zuili I, Gavriel A, Lavi N, Chaimovitz C 1991 Metabolism of 25-OH-vitamin D_3 by peritoneal macrophages from CAPD patients. Kid Int **39**:1005–1011.
174. Barnard J, Newman LS 2001 Sarcoidosis: immunology, rheumatic involvement, and therapeutics. Curr Opin Rheumatol **13**:84–91.
175. Sharma OP 1996 Vitamin D, calcium, and sarcoidosis. Chest **109**:535–539.
176. Studdy PR, Bird R, Neville E, James DG 1980 Biochemical findings in sarcoidosis. J Clin Pathol **33**:528–533.
177. James DG, Neville E, Siltzbach LE, *et al*. 1976 A worldwide review of sarcoidosis. Ann N Y Acad Sci **278**:321–334.
178. James DG, Siltzbach LE, Sharma OP, Carstairs LS 1969 A tale of two cities: a comparison of sarcoidosis in London and New York. Arch Intern Med **123**:187–191.
179. Rizzato G, Montemurro L, Fraioli P 1992 Bone mineral content in sarcoidosis. Semin Resp Med **13**:411–423.
180. Vergnon GM, Chappard D, Mounier D, *et al*. 1988 Phosphocalcic metabolism, bone quantitative histomorphometry, and clinical activity in 10 cases of sarcoidosis. In: Grassi C, Rizzato G, Pozzi E (eds.) Sarcoidosis and other granulomatous disorders. Elsevier: Amsterdam pp. 499–502.
181. Fallon MD, Perry HM III, Teitelbaum SL 1981 Skeletal sarcoidosis with osteopenia. Metab Bone Dis Res **3**:171–174.
182. Need AG, Phillips PJ 1980 Hypercalcemia associated with tuberculosis. Br Med J **280**:831.
183. Felsenfeld AJ, Drezner MK, Llach F 1945 Hypercalcemia and elevated calcitriol in a maintenance dialysis patient with tuberculosis. Arch Intern Med **146**:1941–1945.
184. Gkonos PJ, London R, Hendler ED 1984 Hypercalcemia and elevated 1,25-dihydroxyvitamin D levels in a patient with end-stage renal disease and active tuberculosis. N Engl J Med **311**:1683–1685.
185. Epstein S, Stern PH, Bell NH, Dowdeswell I, Turner RT Evidence for abnormal regulation of circulating 1α,25-dihydroxyvitamin D in patients with pulmonary tuberculosis and normal calcium metabolism. Calcif Tissue Int **36**:541–544.
186. Bell NH, Shary J, Shaw S, Turner RT 1985 Hypercalcemia associated with increased circulating 1,25-dihydroxyvitamin D in a patient with pulmonary tuberculosis. Calcif Tissue Int **37**:588–591.
187. Cadranel J, Garabedian M, Milleron B, Guillozo H, Akoun G, Hance AJ 1990 $1,25(OH)_2D_3$ production by T lymphocytes and alveolar macrophages recovered by lavage from normocalcemic patients with tuberculosis. J Clin Invest **85**: 1588–1593.
188. Cadranel JL, Garabedian M, Milleron B, Guillozzo H, Valeyre D, Paillard F, Akoun G, Hance AJ 1994 Vitamin D metabolism by alveolar immune cells in tuberculosis: correlation with calcium metabolism and clinical manifestations. Eur Res J **7**:1103–1110.
189. Isaacs RD, Nicholson GI, Holdaway IM. Miliary tuberculosis with hypercalcemia and raised vitamin D concentrations. Thorax **42**:555–556.
190. Shai F, Baker RK, Addrizzo JR, Wallach S 1972 Hypercalcemia in mycobacterial infection. J Clin Endocrinol Metab **34**:251–256.
191. Braman SS, Goldman AL, Schwarz MI 1973 Steriod-responsive hypercalcemia in disseminated bone tuberculosis. Arch Intern Med **90**:327–328.
192. Hoffman VH, Korzeniowski OM 1986 Leprosy, hypercalcemia, and elevated serum calcitriol levels. Ann Intern Med **105**:890–891.
193. Ryzen E, Rea TH, Singer FR 1988 Hypercalcemia and abnormal 1,25-dihydroxyvitamin D concentrations in leprosy. Am J Med **84**:325–329.
194. Kantarijian HM, Saad MF, Estey EH, Sellin RV, Samaan NA 1983 Hypercalcemia in disseminated candidiasis. Am J Med **74**:72l–724.
195. Walker JV, Baran D, Yakub YN, Freeman RB 1977 Histoplasmosis with hypercalcemia, renal failure, and papillary necrosis. Confusion with sarcoidosis. JAMA **237**: 1350–1352.
196. Parker MS, Dokoh S, Woolfenden JM, Buchsbaum HW 1984 Hypercalcemia in coccidioidomycosis. Am J Med **76**: 341–343.
197. Ahmed B, Jaspan JB 1993 Case report: hypercalcemia in a patient with AIDS and *Pneumocystis carinii* pneumonia. Am J Med Sci **306**:313–316.
198. Kozeny G, Barbato A, Bansal VK, Vertuno LL, Hano JE 1984 Hypercalcemia associated with silicone-induced granulomas. N Engl J Med **311**:1103–1105.
199. Jurney TH 1984 Hypercalcemia in a patient with eosinophilic granuloma. Am J Med **76**:527–528.
200. Edelson GW, Talpos GB, Bone HG III 1993 Hypercalcemia associated with Wegener's granulomatosis and hyperparathyroidism: etiology and management. Am J Nephrol **13**: 275–277.
201. Al-Ali H, Yabis AA, Issa E, Salem Z, Tawil A, Khoury N, Fuleihan Gel-H 2002 Hypercalcemia in Langerhans' cell granulomatosis with elevated 1,25 dihydroxyvitamin D (calcitriol) level. Bone **30**:331–334.
202. Stoeckle JD, Hardy HL, Weber AL 1969 Chronic beryllium disease. Long-term follow-up of 60 cases and selective review of the literature. Am J Med **46**:545–561.
203. Cook JS, Stone MS, Hansen JR 1992 Hypercalcemia in association with subcutaneous fat necrosis of the newborn: studies of calcium-regulating hormones. Ped **90**:93–96.
204. Bosch X 1998 Hypercalcemia due to endogenous overproduction of 1,25-dihydroxyvitamin D in Crohn's disease. Gastroenterol **114**:1061–1065.
205. Andres PG, Friedman LS 1999 Epidemiology and the natural course of inflammatory bowel disease. Gastroenterol Clin North Am **28**:255–281.
206. Tangpricha V, Flanagan JN, Whitlatch LW, Tseng CC, Chen TC, Holt PR, Lipkin MS, Holick MF 2001 25-hydroxyvitamin D-1α-hydroxylase in normal and malignant colon tissue. Lancet **357**:1673–1674.
207. Bareis P, Bises G, Bischof MG, Cross HS, Peterlik M 2001 25-hydroxy-vitamin D metabolism in human colon cancer cells during tumor progression. Biochem Biophys Res Commun **285**:1012–1017.
208. Ogunkolade BW, Boucher BJ, Fairclough PD, Hitman GA, Dorudi S, Jenkins PJ, Bustin SA 2002 Expression of 25-hydroxyvitamin D-1α-hydroxylase mRNA in individuals with colorectal cancer. Lancet **359**:1831–1832.

209. Zaloga GP, Eil C, Medbery CA 1985 Humoral hypercalcemia in Hodgkin's disease. Arch Intern Med **145**:155–157.
210. Rosenthal N, Insogna KL, Godsall JW 1985 Elevations in circulating 1,25-dihydroxyvitamin D_3 in three patients with lymphoma-associated hypercalcemia. J Clin Endocrinol Metab **60**:29–33.
211. Davies M, Mawer EB, Hayes ME, Lumb GA 1985 Abnormal vitamin D metabolism in Hodgkin's lymphoma. Lancet **1**: 1186–1188.
212. Adams JS, Fernandez M, Endres DB, Gill PS, Rasheed S, Singer FR 1979 Hypercalcemia, hypercalciuria, and elevated serum 1,25-dihydroxyvitamin D concentrations in patients with AIDS- and non-AIDS-associated lymphoma. Blood **73**:235–239.
213. Seymour JF, Gagel RF 1993 Calcitriol: The major humoral mediator of hypercalcemia in Hodgkin's lymphomas. Blood **82**:1383–1394.
214. Seymour JF, Gagel RF, Hagemeister FB, Dimopoulos MA, Cabanillas F 1994 Calcitriol production in hypercalcemic and normocalcemic patients with non-Hodgkin's lymphoma. Ann Intern Med **121**:633–640.
215. Davies M, Hayes ME, Liu Yin JA, Berry JL, Mawer EB 1994 Abnormal synthesis of 1,25-dihydroxyvitamin D in patients with malignant lymphoma. J Clin Endocrinol Metab **78**: 1202–1207.
216. Hewison M, Kantorovich V, Liker HR, Van Herle AJ, Cohan P, Zehnder D, Adams JS 2003 Vitamin D–mediated hypercalcemia in lymphoma: evidence for hormone production by tumor-adjacent macrophages. J Bone Miner Res **18**:579–582.
217. Grote TH, Hainsworth JD 1987 Hypercalcemia and elevated serum calcitriol in a patient with seminoma. Arch Intern Med **147**:2212–2213.
218. Bikle DD, Pillai S, Gee E, Hincenbergs M 1991 Tumor necrosis factor-α regulation of 1,25-dihydroxyvitamin D production by human keratinocytes. Endocrinology **129**:33–38.
219. Bikle DD, Halloran BP, Riviere JE 1994 Production of 1,25 dihydroxyvitamin D_3 by perfused pig skin. J Invest Dermatol **102**:796–798.
220. Schwartz GG, Whitlatch LW, Chen TC, Lokeshwar BL, Holick MF 1998 Human prostate cells synthesize 1,25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3. Cancer Epidemiol Biomarkers Prev **7**:391–395.
221. Zehnder D, Evans KN, Kilby MD, Bulmer JN, Innes BA, Stewart PM, Hewison M 2002 The ontogeny of 25-hydroxyvitamin D_3 1α-hydroxylase expression in human placenta and decidua. Am J Pathol **161**:105–114.
222. Diaz L, Sanchez I, Avila E, Halhali A, Vilchis F, Larrea F 2000 Identification of a 25-hydroxyvitamin D_3 1α-hydroxylase gene transcription product in cultures of human syncytiotrophoblast cells. J Clin Endocrinol Metab **85**:2543–2549.
223. Weisman Y, Harell A, Edelstein S, David M, Spirer Z, Golander A 1979 1α,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 *in vitro* synthesis by human decidua and placenta. Nature **281**:317–319.
224. Segersten U, Correa P, Hewison M, Hellman P, Dralle H, Carling T, Akerstrom G, Westin G 2002 25-hydroxyvitamin D_3–1α-hydroxylase expression in normal and pathological parathyroid glands. J Clin Endocrinol Metab **87**:2967–2972.
225. Zehnder D, Bland R, Chana RS, Wheeler DC, Howie AJ, Williams MC, Stewart PM, Hewison M 2002 Synthesis of 1,25-dihydroxyvitamin D_3 by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. J Am Soc Nephrol **13**:621–629.
226. Merke J, Milde P, Lewicka S, Hugel U, Klaus G, Mangelsdorf DJ, Haussler MR, Rauterberg EW, Ritz E 1989 Identification and regulation of 1,25-dihydroxyvitamin D_3 receptor activity and biosynthesis of 1,25-dihydroxyvitamin D_3. Studies in cultured bovine aortic endothelial cells and human dermal capillaries. J Clin Invest **83**:1903–1915.
227. Hsu JY, Feldman D, McNeal JE, Peehl DM 2001 Reduced 1alpha-hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D_3–induced growth inhibition. Cancer Res **61**:2852–2856.
228. Correa P, Segersten U, Hellman P, Akerstrom G, Westin G 2002 Increased 25-hydroxyvitamin D_3 1α-hydroxylase and reduced 25-hydroxyvitamin D_3 24-hydroxylase expression in parathyroid tumors—new prospects for treatment of hyperparathyroidism with vitamin D. J Clin Endocrinol Metab **87**:5826–5829.
229. Holick MF, Adams JS 1990 Vitamin D metabolism and biological function. In: Avioli LV, Krane SM (eds.) Metabolic Bone Disease and Clinically Related Disorders. W. B. Saunders: Philadelphia, PA pp 155–195.
230. Adams JS 1996 Hypercalcemia due to granuloma-forming disorders. In: Favus MJ, Christakos S (eds.) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Lippincott-Raven: Philadelphia, PA pp. 206–209.
231. Bouillon R, Okamura WH, Norman AW 1995 Structure-function relationships in the vitamin D endocrine system. Endocr Rev **16**:200–256.
232. Feldman D 1986 Imidazole derivatives as inhibitors of steroidogensis. Endocr Rev **7**:409–430.
233. Glass AR, Eil C 1986 Ketoconazole-induced reduction in serum 1,25-dihydroxyvitamin D. J Clin Endocrinol Metab **63**:766–769.
234. Glass AR, Eil C 1988 Ketoconazole-induced reduction in serum 1,25-$(OH)_2D_3$ and total serum calcium in hypercalcemic patients. J Clin Endocrinol Metab **66**:934–938.
235. Saggese G, Bertelloni S, Baroncelli GI, DiNero, G 1993 Ketoconazole decreases the serum ionized calcium and 1,25-dihydroxyvitamin D levels in tuberculosis-associated hypercalcemia. AJDC **147**:270–273.
236. Pont A, Williams PL, Loose DS, Feldman D, Reitz RE, Bochra C, Stevens DA 1982 Ketoconazole blocks adrenal steroid synthesis. Ann Int Med **97**:370–372.
237. Adams JS, Lee G 1997 Recovery of bone mineral density following exogenous vitamin D intoxication. Ann Int Med **127**:203–206.

Vitamin D Analogs: An Overview

J. WESLEY PIKE

The classical function of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is to regulate calcium and phosphorus homeostasis in vertebrate organisms. This regulation is achieved through direct actions by the hormone on the intestine, kidney, and bone, and through its ability to regulate inversely the production of PTH from the parathyroid glands. Intestinal absorption of calcium from the diet and conservation of calcium at the level of the kidney are considered paramount to maintaining blood levels of calcium and phosphorus in the long term, although bone provides an immediate and readily available emergency source, particularly when the dietary mineral content is deficient. These fundamental actions provided the early impetus for the use of vitamin D and its derivatives in a variety of clinical settings of deranged mineral metabolism, many of which are discussed in earlier sections of this book. More recently, it has become clear that $1,25(OH)_2D_3$ exerts additional biologic actions on a wide range of cell and tissue types, primarily as a regulator of growth, differentiation, and general cell function. These actions of vitamin D are also discussed throughout the book from both basic science as well as clinical perspectives. Importantly, these highly pleiotropic actions of $1,25(OH)_2D_3$ have prompted the therapeutic use of this hormone for a wide range of clinical indications. They have also provided the rationale for the development of a broad range of vitamin D analogs with selective biological activities.

It is universally accepted that the primary side effect of $1,25(OH)_2D_3$ is its tendency to raise serum calcium levels and to cause hypercalcemia and hypercalciuria. This effect leads to renal stones, soft tissue calcification and can even be lethal when extreme. Unfortunately, this toxic side effect has narrowed the use of natural vitamin D metabolites for both mineral-related as well as novel indications, many of which are described in the following three sections of this monograph. This reality stimulated a search for alternative approaches that could reduce or alleviate these side effects, and led to the identification by Chugai Pharmaceuticals of novel analogs of vitamin D (see Chapter 86) that only weakly stimulate bone calcium mobilization or intestinal calcium absorption (defined here as analogs with low calcemic potential or LCP). This exciting discovery spurred the development of several pharmaceutical programs, particularly those at Roche and Leo Pharmaceutical Companies (see Chapters 84 and 85), aimed at synthesizing additional vitamin D analogs with even more selective properties. Interestingly, while considerable success has been achieved in the development of new analogs with LCP, neither the structural rules for preparing such analogs nor the mechanisms whereby these compounds exert their selective actions are completely understood. Unfortunately, the intense focus on analogs with LCP has limited efforts to develop additional novel ways of circumventing $1,25(OH)_2D_3$ induced hypercalcemia. Accordingly, it has been only recently that specific alternative approaches such as selective and intermittent treatment regimes and combination therapies have been considered. These approaches in conjunction with new and perhaps more selective vitamin D analogs could provide the means of treating a wide variety of cancers for which therapies are currently unavailable.

The natural actions of $1,25(OH)_2D_3$ in calcium and phosphorus homeostasis have been studied for many decades. Nevertheless, it is perhaps most vividly highlighted in the human syndromes of 25-hydroxyvitamin D_3-1α-hydroxylase (1-OHase) deficiency (Chapter 71) and vitamin D receptor dysfunction (hereditary $1,25(OH)_2D_3$ resistant rickets or HVDRR, see Chapter 72). Importantly, the effects of these genetic alterations in humans have been recapitulated most recently in mice using homologous recombination techniques (see Chapters 7 and 20). Despite the utility of the mouse models for in-depth experimentation, however, it is interesting to note that several of the most important principles of vitamin D actions have been illuminated through the study of the human syndromes. For example, the skeletal abnormalities observed as a consequence of HVDRR in humans are rescued via administration of high levels of calcium and phosphorus. A similar observation has been made in the VDR null mouse. This early discovery in humans and subsequently in mice emphasizes the indirect role of vitamin D in both skeletal formation and mineralization while at the same time highlighting its direct role in the intestine and perhaps kidney. Whether all skeletal abnormalities are fully ameliorated under high dietary calcium and phosphorus conditions is presently the subject of much debate. It seems likely that the VDR

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null mice strain will provide the means to answer this important question.

While high calcium and phosphorus levels rescue many of the phenotypic defects that arise in both the human syndromes and in the two animal models, it is unable to rescue all of them. One of particular interest is a hair follicle abnormality that results in alopecia. Surprisingly, while fully penetrant in VDR-null mice, this defect is seen only in a subset of patients with HVDRR and is not seen in 1-OHase deficiency in either humans or mice. These studies have stimulated a working hypothesis that alopecia results only from mutations in the VDR locus that fully compromise the expression of the VDR itself (see Chapters 13 and 72). If true, it would identify a new and potentially unique role for the VDR in hair production that is either independent of $1,25(OH)_2D_3$ or dependent upon a novel and as yet uncharacterized ligand. Collectively, these findings highlight both the complex interrelationship that exists between calcium and phosphorus homeostasis and vitamin D and how clinical disease can call attention to the most intricate of molecular details.

Why consider these issues in the context of sections on vitamin D analogs and their possible indications? It is clear from the above that the biologic effects of $1,25(OH)_2D_3$ can be direct or indirect through various combinations of regulators. Keratinocyte differentiation, as an example, is regulated by both $1,25(OH)_2D_3$ and local calcium concentrations, the latter operating through the modulation of specific transcription factors that act in concert with the VDR (see Chapter 35). Other biological activities, in contrast, arise directly as a result of either $1,25(OH)_2D_3$ or calcium action. Of note are recent studies in the NOD mouse, a model for human type 1 diabetes and in experimental autoimmune encephalomyelitis (EAE), a model for human multiple sclerosis (MS). Interestingly, $1,25(OH)_2D_3$ suppresses the development of diabetes in the NOD mouse even in the absence of hypercalcemia (see Chapters 98 and 99). Based upon this finding, a rationale for the prevention or treatment of clinical type 1 diabetes with new vitamin D analogs with LCP seems attractive. On the other hand, intervention in MS, for example, where both hypercalcemia and vitamin D may play independent roles, could be more problematic. Clearly, delineating the specific roles of vitamin D in both basic biological processes and various disease states will be a prerequisite to the successful application of vitamin D analogs for effective therapeutic indications.

CHAPTER 80

Overview: Rational Design of 1α,25-Dihydroxyvitamin D_3 Analogs (Deltanoids)

GARY H. POSNER AND MEHMET KAHRAMAN
Department of Chemistry, School of Arts and Sciences, The Johns Hopkins University, Baltimore, Maryland

I. INTRODUCTION

Although the natural hormone 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$, calcitriol, Rocaltrol (Chart I) can inhibit the growth of various human cancers and can regulate the human immune system [1], when used at supraphysiological levels this hormone can produce serious side effects such as severe hypercalcemia and soft tissue calcifications [2]. Therefore, the fundamental challenge to organic and medicinal chemists working on designing analogs of 1,25$(OH)_2D_3$ as new drugs is to incorporate structural changes and functional group modifications leading to new deltanoids (vitamin D analogs) that are still efficacious, but safer and more selective than the natural hormone (see Chapters 82–87). Such new deltanoids often serve also as sensitive probes of the fundamental molecular biology underlying a deltanoid's mechanism of action and its biological activity profile. With several thousand deltanoids having been synthesized and evaluated [3], two generalizations among many are as follows: 1) it is still not possible to predict reliably what specific new structural or functional group change will produce a desirably efficacious but low-calcemic deltanoid and 2) progress has been made by both trial and error and by rational design. This chapter will focus on three important rationales that have guided, and continue to guide, design of new deltanoids as drugs (Chart I) and as drug candidates (Chart II): metabolism, molecular biology, and organic chemistry. Generalized deltanoid Structure **1** (chemical structures are indicated in the text by bold numerals) summarizes the most successful kind of structural modifications of 1,25$(OH)_2D_3$ that have been accomplished already. The chapters immediately following this one provide more detailed discussions of the design rationale and of the therapeutic value of several U.S. government-approved deltanoid drugs, as well as of deltanoid drug candidates that are now in human clinical trials.

II. RATIONALE BASED ON METABOLISM

A. Prodrugs

Biosynthesis of the hormone 1,25$(OH)_2D_3$ from 7-dehydrocholesterol [4] is outlined in Scheme 1 and is fully discussed in Chapters 2–5. Some of the first deltanoid drugs were designed based on this biosynthetic route. For example, 25$(OH)D_3$ is the immediate precursor to 1,25$(OH)_2D_3$ on this biochemical path. Because 25$(OH)D_3$ is less calcemic than the hormone 1,25$(OH)_2D_3$ [5], this prodrug Calderol (Chart I) has been used in humans to treat renal failure and osteoporosis; its slow and steady enzymatic conversion in the kidney into low levels of 1,25$(OH)_2D_3$ [6,7] gives it a good balance of efficacy and safety (i.e. a good therapeutic index). Likewise, the clinically-used drug 1$(OH)D_3$ (One-Alpha, Chart I) is a precursor requiring enzymatic C-25 hydroxylation to produce low and constant amounts of the natural hormone 1,25$(OH)_2D_3$ [8]. Similarly, the drug Hectorol (Chart I) requires liver enzyme-promoted C-25 hydroxylation to form 1,25$(OH)_2D_2$ (the hormonal form of vitamin D_2) [9]. The more recent drug Zemplar (Chart I) is the low-calcemic 19-nor version of 1,25$(OH)_2D_2$ [10]. Also, 1$(OH)D_5$ is being developed as an enzymatically activated prodrug for 1,25$(OH)_2D_5$ [11].

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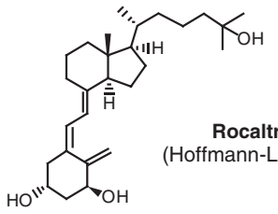
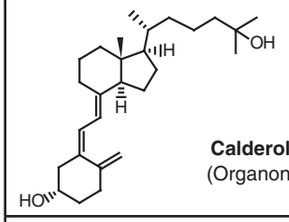
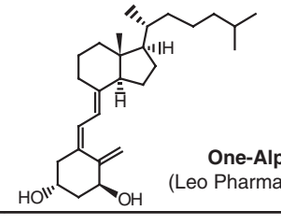
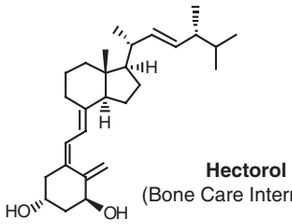
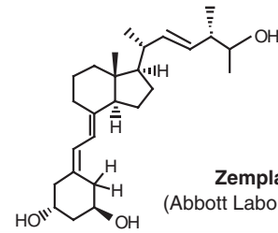
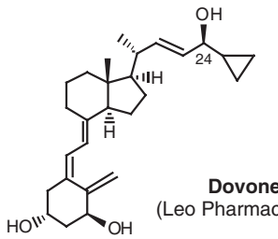
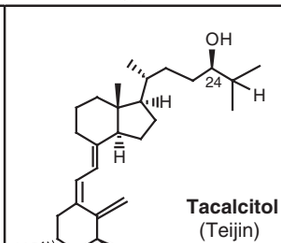
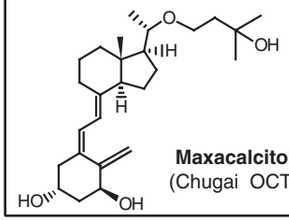
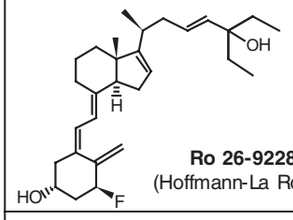
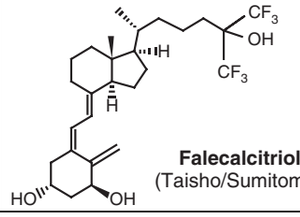
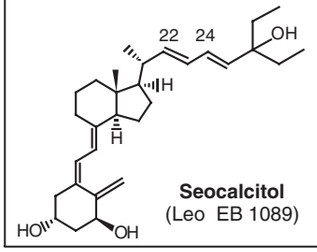
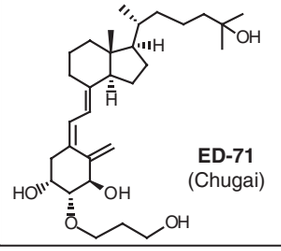
Chart I: DELTANOIDS IN USE AS DRUGS

Deltanoid	Indications	Deltanoid	Indications
 **Rocaltrol** (Hoffmann-La Roche)	Secondary hyperparathyroidism Renal failure Osteoporosis		
 **Calderol** (Organon)	Renal failure Osteoporosis	 **One-Alpha** (Leo Pharmaceutical)	Secondary hyperparathyroidism Renal failure Osteoporosis
 **Hectorol** (Bone Care International)	Secondary hyperparathyroidism	 **Zemplar** (Abbott Laboratories)	Secondary hyperparathyroidism
 **Dovonex** (Leo Pharmaceutical)	Psoriasis	 **Tacalcitol** (Teijin)	Psoriasis
 **Maxacalcitol** (Chugai OCT)	Secondary hyperparathyroidism Psoriasis		

B. Catabolism Inhibitors

The major catabolism of $1,25(OH)_2D_3$ involves enzyme-mediated side chain hydroxylation and, ultimately, side chain fragmentation producing therapeutically inactive calcitroic acid (Scheme 2 and Chapter 81). If such catabolism could be slowed or avoided by small structural and/or functional group changes to the natural hormone, then perhaps very small doses of such new deltanoids could be used therapeutically without causing hypercalcemia [12]. This rationale has led successfully to a variety of new deltanoids in human clinical trials (Chart II) and to others as promising potential drug candidates. Structural changes that inhibit deltanoid catabolism can be divided into two main categories: remote and nearby.

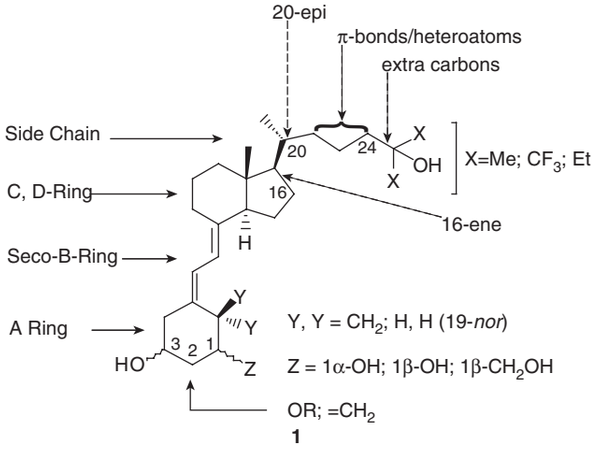
Chart II: DELTANOIDS IN CLINICAL TRIALS

Deltanoid	Indications	Deltanoid	Indications
 **Ro 26-9228** (Hoffmann-La Roche)	Osteoporosis	 **Falecalcitriol** (Taisho/Sumitomo)	Secondary hyperparathyroidism Osteoporosis
 **Seocalcitol** (Leo EB 1089)	Neoplasm Breast tumor Colon tumor Pancreas tumor	 **ED-71** (Chugai)	Osteoporosis

1. REMOTE STRUCTURAL CHANGES

Two publications appearing simultaneously in 1993 were the first reports that remote structural/functional changes can influence the rate of side-chain catabolic oxidation [13,14]. Hoffmann-La Roche's 16-en-24-oxo-1,25$(OH)_2D_3$ (**2**) resists enzymatic 23-hydroxylation [13], and Johns Hopkins' homolog 1-CH_2OH-25$(OH)_2D_3$ **3** resists 24-hydroxylation in human leukemic cells under the conditions in which 1,25$(OH)_2D_3$ is easily 24-hydroxylated [14]. These two reports were the first indications that the presence of the 16-ene modification, as in deltanoid **2**, and the presence of a very remote extra methylene group at the 1-position, as in deltanoid **3**, could alter the ligand-enzyme interaction sufficiently to cause a significant slowing in the rate of the enzymatic side chain oxidation.

Drug Design Alterations

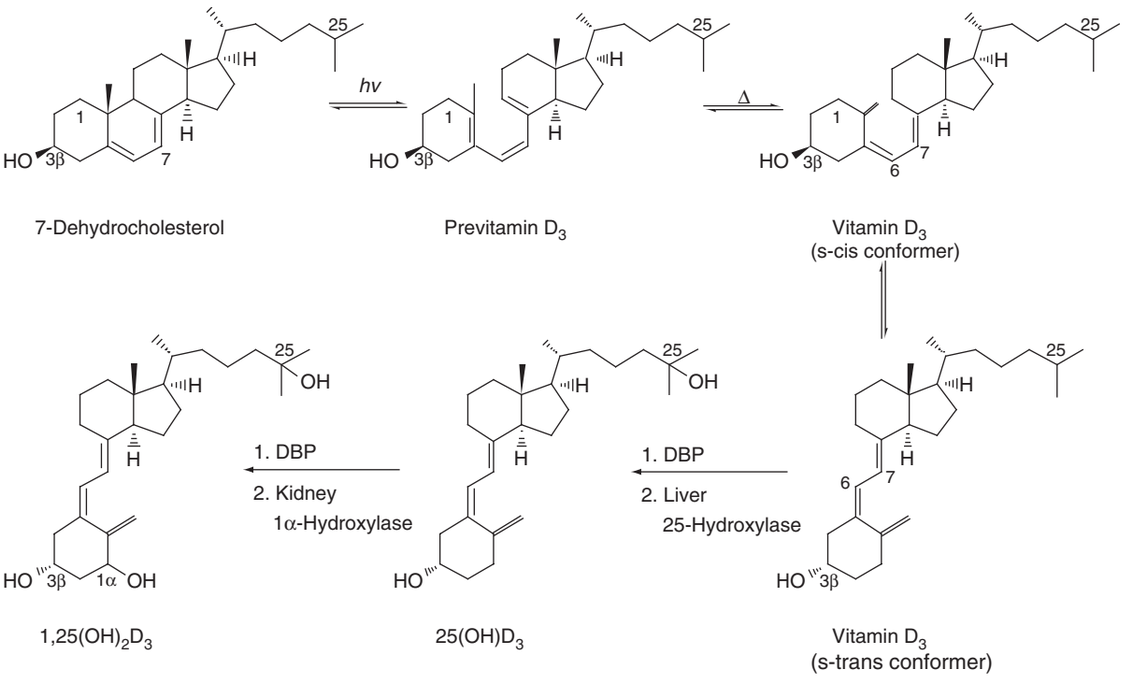


1

STRUCTURE 1

2. NEARBY STRUCTURAL CHANGES

Because catabolism of the hormone 1,25$(OH)_2D_3$ occurs mainly toward the terminus of the side chain (e.g. at C-24 and C-26), retarding such enzymatic hydroxylation and thus prolonging biological half-life has been achieved effectively by incorporating side chain structural changes that operate via steric and electronic effects. Chart II shows Ro 26-9228 [15] and Leo EB 1089 [16], both low-calcemic selective agonists now in human clinical trials, in which unsaturation at C-24 makes the sp^2-hybridized C-24 vinyl-C-H bond stronger and thus less easily oxidized than the normal sp^3-hybridized C-24-H bond [17]. Both Ro 26-9228 and Leo EB 1089 also incorporate an enlarged and therefore sterically protective diethyl carbinol environment near C-24, relative to natural 1,25$(OH)_2D_3$ having a smaller dimethyl carbinol environment near C-24. Also in Chart II, Falecalcitriol incorporates two side chain CF_3 groups that prevent enzymatic C-26 oxidation. Replacing a C-H group by a C-F group, often a miniscule change in a large deltanoid, leads to decreased catabolism because a C-F bond is much stronger and, therefore, less easily broken (i.e. oxidized) than a C-H bond [18]. Thus, in the early 1980s, 24F_2-1,25$(OH)_2D_3$ (**4**) and related 24-fluorinated deltanoids were prepared [19,20]. Although C-24 catabolic hydroxylation was indeed prevented in these compounds, they



SCHEME 1 Biosynthesis of 1α,25-Dihydroxyvitamin D_3

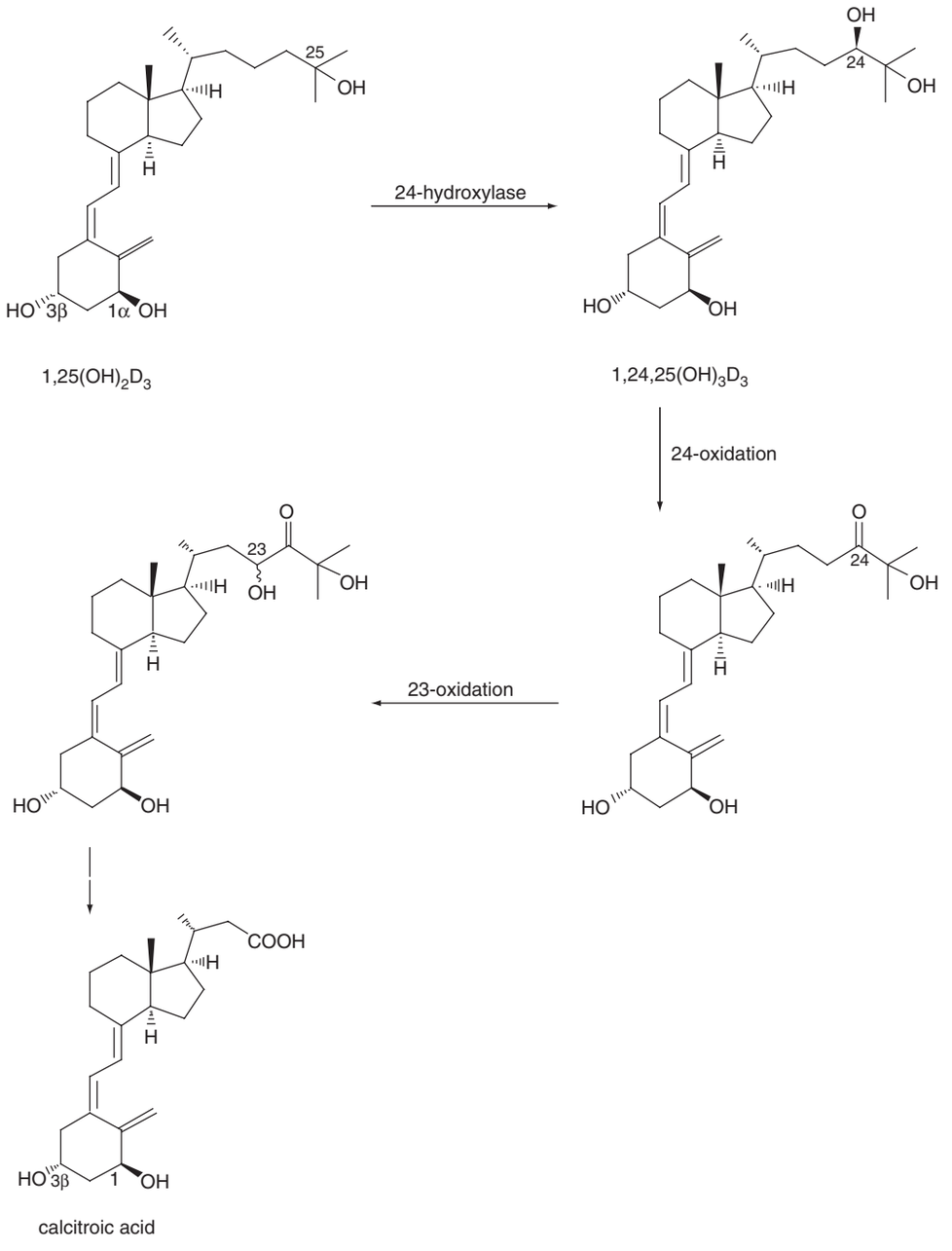
were disappointingly similar to $1,25(OH)_2D_3$ in terms of high calcemic activity [19,20]. In sharp contrast, hybrid deltanoid Hopkins QW $1624F_2$-2 **5** [21], blocked toward C-24 hydroxylation by two fluorine atoms but carrying a calcemia-lowering 1-CH_2OH group, is a selective agonist that inhibits mouse skin tumorigenesis without causing hypercalcemia or animal weight loss over four months of treatment, whereas $1,25(OH)_2D_3$ is lethal under these conditions [22]. Large scale synthesis of 24-difluorinated Hopkins QW $1624F_2$-2 **5** has been completed under the auspices of the NIH RAPID program, and thus this hybrid deltanoid is now available to the scientific community for further study as a molecular probe and as a drug candidate.

C. ACTIVE METABOLITES

When studying the biological profile of any new compounds, it is prudent to check whether any metabolites are physiologically active. Two examples in the vitamin D field are especially noteworthy. Deltanoid 16-ene-24-oxo-$1,25(OH)_2D_3$ (**2**), a C-24 oxidation metabolite of synthetic 16-ene-$1,25(OH)_2D_3$, has some promising therapeutic potential [23]. Also, 3-epimerization has been documented as an important and apparently common metabolic process leading to A-ring cis-oriented 1α,3α-diol deltanoids, many of which are physiologically active [24].

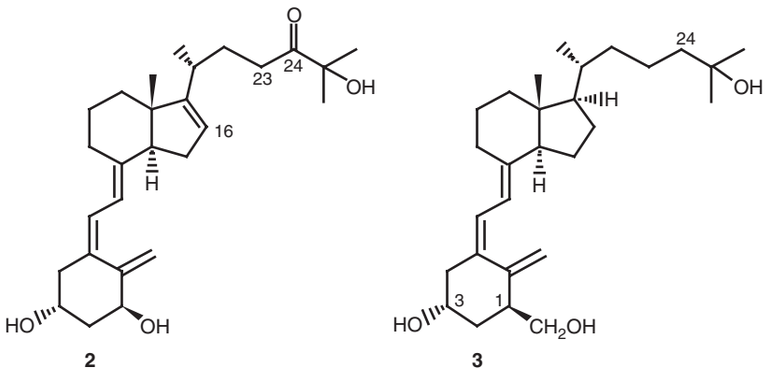
III. RATIONALE BASED ON MOLECULAR BIOLOGY

Rational design of new deltanoids that selectively modulate the nuclear vitamin D receptor (VDR) [25] has been enlightened by X-ray crystal structures of the VDR complexed to $1,25(OH)_2D_2$ and of the VDR complexed also to some other superagonist deltanoids [26–29]. Among the important learnings from these crystal structures are the following: 1) the side chain tertiary OH group of $1,25(OH)_2D_3$ is bound between H-bond acceptor His 305 and H-bond donor His 397; 2) the ligand occupies only 56% of the VDR-binding domain cavity (vs. 66% occupation by retinoic acid); and 3) the ligand-binding domain (LBD) cavity is substantially larger (697 $Å^3$) than in the related hormone receptor RXRγ (412 $Å^3$). Consistent with this relatively large LBD cavity of the VDR, diverse conformationally flexible and yet biologically potent 19-nor deltanoids lacking a D-ring (**6**), or a C-ring (**7**), or both C- and D-rings (**8**) have been reported [30–33]. Impressively, despite the absence of both C- and D-rings, deltanoid **8** has been selected by Hoffmann-La Roche for development as a low-calcemic oral antipsoriatic drug candidate [33]. Based on the reported crystal structure of $1,25(OH)_2D_3$ in the LBD of VDR [26–28], computer-aided molecular modeling and conformational analysis

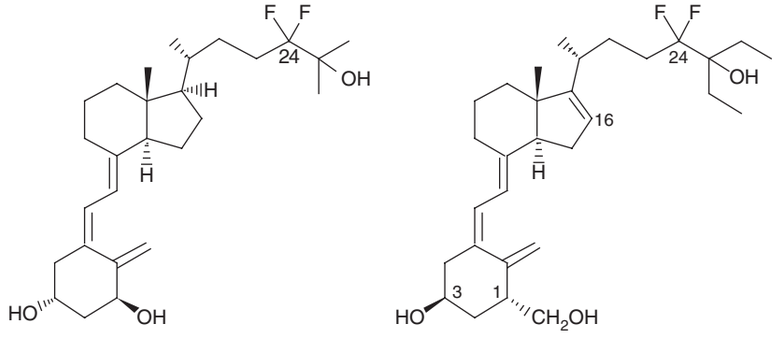


SCHEME 2 Major Catabolism of 1α,25-Dihydroxyvitamin D_3

have assisted rational design of deltanoids that bind to the LBD and that are potent and selective. Among the successful deltanoids designed in this way are the following: 1α-OCH_2Ar deltanoid **9** that selectively restores activity to the VDR mutant associated with rickets [34]; nonsteroidal symmetrical bisphenol derivative **10** (see Chapter 88) that also is a selective agonist for a rickets-associated mutant of the VDR [35]; 22-ethyl deltanoid **11** that is 100 times more potent than the natural hormone in cell-differentiating activity [36–38] and that has a low energy side chain conformation occupying the "active space region" [37–40]; and arocalciferol **12**, one of the first examples of a deltanoid with a conformationally restricted side chain [41].

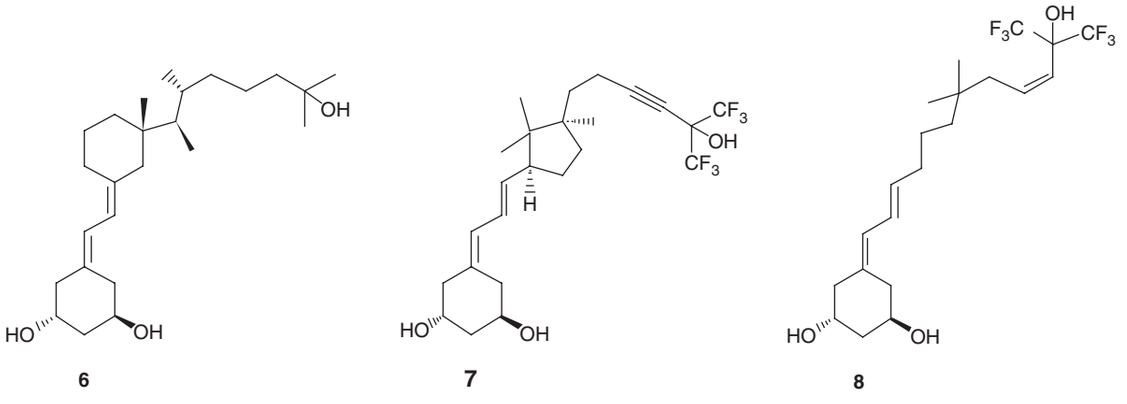


STRUCTURES 1 AND 2

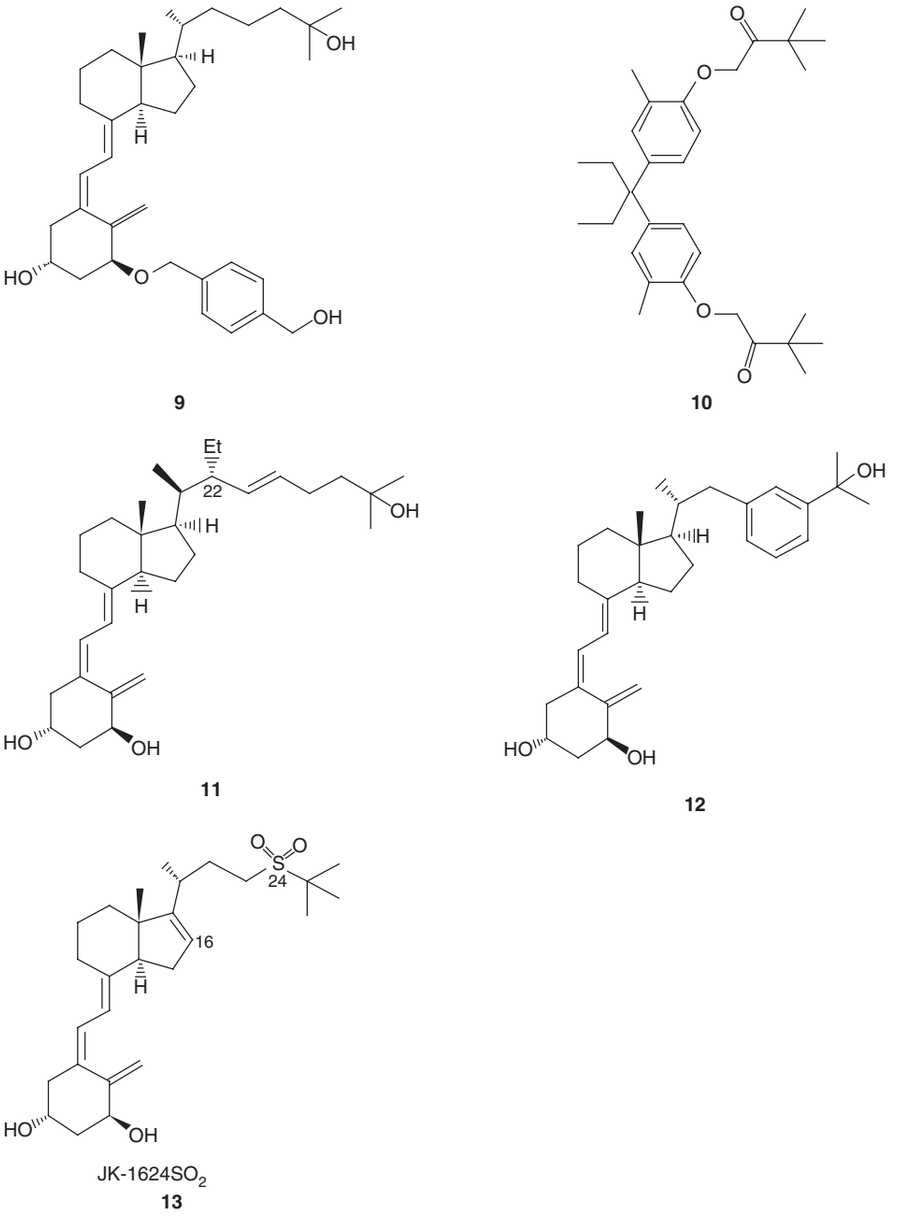


$24F_2$-1,25$(OH)_2D_3$(**4**) Hopkins QW1624F_2-2(**5**)

STRUCTURES 4 AND 5



STRUCTURES 6 AND 8



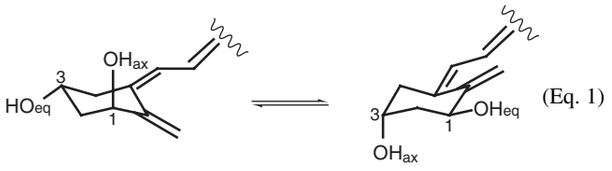
STRUCTURES 9–13

Also, conceptually new 16-ene-24-sulfone deltanoid **13** [42], a transcriptionally potent but low-calcemic deltanoid, docks in the mutant VDR LBD with a sulfone oxygen atom close to both His 305 (2.78 Å) and to His 397 (2.19 Å) [43]; this proximity of the sulfone oxygen to both His 305 and His 397 supports the original design rationale expecting that the sulfone group would act as an H-bond acceptor [42]. DeLuca's group was the first to suggest that even the 25-OH of natural $1,25(OH)_2D_3$ acts as an H-bond acceptor [44].

IV. RATIONALE BASED ON ORGANIC CHEMISTRY

A. Conformation Issues

The cyclohexane A-ring of $1,25(OH)_2D_3$ is conformationally flexible. A-Ring chair-to-chair interconversion via ring flipping occurs at room temperature, thereby interconverting the α-conformer with its 1-axial-OH and 3-equatorial-OH into the corresponding β-conformer

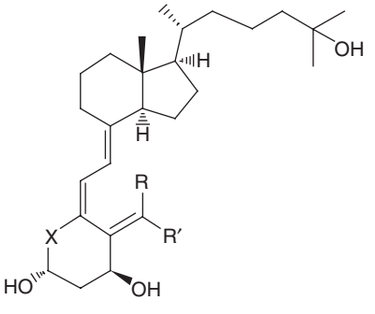


(Eq. 1)

α-conformer β-conformer

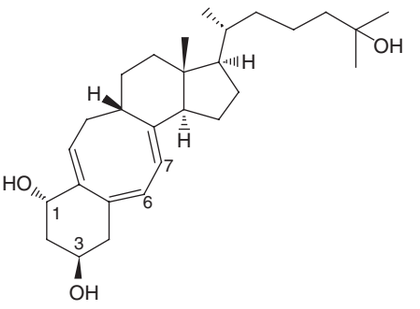
with its 1-equatorial-OH and 3-axial-OH, and *vice versa* (Eq. 1). The A-ring conformation of a deltanoid may change when bound to the VDR. In order to use ^{19}F NMR to probe A-ring conformation in VDR-complexed deltanoids, 4,4-difluoro-1,25$(OH)_2D_3$ (**14**) and 19-fluoro-1,25$(OH)_2D_3$ (**15a** and **15b**) were synthesized [45–47]. Using ^{19}F NMR, 4,4-difluorinated deltanoid **14** was shown to be approximately a 1:1 mixture of A-ring α- and β-conformers, but both geometric isomers **15a** and **15b** of the 19-fluorinated deltanoid exist in only the α-conformer.

The seco-B-ring of 1,25$(OH)_2D_3$ with its conjugated triene system is also conformationally flexible. As shown in Scheme 1 for the biosynthesis of 1,25$(OH)_2D_3$, vitamin D_3 exists as two extreme conformers: 6-*s*-cis and 6-*s*-trans. The "*s*" in these designations refers to the "single bond" between carbon atoms 6 and 7; rotational barriers in carbon-carbon single bonds generally are low, thereby allowing easy interconversions at room temperature between the 6-*s*-cis and 6-*s*-trans single bond conformers of vitamin D_3 and also of 1,25$(OH)_2D_3$. The Riverside group has synthesized and evaluated some deltanoids (e.g. **16**) in which the 6,7-single bond is locked into only the 6-*s*-cis conformation; such exclusive 6-*s*-cis deltanoids have been pivotal in suggesting that the 6-*s*-cis conformer



4,4-difluorocalcitriol (X = CF_2, R = R′ = H, **14**)
(*E*)-19-fluorocalcitriol (X = CH_2, R = F, R′ = H, **15a**)
(*Z*)-19-fluorocalcitriol (X = CH_2, R = H, R′ = F, **15b**)

STRUCTURES 14 AND15



(6-*s*-cis-locked deltanoid)

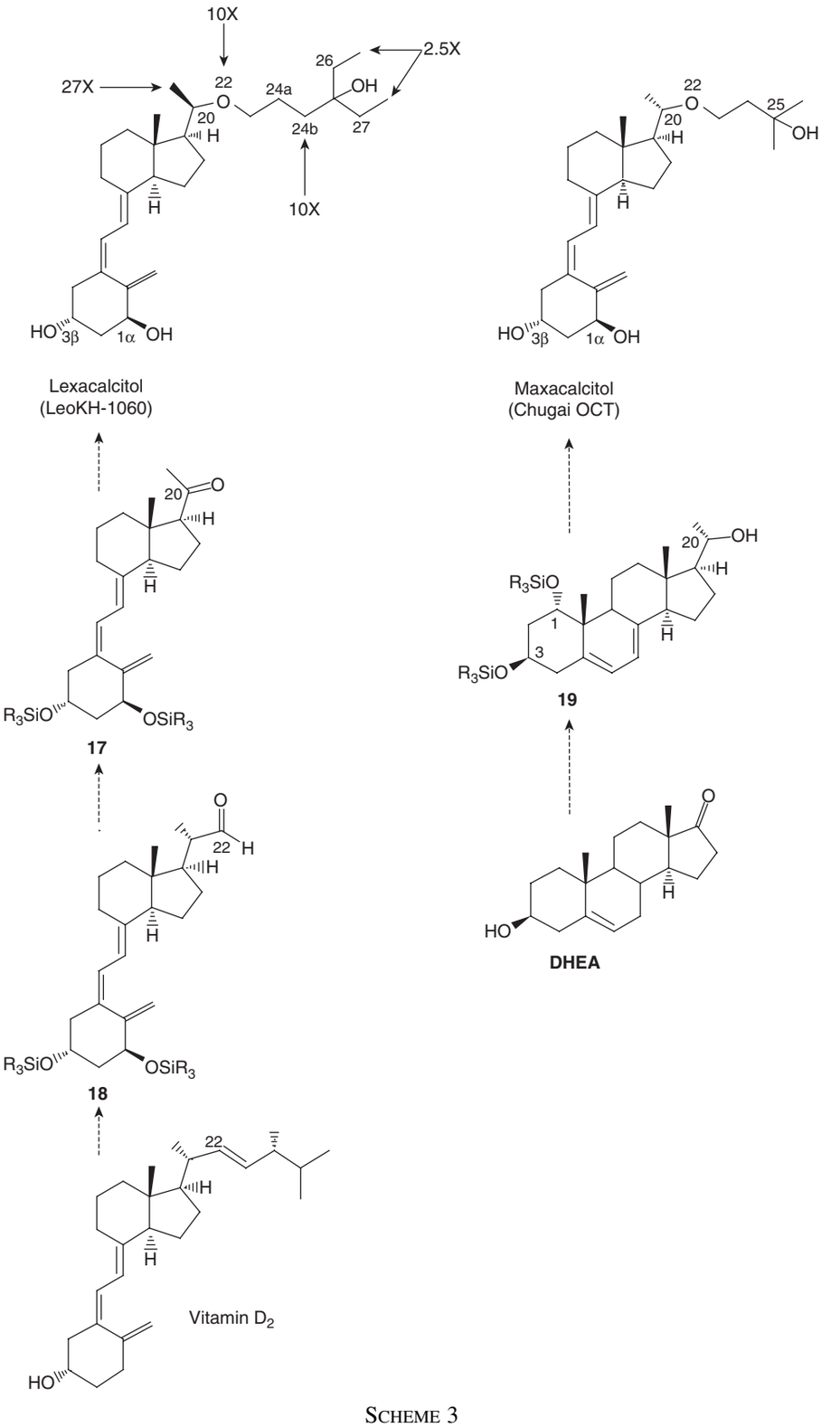
STRUCTURE 16

seems responsible for a deltanoids' nongenomic rapid biological action (Chapter 23), whereas the 6-*s*-trans conformer seems responsible for genomic responses [48–50].

B. Stereoid Precursors

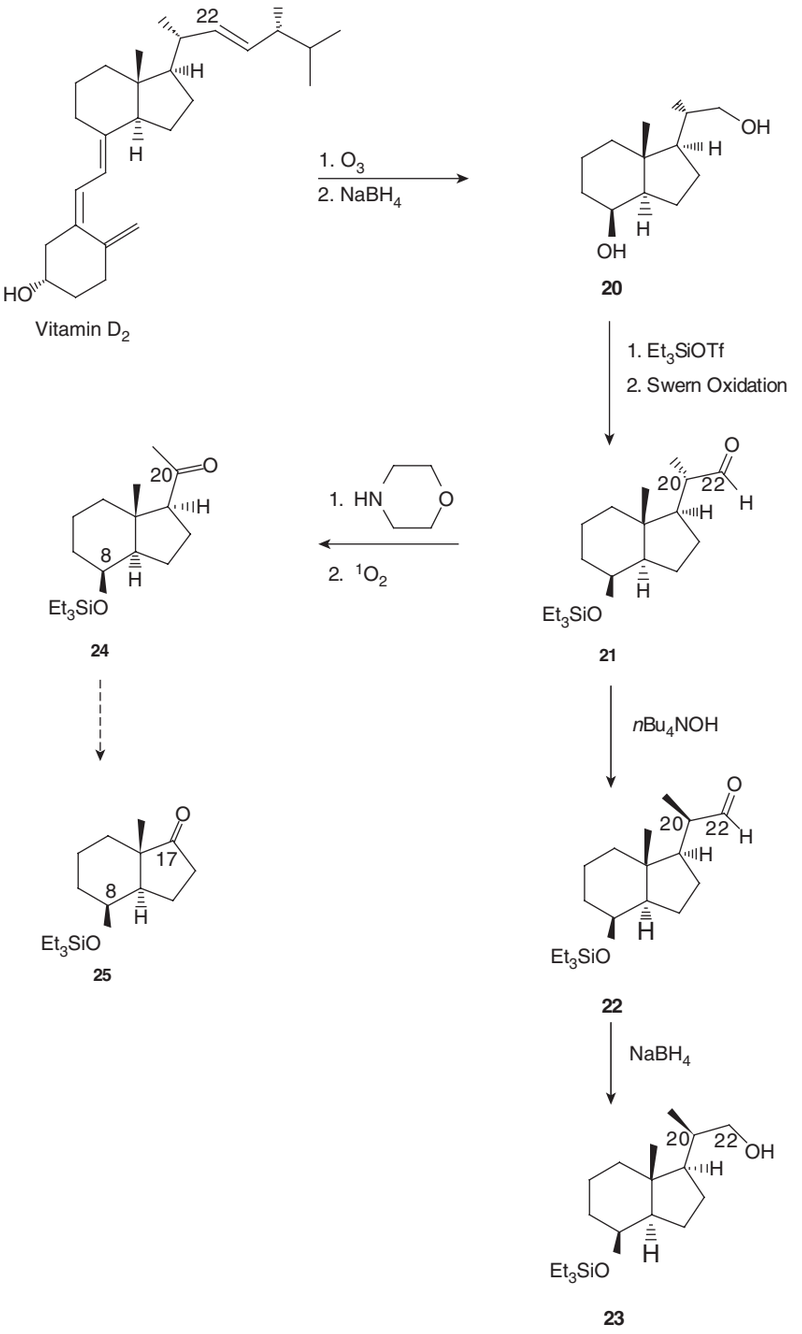
Considerable pharmaceutical company research relating chemical structure to biological activity (structure-activity relationship, SAR) has produced the potent 22-oxa deltanoids Leo KH-1060 (Chapter 84) and Chugai OCT (Chapter 86) (Scheme 3). Such SAR generalizations quantify the antiproliferative potency advantage (Scheme 3, Leo KH-1060, increases in antiproliferative activity are in parentheses) gained by altering the skeleton of 1,25$(OH)_2D_3$ via C-20 epimerization (27X), C-22 oxygenation (10X), and side chain homologation at C-24 and C-27 (2.5X) [3]. Both 22-oxa deltanoids Leo KH-1060 and Chugai OCT are prepared typically from steroid precursors (Scheme 3). Thus, C-20 ketone **17** is derived from C-22 aldehyde **18**, which is prepared via ozonolysis of triene-protected steroidal vitamin D_2 followed ultimately by allylic 1-hydroxylation [51]. Likewise, C-20 alcohol **19** is prepared from the steroid dehydroepiandrosterone (DHEA) [52]. The major advantage of preparing new deltanoids from almost entire steroid precursors is that most of the deltanoid skeleton, including absolute stereochemistry, is available without needing costly and time-consuming multistep synthesis.

The major advantage of using small parts of steroids for construction of new deltanoids is versatility. A shown in Scheme 4, oxidative cleavage and then *in situ* reduction of inexpensive and readily available vitamin D_2 (ergocalciferol) produces versatile, enantiomerically pure deltanoid building block **20** [53]. This C,D-ring unit **20** can be converted easily into diastereomeric

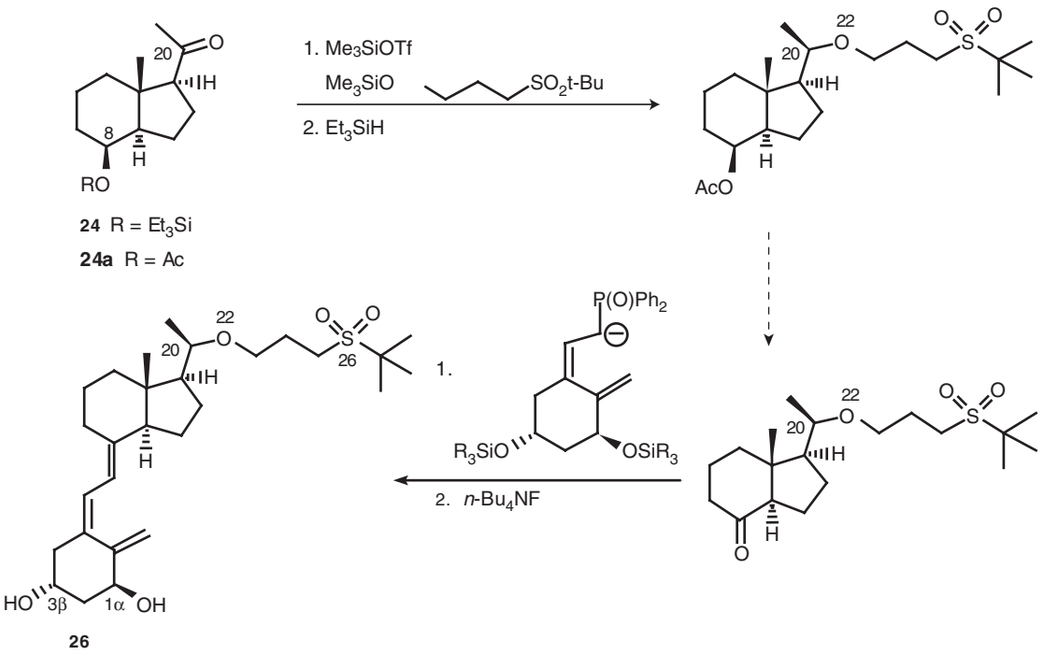

10X
2.5X
27X
22
24a
26
OH
20
O
H
24b
27
10X
HO
3β
1α
OH
Lexacalcitol
(LeoKH-1060)
Maxacalcitol
(Chugai OCT)
25
17
19
18
DHEA
Vitamin D_2
R_3SiO
$OSiR_3$
1
3

SCHEME 3

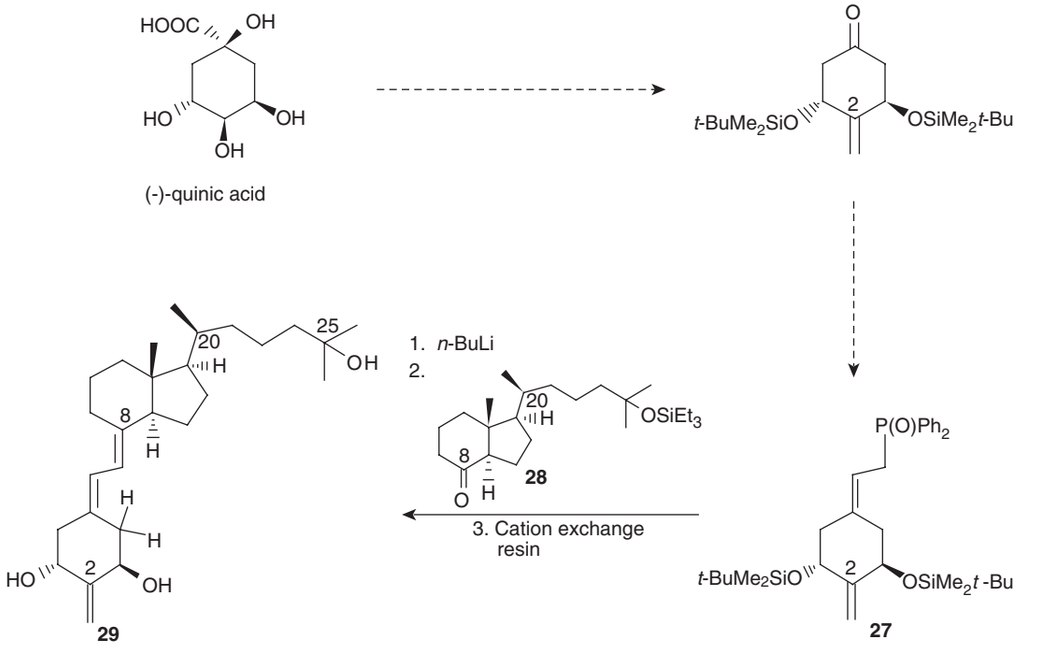
C-22 aldehydes **21** and **22** (differing only by C-20 stereochemistry) and then into C-20-epi C-22 alcohol **23** and into C-20 ketone **24** [53]. Ketone **24** can be converted into C-17 ketone **25** via Baeyer-Villiger oxidation, hydrolysis, and then C-17 oxidation (Scheme 4). These enantiomerically pure C,D-ring building blocks allow versatile attachment of diverse side chains and versatile attachment via Lythgoe coupling [54] also of A-ring portions. Two examples follow.



SCHEME 4



SCHEME 5



SCHEME 6

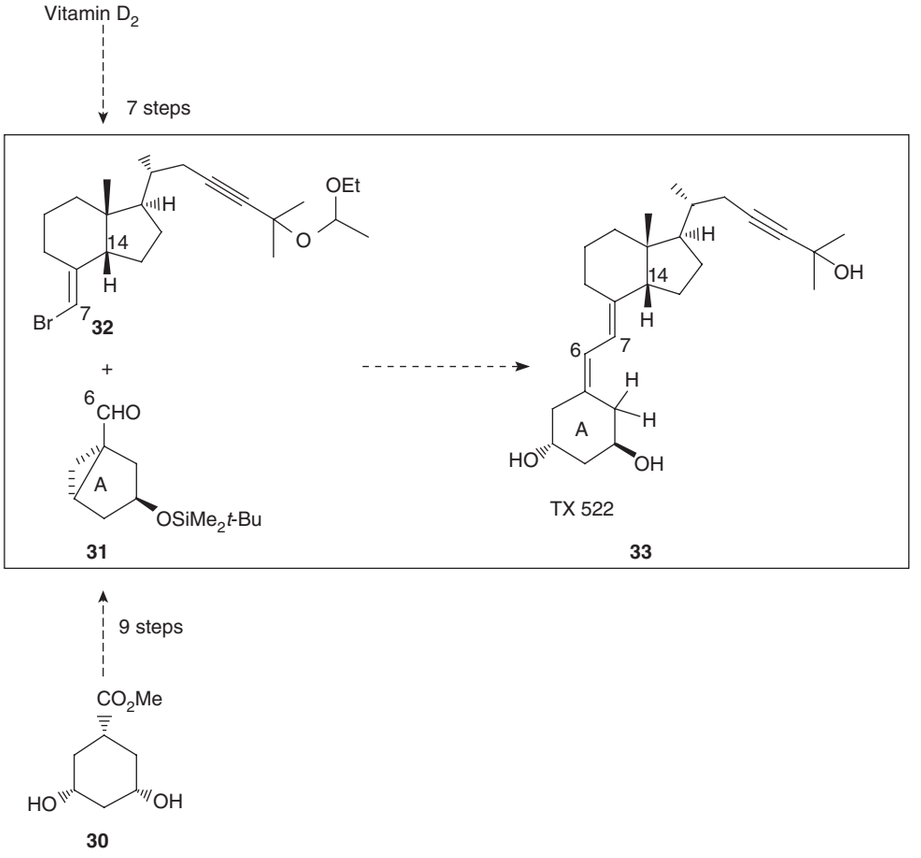
C-20 Ketone **24a** has been converted via reductive etherification into nonclassical side chain sulfone deltanoid **26** (Scheme 5), a powerfully antiproliferative and transcriptionally active but low-calcemic new deltanoid [53]. Also, natural enantiomerically pure (-)-quinic acid has been converted into A-ring phosphine oxide **27** that was joined with (20*S*)-25-oxy Grundmann C-8 ketone **28** via Lythgoe coupling [54] to produce 2-methylene-19-nor-20-epi-1α,25-dihydroxyvitamin D_3 (**29**) (Scheme 6) [55]. This new deltanoid potently and selectively induces bone formation in rats [56] (see Chapter 87).

C. Multistep Synthesis

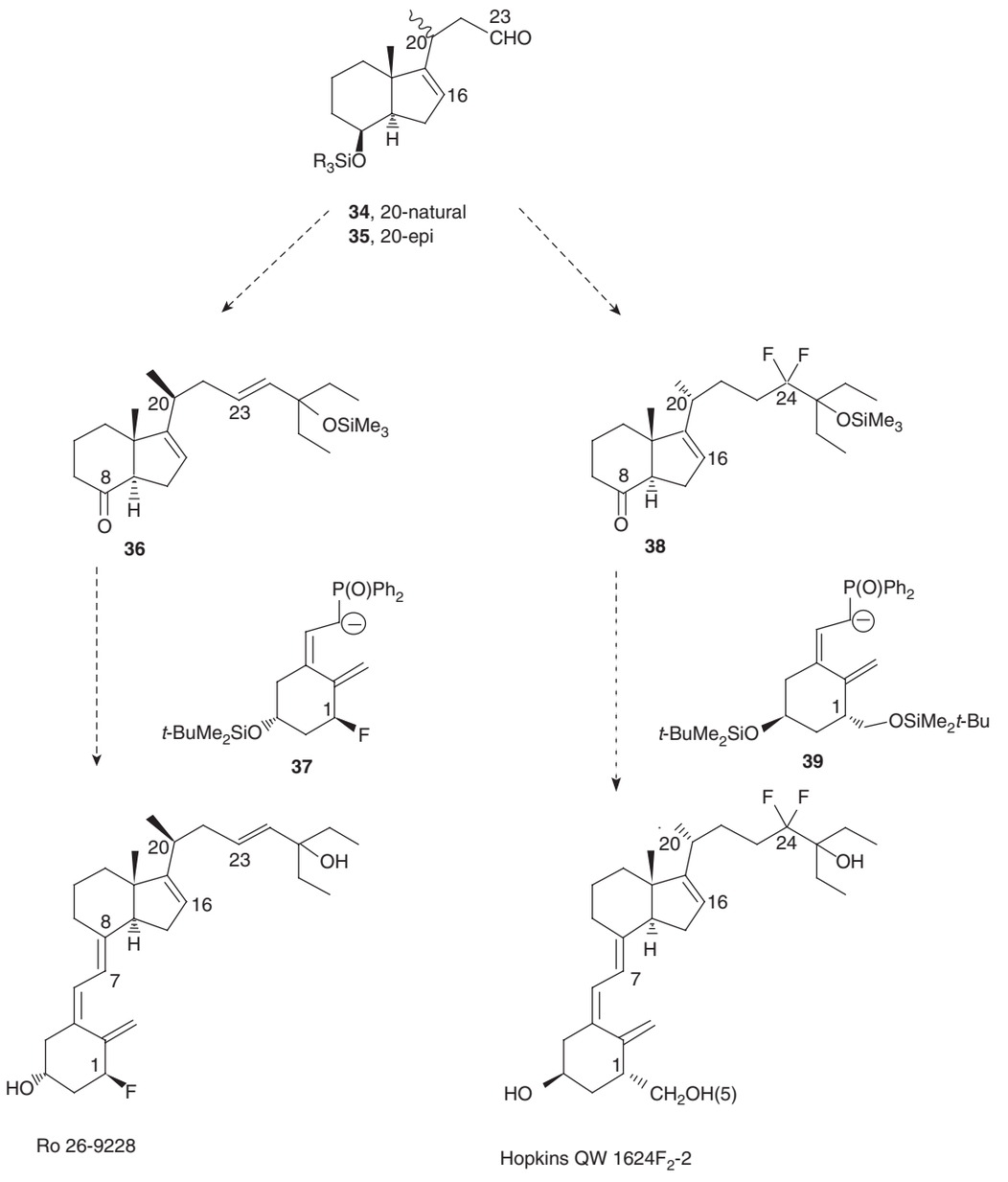
Multistep synthesis of key building blocks that can be joined to form new deltanoids has been reviewed [54,57]. Structurally and stereochemically complex deltanoid building blocks still often offer serious synthetic challenges for organic chemists. Hybrid deltanoids, modified in two remote regions, are best prepared by joining small parts of natural stereoids (typically the enantiomerically pure C,D-ring) with a nonclassical side chain and then with a nonclassical A-ring. Both side chain and A-ring are usually prepared via multistep syntheses. Such convergent synthesis allows for much versatility in selecting components to join and, in principle, allows for small libraries of new deltanoids to be produced [58]. Three examples of multistep syntheses of therapeutically desirable hybrid deltanoids follow.

The Théramex 14-epi-19-nor-23-yne hybrid deltanoid **33** is in advanced clinical trials for treatment of psoriasis [59,60]. It has been prepared via convergent coupling of A-ring cyclopropane aldehyde **31** (prepared from precursor cyclohexanediol **30** in 9 steps) and C,D-ring bromo-olefin **32** (prepared in about 7 steps from vitamin D_2) (Scheme 7).

1α-Fluoro-16,23-diene-20-epi hybrid deltanoid Ro 26-9228 is in human clinical trials for treatment of



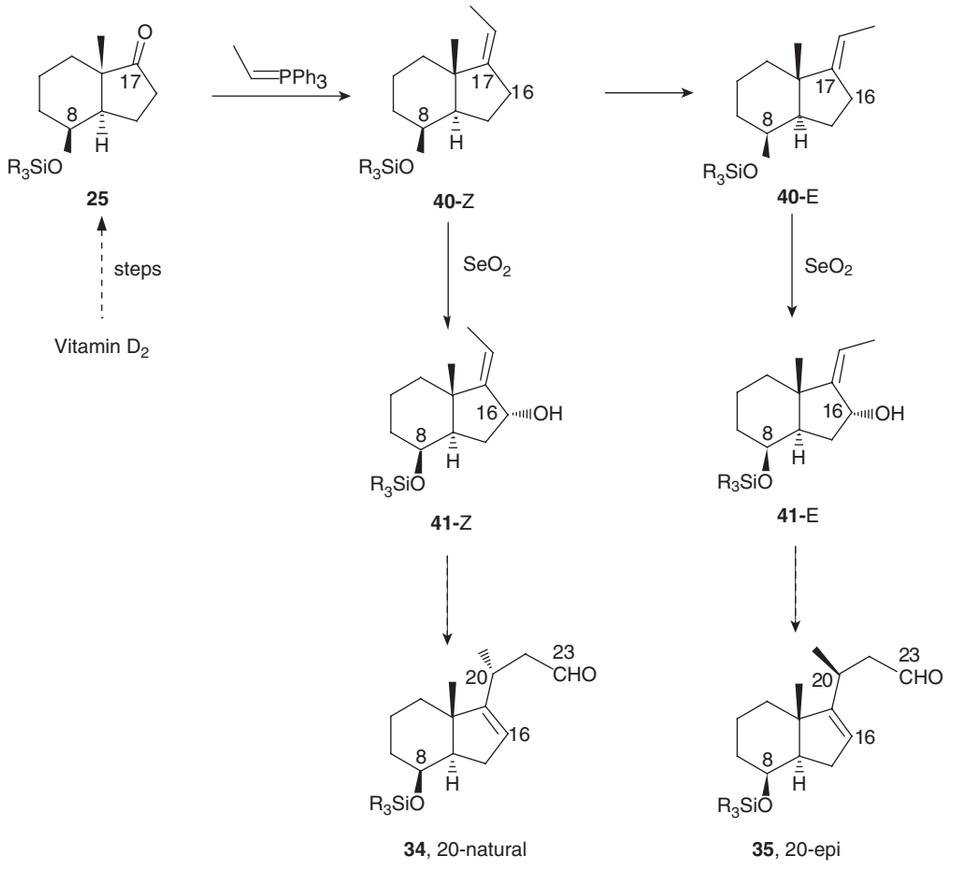
SCHEME 7



SCHEME 8

osteoporosis [61,62]. It has been prepared via convergent coupling of 16,23-diene 8-ketone **36** (prepared from 20-epi-23-aldehyde **35**) and A-ring 1-fluorinated unit **37** (Scheme 8). Also, Hopkins QW-1624F2-2 **5**, a low-calcemic cancer chemopreventive hybrid deltanoid [22], has been synthesized by coupling 24-difluorinated C,D-ring 8-ketone **38** (prepared from 20-natural 23-aldehyde **34**) with A-ring 1-homologated unit **39** (Scheme 8) [21].

Scheme 9 is provided to illustrate the multistep preparation from vitamin D_2 of diastereomeric C,D-ring building blocks **34** and **35** used in Scheme 8 to prepare hybrid deltanoids Ro 26-9228 and Hopkins QW-1624F2-2 **5**. Wittig olefination of C-17 ketone **25** produces 17-ethylidene derivative **40**-Z that can be isomerized into **40**-E [63]. Allylic oxygenation at C-16 produces allylic alcohol **41**-Z and separately allylic alcohol **41**-E. Then, concerted and highly



SCHEME 9

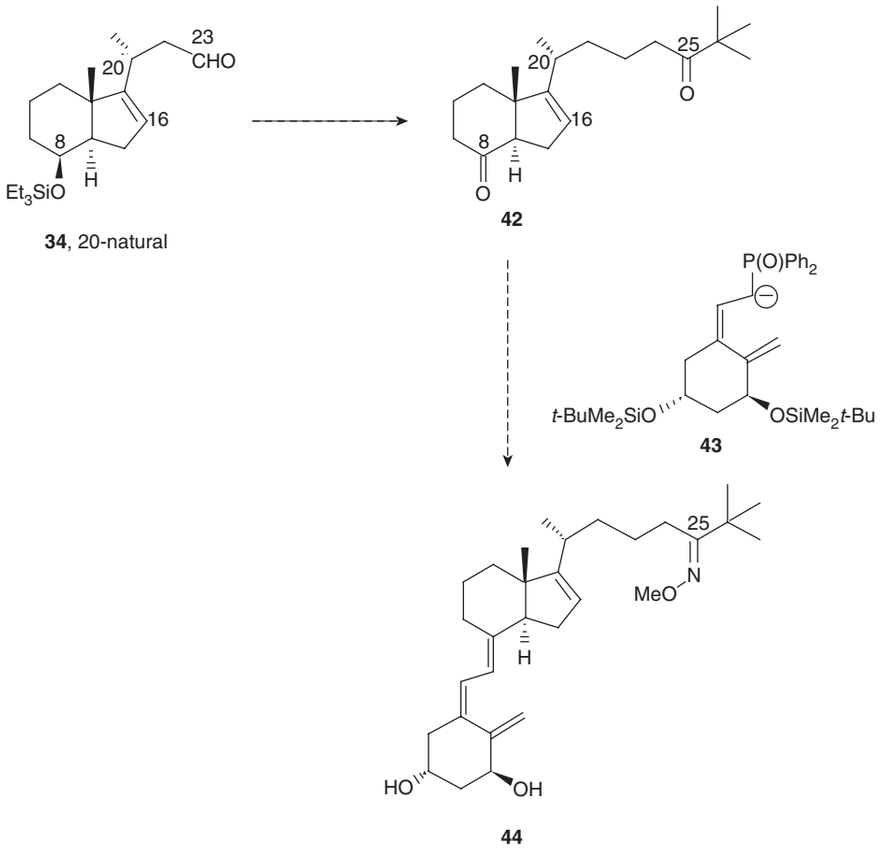
stereocontrolled [3,3]-sigmatropic rearrangement of the corresponding allyl vinyl ethers produces the required 16-ene C-23 aldehydes **34** and **35** (Scheme 9) [64,65].

Although not a hybrid deltanoid, conceptually new 16-ene-25-oxime deltanoid **44** is low-calcemic but antiproliferatively and transcriptionally potent. Oxime deltanoid **44** has been prepared also from key 16-ene-23-aldehyde building block **34** (Scheme 10) [66]. Noteworthy is the regiospecific joining of the A-ring nucleophile **43** with only the C-8 ketone group in C-8,C-25-diketone **42**, and noteworthy also is oximation of the C-25 ketone group without disturbing the sensitive conjugated triene unit.

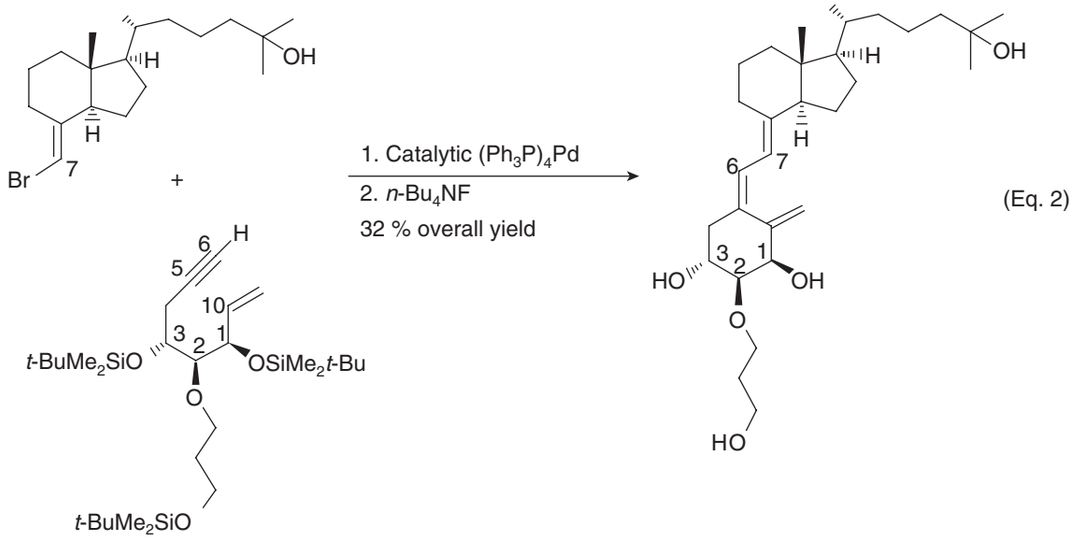
An important advance in synthetic methodology for efficient construction of A-ring cyclohexane units directly via C-10 to C-5 bond formation in one operation from noncyclic precursors has been reported (Eq. 2) [67]. This organometallic palladium-mediated approach to a C-6 vinylpalladium intermediate that couples *in situ* with C-7 vinylic bromides has been used successfully in some deltanoid syntheses (Eq. 2) [68].

V. Conclusions

Rational design of new generations of deltanoids will continue to rely on understanding the metabolism and the molecular biology of existing deltanoids and also on the power of modern organic chemistry for synthesis of complex molecules. Progress will be facilitated especially by good communication and cooperation between molecule-makers and molecule-testers [69]. Identifying the best new deltanoid drug candidates ideally will involve direct head-to-head comparisons of leading deltanoid new chemical entities, irrespective of their industrial or academic origin.



SCHEME 10



(Eq. 2)

References

1. Reichle H, Koeffler HP, Norman AW 1989 The role of the vitamin D endocrine system in health and disease. N Engl. J Med **320**:980–991.
2. Vieth R 1990 The mechanisms of vitamin D toxicity. Bone Miner **11**:267–272.
3. Bouillon R Okamura WH, Norman AW 1995 Structure-function relationships in the vitamin D endocrine system. Endocr Rev **16**:200–257.
4. Jones G, Strugnell SA, DeLuca HF 1998 Current understanding of the molecular actions of vitamin D. Physiol Rev **78**: 1193–1231.
5. Barger-Lux MJ, Heaney RP, Lanspa SJ, Healy JC, DeLuca HF 1995 An investigation of sources of variation in calcium absorption efficiency. J Clin Endocrinol Metab **80**:406–411.
6. Rasmussen H, Wong M, Bikle D, Goodman DBP 1972 Hormonal control of the renal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol. J Clin Invest **51**:2502–2504.
7. Chen TC, Schwartz GC, Burnstein KL, Lokeshwar BL, Holick MF 2000 The *in vitro* evaluation of 25-hydroxyvitamin D_3 and 19-nor-1α,25-dihydroxyvitamin D_2 as therapeutic agents for prostate cancer. Clin Cancer Res **6**:901–908.
8. Crocker JS, Muhtadie SF, Hamilton DC, Cole DE 1985 The comparative toxicity of vitamin D metabolites in the weanling mouse. Biol Appl Pharmacol **80**:119–126.
9. Gallagher JC, Bishop CW, Knutson JC, Mazess RB, DeLuca HF 1994 Effects of increasing doses of 1α-hydroxyvitamin D_2 on calcium homeostasis in postmenopausal osteopenic women. J Bone Miner Res **9**:607–614.
10. Slatopolsky E, Finch J, Ritter C, Denda M, Morrissey J, Brown A, DeLuca HF 1995 A new analog of calcitriol, 19-nor-1,25-$(OH)_2D_2$, suppresses parathyroid hormone secretion in uremic rats in the absence of hypercalcemia**.** Am J Kidney Dis **26**:852–860.
11. Mehta RG, Moriarty RM, Mehta RR, Penmasta R, Lazzaro G, Constantinou A, Guo L 1997 Prevention of preneoplastic mammary lesion development by a novel vitamin D analog, 1α-hydroxyvitamin D_5. J Natl Cancer Inst **89**:212–218.
12. Posner GH, Crawford KR, Yang HW, Kahraman M, Jeon HB, Li H, Lee JK, Suh BC, Hatcher MA, Labonte T, Usera A, Dolan PM, Kensler TW, Peleg S, Jones G, Zhang A, Korczak B, Saha U, Chuang SS 2004 Potent, Low-Calcemic, Selective Inhibitors of CYP24 Hydroxylase: 24-Sulfone Analogs of the Hormone 1α,25-Dihydroxyvitamin D_3. J Steroid Biochem Mol Biol, **89–90**:5–12.
13. Reddy GS, Clark JW, Tserng K-Y, Uskokovic MR, McLane JA 1993 Metabolism of 1,25(OH)216-ene D_3 in kidney: Influence of structural modification of D-ring on side chain metabolism. Bioorg Med Chem Lett **3**:1879–1884.
14. Posner GH, Guyton KZ, Kensler TW, Barsony J, Lieberman ME 1993 1-(Hydroxyalkyl)-25-hydroxyvitamin D_3 analogs of calcitriol 2. Preliminary biological evaluation. Bioorg Med Chem Lett **3**:1835–1840.
15. Peleg S, Uskokovic M, Ahene A, Vickery B, Avnur Z 2002 Cellular and molecular events associated with the bone-protecting activity of the noncalcemic vitamin D analog Ro-26-9228 in osteopenic rats. Endocrin **143**:1625–1636.
16. Hansen CM, Hamberg KJ, Binderup E, Binderup L 2000 Seocalcitol (EB 1089): A vitamin D analog of anticancer potential. Background, design, synthesis, preclinical, and clinical evaluation. Curr Pharm Design **6**:803–828.
17. Smith MB, March J. "Advanced Organic Chemistry." Wiley-Interscience:New York, NY, Fifth Edition, 2001, Chapter 1.
18. Banks RE, Smart BE, Tatlow JC (Eds.) "Organofluorine Chemistry: Principles and Commercial Applications" Plenum Press: New York, 1994.
19. Okamoto S, Tanaka Y, DeLuca HF, Kobayashi Y, Ikekawa N 1983 Biological activity of 24,24-difluoro-1,25-dihydroxyvitamin D_3. Am J Physiol **244**:E159–E163.
20. Gill HS, Londowski JM, Corradino RA, Zinsmeister AR, Kumar R 1990 Synthesis and biological activity of novel vitamin D analogs: 24,24-Difluoro-25-hydroxy-26,27-dimethylvitamin D_3 and 24,24-difluoro-1α,25-dihydroxy-26,27-dimethylvitamin D_3. J Med Chem **33**:480–490.
21. Posner GH, Lee JK, Wang Q, Peleg S, Burke M, Brem H, Dolan PM, Kensler TW 1998 Noncalcemic, antiproliferative, transcriptionally active, 24-fluorinated hybrid analogs of the hormone 1α,25-dihydroxyvitamin D_3. Synthesis and preliminary biological evaluation. J Med Chem **41**:3008–3014.
22. Kensler TW, Dolan PM, Gange SJ, Lee J-K, Wang Q, Posner GH 2000 Conceptually new deltanoids (vitamin D analogs) inhibit multistage skin tumorigenesis. Carcinogen **21**:1341–1345.
23. Siu-Caldera ML, Clark JW, Santos-Moore A, Peleg S, Liu YY, Uskokovic MR, Sharma S, Reddy GS 1996 1α,25-Dihydroxy-24-oxo-16-ene vitamin D_3, a metabolite of a synthetic vitamin D_3 analog, 1α,25-dihydroxy-16-ene vitamin D_3, is equipotent to its parent in modulating growth and differentiation of human leukemic cells. J Steroid Biochem Mol Biol **59**: 405–412.
24. Brown AJ, Ritter C, Slatopolsky E, Muralidharan KR, Okamura WH, Reddy GS 1999 1α,25-Dihydroxy-3-epi-vitamin D_3, a natural metabolite of 1α,25-dihydroxyvitamin D_3, is a potent suppressor of parathyroid hormone secretion. J Cell Biochem **73**:106–113.
25. Peleg S, Posner GH 2003 Vitamin D analogs as modulators of vitamin D receptor action. Curr Trends Med Chem **3**:1555–1572.
26. Rochel N, Wurtz JM, Mitschler A, Klaholz B, Moras D 2000 The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. Molecular Cell **5**:173–179.
27. Tocchini-Valentini G, Rochel N, Wurtz JM, Mitschler A, Moras D 2001 Crystal structures of the vitamin D receptor complexed to superagonist 20-epi ligands. Proc Natl Acad Sci USA, **98**:5491–5496.
28. Verboven C, Rabijns A, De Maeyer M, Van Baelen H, Bouillon R, De Ranter C 2002 A structural basis for the unique binding features of the human vitamin D–binding protein. Nat Struct Biol **9**:131–136.
29. Choi M, Yamamoto K, Masuno H, Nakashima K, Taga T, Yamada S 2001 Ligand recognition by the vitamin D receptor. Bioorg Med Chem **9**:1721–1730.
30. Zhou X, Zhu G-D, Van Haver D, Vandewalle M, De Clercq PJ, Verstuyf A, Bouillon R 1999 Synthesis, biological activity, and conformational analysis of four seco-D-15,19-bisnor-1α,25-dihydroxyvitamin D analogs, diastereomeric at C17 and C20. J Med Chem **42**:3539–3556.
31. Wu Y, Sabbe K, De Clercq P, Vandewalle M, Bouillon R, Verstuyf A 2002 Vitamin D_3: Synthesis of seco C-9,11,21-trisnor-17-methyl-1α,25-dihydroxyvitamin D_3 analogs. Bioorg Med Chem Lett **12**:1629–1632.
32. Verstuyf A, Verlinden L, Van Baelen H, Sabbe K, D'Hallewyn C, De Clercq P, Vandewalle M, Bouillon R 1998 The biological activity of nonsteroidal vitamin D hormone analogs lacking both the C- and D-rings. J Bone Miner Res **13**:549–558.

33. Bauer FW, Barbier P, Mohr P, Phister T, Pirson W, Theil F-P 2000 Preclinical profile of the cyclohexanediol Ro 65-2299, a potential oral antipsoriatic. In: Norman AW, Bouillon R, Thomasset M (eds) Vitamin D Endocrine System: Structural, Biological, Genetic, and Clinical Aspects. University of California, Riverside: Printing and Reprographics, pp. 615–618.
34. Swann SL, Bergh JJ, Farach-Carson MC, Koh JT 2002 Rational design of vitamin D_3 analogs, which selectively restore activity to a vitamin D receptor mutant associated with rickets. Org Lett **4**:3863–3866.
35. Swann SL, Bergh J, Farach-Carson MC, Ocasio CA, Koh JT 2002 Structure-based design of selective agonists for a rickets-associated mutant of the vitamin D receptor. J Am Chem Soc **124**:13795–13805.
36. Masuno H, Yamamoto K, Wang X, Choi M, Ooizumi H, Shinki T, Yamada S 2002 Rational design, synthesis, and biological activity of novel conformationally-restricted vitamin D analogs, (22*R*)- and (22*S*)-22-ethyl-1,25-dihydroxy-23,24-didehydro-24a,24b-dihomo-20-epivitamin D_3. J Med Chem **45**:1825–1834.
37. Yamada S, Yamamoto K, Masuno H, Ohta M 1998 Conformation function relationship of vitamin D: Conformational analysis predicts potential side-chain structure. J Med Chem **41**:1467–1475.
38. Yamada S, Yamamoto K, Masuno H 2000 Structure function analysis of vitamin D and VDR model. Curr Pharm Des **6**:733–748.
39. Gabriëls S, Van Haver D, Vandewalle M, De Clercq P, Verstuyf A, Bouillon R 2001 Development of analogs of 1α,25-dihydroxyvitamin D_3 with biased side-chain orientation: Methylated des-C, D-homo analogs. Chem Eur J **7**:520–532.
40. Okamura WH, Palenzuela JA, Plumet J, Midland MM 1992 Vitamin D: Structure function analyses and the design of analogs. J Cell Biochem **49**:1–9.
41. Figadere B, Norman AW, Henry HL, Koeffler HP, Zhou J-Y, Okamura WH 1991 Studies of vitamin D (calciferol) and its analogs. 39. Arocalciferols: synthesis and biological evaluation of aromatic side-chain analogs of 1α,25-dihydroxyvitamin D_3. J Med Chem **34**:2452–2463.
42. Posner GH, Wang Q, Han G, Lee JK, Crawford K, Zand S, Brem H, Peleg S, Dolan P, Kensler TW 1999 Conceptually new sulfone analogs of the hormone 1α,25-dihydroxyvitamin D_3: Synthesis and preliminary biological evaluation. J Med Chem **42**:3425–3435.
43. Unpublished results of Drs. H. Masuno and S. Yamada, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo, Japan. We thank Drs. Masuno and Yamada for these docking data.
44. Ostrem VK, Lau WF, Lee SH, Perlman K, Prahl J, Schnoes HK, DeLuca, HF 1987 Induction of monocytic differentiation of HL-60 cells by 1,25-dihydroxyvitamin D analogs. J Biol Chem **262**:14164–14171.
45. Shimizu M, Iwasaki Y, Ohno A, Yamada S 1997 Synthesis of fluorovitamin D analogs for conformational analysis of ligand bound to vitamin D receptor. In: Norman AW, Bouillon R, Thomasset, M (eds.) Vitamin D: Chemistry, Biology, and Clinical Applications of the Steroid Hormone: Proceedings from the Tenth Workshop on Vitamin D, Strasbourg, France—May 24–29. University of California, Riverside: Printing and Reprographics, pp. 24–25.
46. Shimizu M, Iwasaki Y, Ohno A, Yamada S 1999 4,4-Difluoro-1α,25-dihydroxyvitamin D_3: Analog to probe A-ring conformation in vitamin D–receptor complex. Tett Lett **40**:1697–1700.
47. Shimizu M, Ohno A, Iwasaki Y, Yamada S, Ooizumi H, DeLuca HF 2000 A-ring conformation and biological activity: Based on studies of fluorovitamin D analogs. In: Norman AW, Bouillon R, Thomasset M (eds.) Vitamin D Endocrine System: Structural, Biological, Genetic and Clinical Aspects: Proceedings from the Eleventh Workshop on Vitamin D, Nashville, TN, USA—May 27–June 1. University of California, Riverside: Printing and Reprographics, pp. 45–48.
48. Okamura WH, Do S, Kim H, Jeganathan S, Vu T, Zhu G-D, Norman AW 2001 Conformationally-restricted mimics of vitamin D rotamers. Steroids **66**:239–247.
49. Hayashi R, Fernández S, Okamura WH 2002 An 8π-electron electrocyclization leading to a 9,19-methano-bridged analog of 1α,25-dihydroxyvitamin D_3. Org Lett **4**:851–854.
50. Norman AW, Bishop JE, Bula CM, Olivera CJ, Mizwicki MT, Zanello LP, Ishida H, Okamura WH 2002 Molecular tools for study of genomic and rapid signal transduction responses initiated by 1α,25$(OH)_2$ vitamin D_3. Steroids **67**:457–466.
51. Andrews DR, Barton DHR, Hesse RH, Pechet MM 1986 Synthesis of 25-hydroxy- and 1α,25-dihydroxyvitamin D_3 from vitamin D_2 (calciferol). J Org Chem **51**:4819–4828.
52. Kubodera N, Watanabe H, Kawanishi T, Matsumoto M 1992 Synthetic studies of vitamin D analogs. XI. Synthesis and differentiation-inducing activity of 1α,25-dihydroxy-22-oxavitamin D_3 analogs. Chem Pharm Bull **40**:1494–1499.
53. Posner GH, Crawford K, Siu-Caldera M-L, Reddy GS, Sarabia SF, Feldman D, Etten EV, Mathieu C, Gennaro Vouros P, Peleg S, Dolan PM, Kensler TW 2000 Conceptually new 20-epi-22-oxa sulfone analogs of the hormone 1α,25-dihydroxyvitamin D_3: Synthesis and biological evaluation. J Med Chem **43**:3581–3586.
54. Dai H, Posner GH 1994 Synthetic approaches to vitamin D. Synthesis 1383–1398.
55. Sicinski RR, Prahl JM, Smith CM, DeLuca HF 1998 New 1α,25-dihydroxy-19-norvitamin D_3 compounds of high biological activity: Synthesis and biological evaluation of 2-hydroxymethyl, 2-methyl and 2-methylene analogs. J Med Chem **41**:4662–4674.
56. Shevde NK, Plum LA, Clagett-Dame M, Yamamoto H, Pike JW, DeLuca HF 2002 A potent analog of 1α,25-dihydroxyvitamin D_3 selectively induces bone formation. Biochem **99**:13487–13491.
57. Zhu G, Okamura WH 1995 Synthesis of vitamin D (calciferol). Chem Rev **95**:1877–1952.
58. Hijikuro I, Doi T, Takahashi T 2001 Parallel synthesis of a vitamin D_3 library in the solid-phase. J Am Chem Soc **123**: 3716–3722.
59. Wu Y, Zhao Y, Tian H, Clercq PD, Vandewalle M, Berthier M, Pellegrino G, Maillos P, Pascal J-C 2001 A practical synthesis of 14-*epi*-19-*nor*-1α,25-dihydroxyvitamin D_3 analogs and their A-ring epimers. Eur J Org Chem 3779–3788.
60. Verlinden L, Verstuyf A, Camp MV, Marcelis S, Sabbe K, Zhao X-Y, Clercq PD, Vandewalle M, Bouillon R 2000 Two novel 14-*epi*-analogs of 1α,25-dihydroxyvitamin D_3 inhibit the growth of human breast cancer cells *in vitro* and *in vivo*. Cancer Res **60**:2673–2679.
61. Kabat MM, Radinov R 2001 The practical synthesis of vitamin D analogs: A challenge for process research. Curr Opin Drug Discovery Dev **4**:808–833.
62. Kabat MM, Garofalo LM, Daniewski AR, Hutchings SD, Liu W, Okabe M, Radinov R, Zhou Y 2001 Efficient synthesis of 1α-fluoro A-ring phosphine oxide, a useful building block for vitamin D analogs, from (*S*)-carvone via a highly selective palladium-catalyzed isomerization of dieneoxide to dieneol. J Org Chem **66**:6141–6150.
63. Blæhr LKA, Bjorkling F, Calverley MJ, Binderup E, Begtrup M 2003 Synthesis of Novel Hapten Derivatives of 1α,25-dihydroxy vitamin D_3 and its 20-epi analogs. J Org Chem **68**:1367–1375.

64. Uskokovic MR, Studzinski GP, Gardner JP, Reddy GS, Campbell MJ, Koeffler HP 1997 The 16-ene vitamin D analogs. Curr Pharm Des **3**:99–123.
65. Hatcher MA, Posner GH 2002 [3,3]-Sigmatropic rearrangements: Short, stereocontrolled synthesis of functionalized vitamin D_3 side-chain units. Tett Lett **43**: 5009–5012.
66. Posner GH, Halford BA, Peleg S, Dolan PM, Kensler TW 2002 Conceptually new low-calcemic oxime analogs of the hormone 1α,25-dihydroxyvitamin D_3: Synthesis and biological testing. J Med Chem **45**:1723–1730.
67. Trost BM, Dumas J, Villa M 1992 New strategies for the synthesis of vitamin D metabolites via Pd-catalyzed reactions. J Am Chem Soc **114**:9836–9845.
68. Kittaka A, Suhara Y, Takayanagi H, Fujishima T, Kurihara M, Takayama H 2000 A concise and efficient route to 2α-(ω-hydroxyalkoxy)-1α,25-dihydroxyvitamin D_3: Remarkably high affinity to vitamin D receptor. Org Lett **2**:2619–2622.
69. Posner GH, Jeon HB, Sarjeant A, Riccio ES, Doppalapudi RS, Kapetanovic IM, Dolan P, Kensler TW 2004 Low-calcemic, efficacious, 1α,25-dihydroxyvitamin D_3 analog QW-1624F_2-2: Calcemic dose-response determination, preclinical genotoxicity testing, and revision of A-ring stereochemistry, Steroids, submitted.

CHAPTER 81

Analog Metabolism

GLENVILLE JONES Departments of Biochemistry and Medicine, Queen's University, Kingston, Ontario, Canada

I. GENERAL CONSIDERATIONS

A. Vitamin D Metabolism

No mention of the metabolism of vitamin D analogs can ignore the rich and varied history of the metabolism of vitamin D itself over the last few decades [1]. (See Chapter 1 of this treatise.) Metabolic investigations over the last few decades have revealed not only the nature of the hydroxylation/oxidation steps important in the activation of vitamin D but also much about the cytochrome P450-based enzymes involved. In fact, it was the elucidation of the metabolism of vitamin D that sparked the synthesis of the first vitamin D analogs back in the early 1970s. See Chapter 2 for details of natural metabolites and Chapters 4, 5, 6 for details of the 25-, 1α-, and 24-hydroxylase enzymes, since this knowledge is important background for a fuller appreciation of the metabolism of vitamin D analogs.

Any review of the metabolism of vitamin D analogs should make an important distinction between:1) that metabolism providing activation of the analog which thereby leads to a more biologically active molecule and 2) that metabolism, otherwise known as catabolism, which deactivates the molecule leading to its destruction and excretion. In fact, this distinction allows for a basis for a classification of vitamin D analogs into two distinct groups:

a) Prodrugs (analogs) requiring one or more step(s) of activation (e.g. one step: 1α-OH-D_2, 1α-OH-D_3, 1α-OH-D_4, 1α-OH-D_5, or 25-OH-D_3; multiple steps: vitamin D_2 and dihydrotachysterol)
b) Analogs of 1α,25-dihydroxyvitamin D_3 (calcitriol) requiring NO activation (e.g. calcipotriol, OCT, EB1089, KH1060, 19-nor-1α,25-$(OH)_2$ D_3)

It is clear that many of the early generations of vitamin D analogs were prodrugs, which were designed to take advantage of the enzymes already in place for the metabolic activation of vitamin D itself. Most of these analogs showed a close resemblance to vitamin D since they required sufficient similarity to the natural substance to ensure activation. More recently, chemists in the pharmaceutical industry and university domain have designed more exotic vitamin D analogs, which deviate significantly from the basic vitamin D/calcitriol structure. In the majority of these cases, it is the vitamin D side chain which has been changed in these analogs, but there now exists a significant number of examples stemming from excursions into A-ring or C/D-ring modification. Three reviews [2,3,148] and Chapters 80 and 84–88 offer comprehensive lists of the analogs of vitamin D synthesized to date. In Tables I and II, we have selected, respectively, some of the more interesting prodrugs and calcitriol analogs that are currently marketed or for which metabolic data are available.

Prodrug activation (e.g., for vitamin D_2 or 1α-OH-D_3) may involve the same cytochrome P450-based enzymes (namely the 25- and 1α-hydroxylases) as for vitamin D_3, and consequently this allows us to explore the substrate specificity of these vitamin D–related enzymes. Current dogma suggests that 25-hydroxylation of endogenous vitamin D_3 is carried out by a bifunctional enzyme also capable of the 25- and 27-hydroxylation steps within pathways of bile acid metabolism [4]. Indeed, this liver mitochondrial cytochrome P450, known as *CYP27A* (see Chapter 4), when transfected into COS-1 cells has also been shown to be capable of the 25-hydroxylation (and other hydroxylations at C-24 and C-27) of a number of vitamin D analogs (including vitamin D_3), when provided at high substrate concentrations [5]. However, there remains some doubt that CYP27A exclusively carries out the 25-hydroxylation of vitamin D_3 at the substrate concentrations observed in the human *in vivo* [6]. There is some experimental data [collected in ref. 6] to support the idea that another enzyme CYP2R1, in the liver microsomal fraction, is also responsible for

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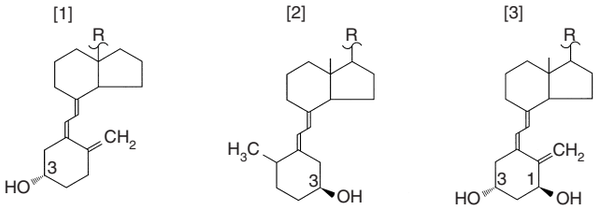
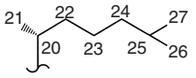
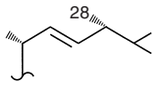
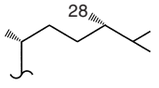
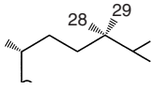
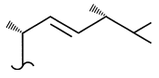
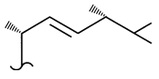
Table I Vitamin D Prodrugs



Vitamin D prodrugs [ring structure]	Side chain structure R	Company	Possible target diseases	Mode of delivery	Ref.
1αOHD$_3$ [3]		Leo	Osteoporosis	Systemic	[134]
1αOHD$_2$ [3]		Bone Care Int.	Hyperparathyroidism	Systemic	[135]
1αOHD$_4$ [3]		Bone Care Int.	Psoriasis Cancer	Topical Systemic	[56]
1αOHD$_5$ [3]		NCI	Cancer	Systemic	[57]
Dihydrotachysterol [2]		Solvay-Duphar	Renal failure	Systemic	[29]
Vitamin D$_2$ [1]		Various	Rickets Osteomalacia	Systemic	[136]

25-hydroxylation of low concentrations of endogenous vitamin D_3 [6a]. Similarly, 1α-hydroxylation of vitamin D analogs lacking a 1α-hydroxyl function may not be the exclusive domain of the renal 1α-hydroxylase enzyme (containing CYP27B) described in Chapter 5. It is possible that the extra-renal forms of the 1α-hydroxylase [7] (see also Chapter 79) or other CYPs may activate vitamin D analogs (e.g. dihydrotachysterol), though this reviewer knows of no example where this is the underlying strategy of a clinical treatment protocol.

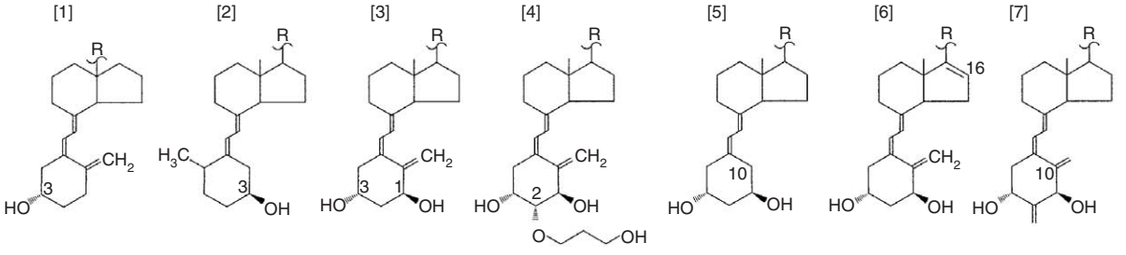
B. Calcitriol Catabolism

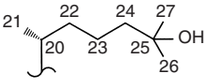
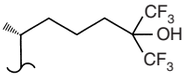
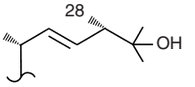
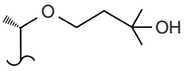
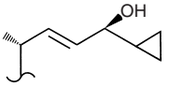
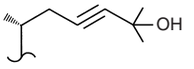
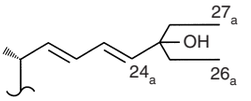
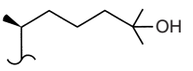
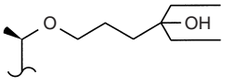
The other side of metabolism is catabolism and this is where the majority of the emphasis of this chapter will be placed. Calcitriol is subject to catabolism by two different pathways.

1. C-24 Oxidation Pathway to Calcitroic Acid

The C-24 oxidation pathway seems to predominate in most target cells because of the inducible nature of

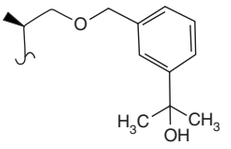
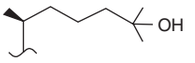
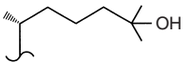
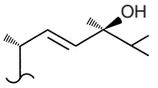
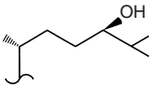
TABLE II Analogs of $1,25(OH)_2D_3$



Vitamin D analog [ring structure]	Side chain structure (R)	Company	Possible target diseases	Mode of delivery	Ref.
$1\alpha,25(OH)_2D_3$ [3]		Roche, Solvay-Duphar	Hypocalcemia Psoriasis	Systemic Topical	[137]
$26,27\text{-}F_6\text{-}1\alpha,25(OH)_2D_3$ [3]		Sumitomo-Taisho	Osteoporosis Hypoparathyroidism	Systemic Systemic	[107]
19-Nor-$1\alpha,25(OH)_2D_2$ [5]		Abbott	Hyperparathyroidism	Systemic	[116]
22-Oxacalcitriol (OCT) [3]		Chugai	Hyperparathyroidism Psoriasis	Systemic Topical	[88]
Calcipotriol (MC903) [3]		Leo	Psoriasis	Topical	[59]
$1\alpha,25(OH)_2$-16-ene-23-yne-D_3 (Ro 23-7553) [6]		Roche	Leukemia	Systemic	[138]
EB1089 [3]		Leo	Cancer	Systemic	[78]
20-Epi-$1\alpha,25(OH)_2D_3$ [3]		Leo	Immune diseases	Systemic	[62]
KH1060 [3]		Leo	Immune diseases	Systemic	[63]

Continued

TABLE II Analogs of $1,25(OH)_2D_3$—Cont'd

Vitamin D analog [ring structure]	Side chain structure (R)	Company	Possible target diseases	Mode of delivery	Refs.
KH1650 [3]		Leo	Psoriasis	Topical	[97]
2-μethylene-19-Nor-20-Epi-1α,25$(OH)_2D_3$ [7]		Deltanoids	Osteoporosis	Systemic	[64]
ED-71 [4]		Chugai	Osteoporosis	Systemic	[105]
1α,24(*S*)$(OH)_2D_2$ [3]		Bone Care Int.	Psoriasis	Systemic	[47]
1α,24*R*$(OH)_2D_3$ (TV-02) [3]		Teijin	Psoriasis	Topical	[139]

the mitochondrial cytochrome P450 involved, known as CYP24, which is the substrate-binding component of the 24-hydroxylase complex [8]. The C-24 oxidation pathway comprises 5 enzymatic steps (Fig. 1) involving successive hydroxylation/oxidation reactions at C-24 and C-23 followed by cleavage of the molecule between C-23 and C-24 and oxidation of the resultant truncated product to calcitroic acid [9,10]. Targeted disruption of the murine CYP24 gene gives 50% lethality at weaning due to hypercalcemia and

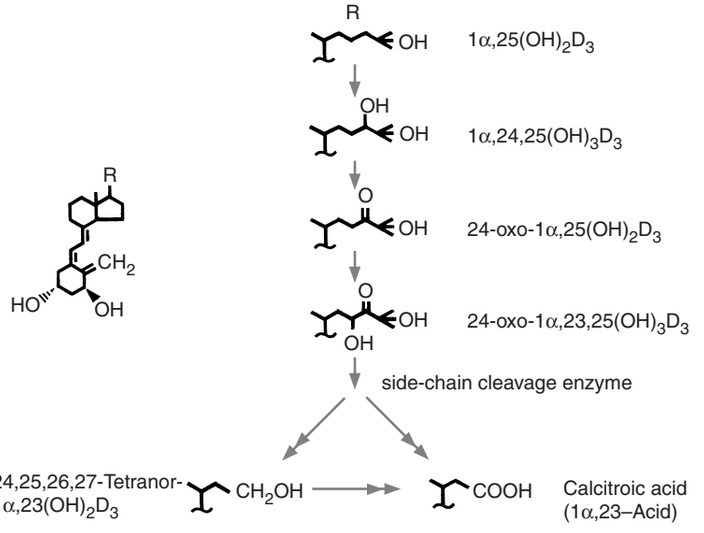


FIGURE 1 C24-Oxidation pathway. (Reproduced from ref. 9 with permission.)

nephrocalcinosis; survivors showing much reduced capacity to clear a bolus of 1α,25-$(OH)_2D_3$ from the bloodstream [11,12]. These results suggest an important role for CYP24 in catabolism. Reconstitution assays using recombinant CYP24 produced in *E. coli* and bacculovirus systems have shown that the first three enzyme activities of the pathway, and possibly more, reside in a single cytochrome P450 chain [13–15]. The fact that this C-24 oxidation pathway has been connected to a known excretory product of calcitriol, in the form of calcitroic acid, adds credence to the view that the pathway is catabolic in nature. Consistent with this viewpoint is the finding that the mRNA for CYP24 and C-24-hydroxylation activity have been found in classical vitamin D target tissues including intestine, kidney, bone, as well as a variety of primary cells and cultured cell lines such as CaCo-2 (colon), UMR-106 (bone), LLC-PK1 (kidney), HPK1A-ras (keratinocyte) [16–19]. Depending upon the structure of their side chain, vitamin D analogs can also be metabolized by these same C-24 oxidation pathway enzymes, usually to molecules with reduced biological activity. Therefore, studies concerned with metabolism of vitamin D analogs have the dual role of defining metabolic products of the analog and allowing for exploration of the substrate preferences of CYP24 or other enzyme(s) involved.

2. C-26 HYDROXYLATION/26,23-LACTONE FORMATION

The role of 26-hydroxylation of vitamin D compounds is unknown. Equally obscure is the importance or the function of the lactone pathway to either 25-OH-D_3-26,23-lactone or 1α,25-$(OH)_2D_3$-26,23-lactone derived from 25-OH-D_3 or 1α,25-$(OH)_2D_3$, respectively. The evidence for the formation of these metabolites is irrefutable; the pathway to the lactone well defined [20] (see also Fig. 2); and recent evidence suggests that CYP24 is again responsible since the recombinant enzyme catalyzes the five-step process to the lactone, which starts with the 23-hydroxylated metabolite: 23,25-$(OH)_2D_3$ or 1α,23,25-$(OH)_3D_3$ [12,21] On the other hand, it is not clear why the alternative metabolites 25,26-$(OH)_2D_3$ and 1α,25,26-$(OH)_3D_3$ are formed at all since 26-hydroxylated derivatives do not seem to be the initial precursors to either observed lactone [22]. Pathway idiosyncrasies aside, it seems clear that both 26-hydroxylation and 26,23-lactone formation result in molecules with reduced biological activity, suggesting but not conclusively proving that they represent catabolites.

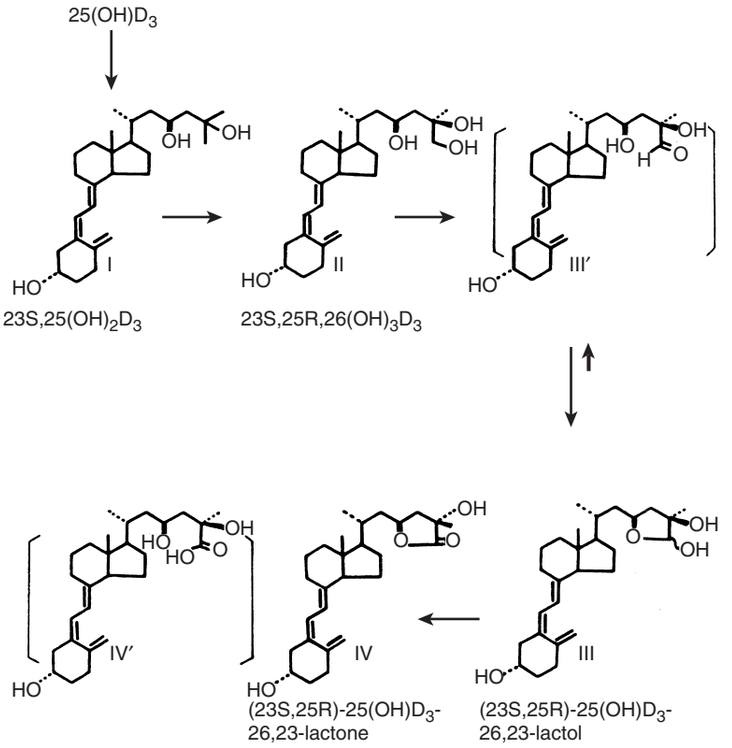


FIGURE 2 26,23-Lactone pathway. (Reproduced from ref. 22 with permission).

C. Nonvitamin D–Related Metabolism and General Methodology

As the vitamin D molecule is increasingly modified by the organic chemist, it becomes more and more susceptible to metabolism by enzymes that are distinct from those specifically involved in vitamin D metabolism. It is the analogs of calcitriol that are the most likely to be subject to metabolism by nonvitamin D–related enzyme systems. This is because analogs of calcitriol incorporate the greatest number of structural changes and are modified mainly in that part of the molecule that is subject to metabolic alteration *in vivo*, specifically the side chain. In the specific examples of vitamin D analogs that follow, note that there is strong evidence for involvement of other (thus far undefined) cytochrome P450-based enzyme systems. Given the uncertainty about the identity of the enzymes involved in the metabolism of vitamin D analogs or even their tissue source, it is not surprising that most investigations involve a cross-section of metabolic systems in order to identify the metabolic products and to focus on the enzymes responsible [23]. Metabolic studies utilize a variety of species and *in vivo* and *in vitro* models from broken cell to intact cell systems (Table III). The predominant models used for metabolic studies are liver-based systems and have revealed that this organ is a primary site of vitamin D analog metabolism. As with calcitriol, many vitamin D analogs can also be metabolized by vitamin D–target cells. The cloning of most if not all of the vitamin D–related cytochromes P450 has enabled us to generate cell-free and cell expression systems that allow us to study the CYPs in isolation. This has been achieved for CYP24, CYP27A, CYP2R1, and CYP27B [13–15]. These novel metabolic systems are valuable in that they allow the study of developing specific inhibitors for any of the known CYPs, or alternatively, of studying the substrate preferences of the target cell enzymes in order to design better catabolism-sensitive or catabolism-resistant vitamin D analogs for use as drugs.

Methodology for studying vitamin D analog metabolism not only involves a wide variety of biological systems (Table III), but also usually depends upon the

TABLE III Biological Systems Used in the Study of the Metabolism of Vitamin D and Its Analogs

Biological system	Enzyme activity	Ref.
Isolated perfused organ		
Rat liver	25-Hydroxylation	[140]
Rat kidney	1α- and 24-hydroxylation	[141,142]
Primary cells		
Chick kidney	1α- and 24-hydroxylation	[143]
Bovine parathyroid	24-Oxidation pathway	[25]
Mouse neonatal keratinocyte from wildtype αCYP24 null mice	24-Oxidation pathway (with and without CYP24)	[15]
Cultured cell lines[a]		
CaCo-2, human colon (“intestinal”)	24-Oxidation pathway	[17]
UMR-106, rat osteosarcoma	24-Oxidation pathway	[18]
LLC-PK1, pig kidney	24-Oxidation pathway	[144]
HPK1A-ras, human keratinocyte	24-Oxidation pathway	[19]
SW900, human lung	Extra-renal 1-hydroxylation	[149]
HD-11, chick myelomonocyte	Extra-renal 1-hydroxylation	[35,36]
Broken cell systems		
Rat liver mitochondria	25-Hydroxylation	[33]
Rat kidney mitochondria	1α- and 24-hydroxylation	[145]
Rat, human, and minipig postmitochondrial supernatant	General metabolism	[28]
Reconstituted system with cytochrome P450, ferridoxin, reductase	1α- and 25-hydroxylation	[129]
Transfected cell systems		[5]
CYP27A transfected into COS-1 cells	25-Hydroxylation	
CYP24 transfected into *E. coli*	24-Oxidation pathway	[13]
CYP24 transfected into insect cells	24-Oxidation pathway	[14]
CYP24 transfected into V79 Chinese hamster lung cell line[b]	24-Oxidation pathway	[15]

[a]Only a few examples are given.
[b]79 host cell line lacks endogenous CYP24 or CYP27A or CYP27B.

availability of a suitably-radioactive vitamin D analog. This radioactive analog must possess a sufficiently-high specific activity to allow detection of nanomolar concentrations of analog in addition to a label location that is in a metabolically-resistant region of the molecule. This usually means that [^{3}H]-labeling is the preferred isotope, and a nuclear-location of the radioactive tag is best if the label is not to be lost. Such is the case with [1β-^{3}H] 1α,25-$(OH)_2D_3$ where the label is retained by the molecule even on truncation of the side chain to [1β-^{3}H] calcitroic acid, which can be conveniently separated from other metabolites by a simple Bligh and Dyer extraction [24]. Occasionally, the susceptibility of the [^{3}H]-label to metabolic attack is judiciously used as an indicator of the type of metabolism occurring. An excellent example of this is the ingenuity of Chugai chemists working with Slatopolsky's group to compare the metabolism of both [26-^{3}H]OCT and [2β-^{3}H]OCT in their biological systems. Using such a combination of labels, these researchers were able to confirm the loss of a portion of the side chain of OCT during metabolism, thereby suggesting the formation of side-chain truncated metabolites of OCT [25,26]. Although the availability of the costly radioactive analog has not been a significant problem when studying most of the well-established compounds developed to date, it remains a significant barrier to the widespread screening of the hundreds of vitamin D analogs currently available.

If radioactive analogs are not readily available, another technique involving detection of metabolites by diode-array spectrophotometry can be used, but at the disadvantage of being forced to employ high substrate concentrations [27]. Although this technique is restricted to high concentrations of analogs, the analysis is quicker and cheaper than using radioactive analogs, results in the generation of larger amounts of metabolites, and this, in turn, permits a more rigorous identification of the metabolic products. Our laboratory has used such a procedure very effectively over the past 10 years to identify the products of both natural and synthetic vitamin D compounds incubated with a range of *in vitro* biological systems. The quantitative and qualitative answers that diode-array spectrophotometry provides are, in large part, consistent with those answers resulting from studies employing radioactive vitamin D analogs (*cf.* Sorensen *et al.* [28] and Masuda *et al.* [19]).

Most recently, metabolic studies of vitamin D analogs have been revolutionized by the emergence of a new analytical technique for the detection of all metabolites based upon liquid chromatography/mass spectrometry (LC/MS) [15]. Though this technique lacks the exquisite sensitivity offered by radioisotopically-labeled molecules, it is still one or two orders of magnitude more sensitive than conventional LC detectors (e.g. diode-array detector). Thus, LC/MS-based method can detect as little as 10 pg of a vitamin D analog, whereas most LC detectors have a detection limit of 1 nanogram. Furthermore, LC/MS-based methods are quick and convenient, providing a selectivity unmatched by conventional LC detectors by incorporating spectral focusing routines (e.g. MRM—focus on transitions between major MS1 ions and their daughter ions), which dramatically reduce the background noise. As a consequence, LC/MS-based technologies are rapidly taking over as the method of choice in metabolic studies in all small molecule research, including vitamin D. Thus, while there are few current examples of the use of LC/MS with vitamin D, it can be safely assumed that in the future, LC/MS-based methods will become metabolic screening tools for all nonradioactive vitamin D analogs. The specific metabolic studies described below will provide examples of the use of all three of these approaches to the study of the metabolism of vitamin D analogs.

II. EXAMPLES OF THE METABOLISM OF ANALOGS OF VITAMIN D

A. Dihydrotachysterol

This example of a vitamin D prodrug represents the oldest vitamin D analog and was developed in the 1930s as a method of stabilizing the triene structure of one of the photoisomers of vitamin D. The structure of dihydrotachysterol$_2$ shown in Table I contains an A-ring rotated through 180°, a reduced C10-19 double bond, and the side chain structure of ergosterol/vitamin D_2. This side chain is depicted because the clinically approved drug form of dihydrotachysterol is dihydrotachysterol$_2$. However, it should be noted that dihydrotachysterol$_3$ (DHT_3) can also be synthesized with the side chain of vitamin D_3. The metabolism of both dihydrotachysterol$_2$ (DHT_2) and dihydrotachysterol$_3$ (DHT_3) have been extensively studied over the past three decades [29–32]. Initial studies performed in the early 1970s showed that both DHT_2 and DHT_3 are efficiently converted to their 25-hydroxylated metabolites [33].

The effectiveness of DHT to relieve the hypocalcemia of chronic renal failure in the absence of a functional renal 1α-hydroxylase led to the hypothesis [34] that 25-OH-DHT might represent the biologically-active form of DHT, by virtue of its 3β-hydroxy group being rotated 180° into a "pseudo 1α-hydroxyl position."

It was thus believed that 1α-hydroxylation of 25-OH-DHT was unnecessary. This viewpoint prevailed for at least a decade, but debate was renewed when Bosch *et al.* [30] were able to provide evidence for the existence of a mixture of 1α- and 1β-hydroxylated products of 25-OH-DHT_2 in the blood of rats dosed

with DHT_2. Studies involving the perfusion of kidneys from vitamin D–deficient rats with an incubation medium containing 25-OH-DHT_3 and using diode-array spectrophotometry to analyze the extracts showed this molecule to be subject to extensive metabolism by renal enzymes, but failed to give the expected 1-hydroxylated metabolites, opening up the possibility that the 1α- and 1β-hydroxylated metabolites observed by Bosch *et al.* might be formed by an extra-renal 1-hydroxylase [31] (see also Fig. 3). Following the synthesis of appropriate authentic standards, subsequent research [35] has rigorously confirmed the *in vivo* formation and identity of 1α,25-$(OH)_2$DHT and 1β,25-$(OH)_2$DHT in both rat and human. The ability of these 1α-hydroxylated forms of both DHT_2 and DHT_3 to stimulate a VDRE-inducible growth hormone reporter system exceeded that of 25-OH-DHT, and in the process established 1α,25-$(OH)_2$DHT and 1β,25-$(OH)_2$DHT as the most potent derivatives of DHT identified to date. The formation of these metabolites also brings into question the importance of the "pseudo 1α-hydroxyl group" hypothesis, although current findings do not rule out that the biological activity of DHT might be due to the collective action of a group of metabolites including 25-OH-DHT, 1α,25-$(OH)_2$DHT and 1β,25-$(OH)_2$DHT. The latest information on the site of biosynthesis of 1-hydroxylated DHTs comes from studies using the cultured chicken myelomonocytic cell line, HD-11 [35]. This cell line, which has been documented previously as a rich source of the extra-renal 1α-hydroxylase [36], has also been shown to be capable of the 1-hydroxylation of 25-OH-DHT [35]. These results are consistent with DHT being 25-hydroxylated in the liver and then subject to 1-hydroxylation by an extra-renal source of CYP27B of bone marrow origin *in vivo*.

Though the enzymes involved in the activation of DHT, especially the 1-hydroxylation step, have an altered specificity toward this molecule, the enzymes involved in the catabolism of DHT_3 appear to treat the molecule as they would 25-OH-D_3 or 1α,25-$(OH)_2D_3$. Side chain hydroxylated derivatives of both 25-OH-DHT_3 and 1,25-$(OH)_2DHT_3$ have been identified and appear to be analogous to intermediates of the C-24 oxidation and 26,23-lactone pathways of vitamin D_3 metabolism [37,38]. The major difference between the catabolism of DHT_3 and vitamin D_3 is that the 26,23-lactone formation from DHT_3 appears exaggerated, suggesting that DHT_3 is either a better substrate for the enzymes involved in 26,23-lactone formation, or else it is discriminated against by CYP24 or other enzymes of the alternative C-24 oxidation pathway.

B. Vitamin D_2 Derivatives

Though vitamin D_2 can be synthesized naturally by irradiation of ergosterol, little finds its way into the human diet unless it is provided as a dietary supplement. Thus, one could make a case for considering vitamin D_2 as a prodrug. The complex metabolism of vitamin D_2 has been included as part of Chapter 2 and will not be repeated here. However, it is worth noting that vitamin D_2 gives rise to several analogous metabolites to those of vitamin D_3 in the form of 25-OH-D_2 [39], 1α,25-$(OH)_2D_2$ [40], and 24,25-$(OH)_2D_2$ [41], as well as several unique metabolites including 24-OH-D_2 [42],

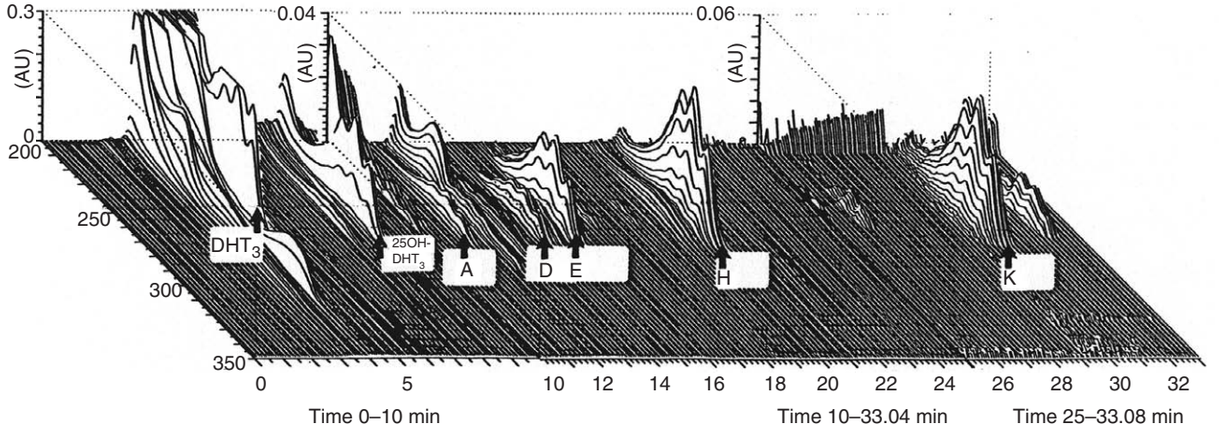


FIGURE 3 *In vivo* metabolism of dihydrotachysterol$_3$ in the rat. Diode-array HPLC of the plasma extract of a rat administered 1 mg DHT_3 18 hr prior to sacrifice. Metabolites are labeled 25-OH-DHT_3 and peaks A–L. All possess the distinctive tricuspid UV spectrum (λ_{max} 242.5 nm, 251nm, and 260.5 nm). Metabolites A–L were subsequently identified as side chain modified compounds analogous to vitamin D metabolites of the C-24 oxidation and 26,23-lactone pathways depicted in Figs. 1 and 2. (Reproduced from ref. 31 with permission.)

1α,24*S*-$(OH)_2D_2$ [43], 24,26-$(OH)_2D_2$ [44], and 1α,25,28-$(OH)_3D_2$ [45]. These differences in the metabolism of vitamin D_2 have been exploited by pharmaceutical companies synthesizing and using the metabolites unaltered or else creating slightly modified versions (e.g. Roche compound 1α,25,28-$(OH)_3D_2$; Bone Care compound 1α,24*S*-$(OH)_2D_2$). Furthermore, features of the vitamin D_2 side chain, namely the C22-23 double bond or the C-24 methyl group (see Table I), have been successfully incorporated into the structure of other analogs (e.g. calcipotriol).

Even a prodrug based upon vitamin D_2 has been designed in the form of 1α-OH-D_2 [46]. This molecule is a valuable tool in studying hydroxylation reactions in the liver. At low substrate concentrations, 1α-OH-D_2, like 1α-OH-D_3, is 25-hydroxylated by liver hepatomas, Hep3B and HepG2, producing the well-established, biologically-active compound 1α,25-$(OH)_2D_2$. However, when the substrate concentration is increased to micromolar values, the principal site of hydroxylation of 1α-OH-D_2 becomes the C-24 position, the product being 1α,24*S*-$(OH)_2D_2$, another compound with significant biological activity [47,48]. This metabolite has been previously reported in cows receiving massive doses of vitamin D_2 [43]. Transfection studies using the liver cytochrome P450, CYP27A, expressed in COS-1 cells suggest that 1α,24*S*-$(OH)_2D_2$ is a product of this cytochrome [5]. Whether the formation of this unique metabolic product of 1α-OH-D_2 is the reason for the relative lower toxicity of 1α-OH-D_2, as compared to 1α-OH-D_3 [49], has not been established definitively.

Active vitamin D_2 compounds, such as 1α,25-$(OH)_2D_2$, 19-nor-1α,25-$(OH)_2D_2$, and 1α,24*S*-$(OH)_2D_2$, are also subject to further metabolism, although it differs from that of calcitriol, essentially because on the face of it, the modifications in the vitamin D_2 side chain prevent the C23-C24 cleavage observed during calcitroic acid production. Instead, the principal products are more polar tri- and tetra-hydroxylated metabolites such as: 1α,24,25-$(OH)_3D_2$, 1α,25,28-$(OH)_3D_2$, and 1α,25,26-$(OH)_3D_2$ from 1α,25-$(OH)_2D_2$ [45,50,51]; 19-nor-1α,24,25-$(OH)_3D_2$, 19-nor-1α,24,25,28-$(OH)_4D_2$, and 19-nor-1α,24,25,26-$(OH)_4D_2$ from 19-nor-1α,25-$(OH)_2D_2$ [52]; and 1α,24,26-$(OH)_3D_2$ from 1α,24*S*-$(OH)_2D_2$ [48]. These poly-hydroxylated metabolites are accompanied by side-chain cleaved products [52a], and there are even suggestions that calcitroic acid can be formed from 1α,25-$(OH)_2D_2$ [53]. This is not surprising when one considers that recent data using recombinant protein suggests that CYP24 is again responsible for these multiple hydroxylations of the D_2 side chain [54,55]. However, at least in the case of 1α,24*S*-$(OH)_2D_2$ metabolism by CYP24, the rate of metabolism appears slower than that of 1α,25-$(OH)_2D_3$ [48]. Some D_2 catabolites retain considerable biological activity and at least one, 1α,25,28-$(OH)_3D_2$, is patented for use as a drug. See Chapter 2 for further discussion of vitamin D_2 metabolism.

It is interesting to note that two newer families of vitamin D compounds under development are based upon minor modifications of the vitamin D_2 side chain. These are the D_4 series, which retain the C24(C28)-methyl group but lack the C22=C23 double bond [56], and the D_5 series, which possess a C24(C28,29)-ethyl group but lack the C22=C23 double bond [57].

C. Cyclopropane-ring Containing Analogs of Vitamin D

These analogs are modified in their side chains such that C-26 is joined to C-27 to give a cyclopropane ring consisting of C-25, C-26, and C-27. The simplest member of this series is MC 969, which possesses the vitamin D_3 side chain except for the presence of the cyclopropane ring together with a 1α-OH-D nucleus [58]. The best-known member of this group of compounds is MC 903 or calcipotriol, the structure of which is shown in Table II. In addition to the cyclopropane ring, calcipotriol features a C22=C23 double bond and a 24*S*-hydroxyl group [59]. As is presented in Chapter 101, calcipotriol was the first vitamin D analog to be approved for topical use in psoriasis, and is currently used worldwide for the successful control of this skin lesion [60].

When MC 969 is incubated with the hepatoma Hep3B, it is hydroxylated, not at the C-25 position as is 1α-OH-D_3, but at the C-24 position, as is 1α-OH-D_2, and then further oxidized to a 24-ketone [58]. 25-Hydroxylation of vitamin D analogs containing cyclopropane ring structures is feasible, and indeed the molecule has been synthesized chemically, but it is not produced enzymatically from MC969. It thus appears that the cyclopropane ring directs the hydroxylation site to the C-24 position.

Of course, the other cyclopropane-ring containing analog, calcipotriol, contains a pre-existing C-24 hydroxyl, which has been proposed to act as a surrogate C-25 hydroxyl in interactions of the molecule with the VDR. Pharmacokinetic data acquired for calcipotriol showed that it had a very short $t_{1/2}$, in the order of minutes; results that are consistent with the lack of a hypercalciuric/hypercalcemic effect when administered *in vivo* [61]. The first metabolism studies [28] revealed that calcipotriol was rapidly metabolized by a variety of different liver preparations from rat, minipig, and human to two novel products. Sorensen *et al*. [28] were able to isolate and identify the two principal products as a C22=C23 unsaturated, 24-ketone

(MC1046) and a C22-C23 reduced, 24-ketone (MC1080). Coincidentally, this is the same product as is formed from MC969. These results were confirmed and extended by Masuda *et al*. [19], who showed that calcipotriol metabolism was not confined to liver tissue, but could be carried out by a variety of cells including those cells exposed to topically-administered calcipotriol *in vivo*, namely keratinocytes. Furthermore, Masuda *et al*. [19] proposed further metabolism of the 24-ketone in these vitamin D target cells to side-chain cleaved molecules including calcitroic acid (Fig. 4). The main implications of this work are that calcipotriol is subject to rapid metabolism initially by nonvitamin D–related enzymes, and then by vitamin D-related pathways to a side-chain cleaved molecule. The catabolites are produced in a variety of tissues and appear to have lower biological activity than the parent molecule.

The reduction of the C22=C23 double bond during the earliest phase of calcipotriol catabolism was an unexpected event, given that the C22=C23 double bond in vitamin D_2 compounds is extraordinarily stable to metabolism. It thus appears that metabolism of calcipotriol provides evidence that the C-24 methyl group in the vitamin D_2 side chain must play a stabilizing role, preventing the formation of the 24-ketone, which facilitates the reduction of the C22=C23 double bond.

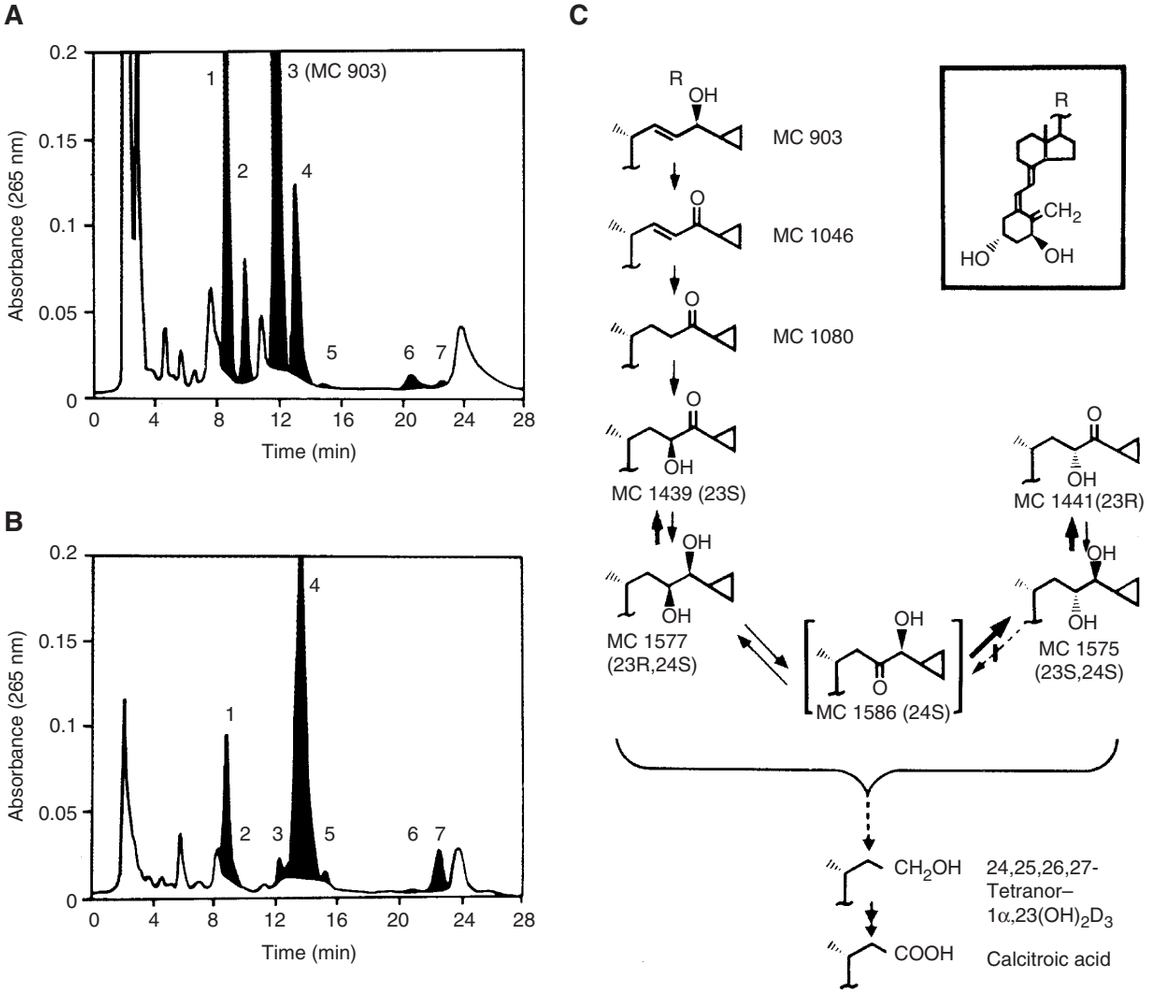


FIGURE 4 *In vitro* metabolism of calcipotriol (MC903) by HPK1A-ras cells. HPLC of lipid extracts following incubation of MC903 with (**A**) HPK1A human keratinocytes and (**B**) HPK1A-ras human keratinocytes. Peak 1=MC1080; Peak 2=MC1046; Peak 3=MC903 (calcipotriol); Peak 4=mixture of MC1439 and MC1441; Peak 5=Tetranor-1α,23(OH)$_2$D$_3$; Peak 6=MC1577; Peak 7=MC1575. (**C**) Proposed pathway of calcipotriol metabolism in cultured keratinocytes. (Reproduced from ref. 19 with permission.)

However, it is still unknown which enzyme is responsible for this reduction in the side chain of calcipotriol.

D. 20-Epi- and 20-Methyl Analogs

In the early 1990s, Leo organic chemists were first to change the stereochemistry of the side chain at the C-20 position [62,63]. As a result, they were in a position to synthesize a novel class of compounds, which were 20-epimers of existing analogs, the simplest being 20-epi-1α,25-$(OH)_2D_3$ and the most complex being KH1060 (see Table II for structures). Some of these 20-epi-analogs are extremely potent in cell differentiation and anti-proliferation assays, and are thus under development for use in hyperproliferative conditions. Another 20-epi analog 2-methylene-19-nor-20S-1α,25-$(OH)_2D_3$ (known as 2MD) is reported to be highly bone-specific and is thus under development as an anti-osteoporosis drug [64; see Chapter 87].

These 20-epi-molecules have been particularly well studied, not only for their susceptibility to metabolism, but also for their ability to transactivate model genes (see Chapter 83). Dilworth *et al.* [65] showed that 20-epi-1,25-$(OH)_2D_3$ (MC 1288) has virtually no DBP binding, slightly improved affinity for the bovine thymus VDR, and an altered rate of metabolism through certain steps of the C-24 oxidation pathway. Dilworth *et al.* [65] concluded that all of these factors: DBP affinity, VDR affinity, and rate of catabolism in target cells contribute to the biological activity advantages that 20-epi-1α,25-$(OH)_2D_3$ appears to possess over 1α,25-$(OH)_2D_3$ *in vitro* (Fig. 5A–D). The influence of DBP (in FCS) on 20-epi-1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-induced-hGH reporter gene expression is illustrated in Fig. 5C and D. Though the curve for 20-epi-1,25-$(OH)_2D_3$ remains unchanged by the presence

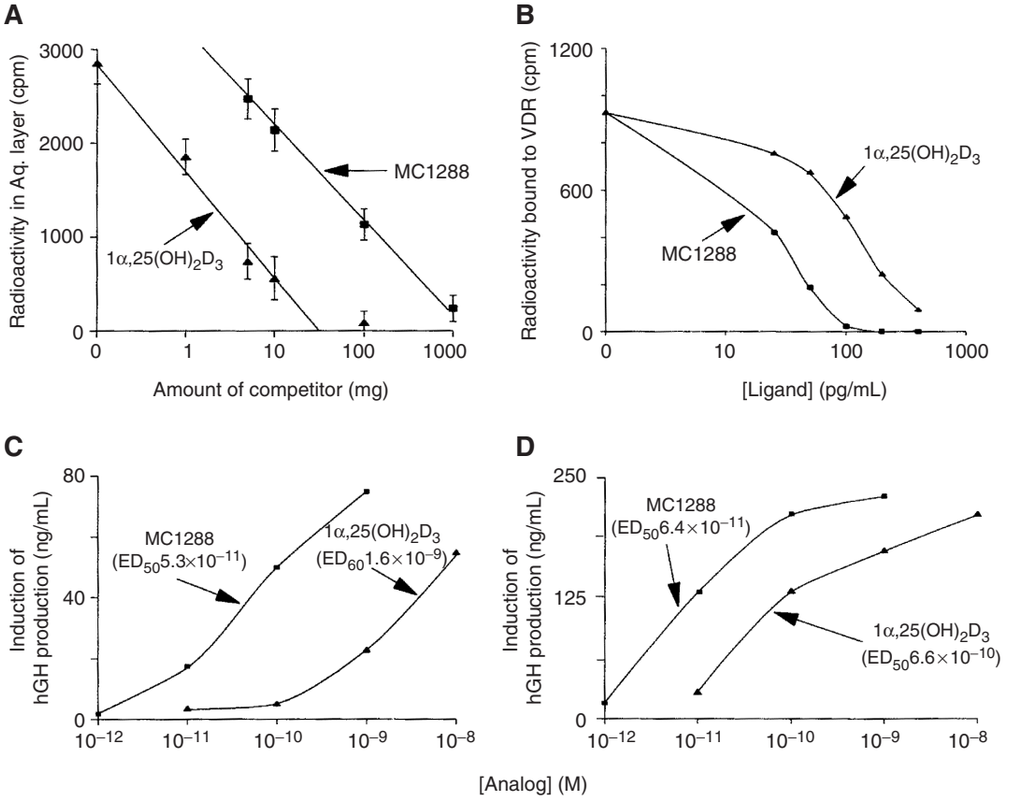


FIGURE 5 Biological parameters for 20-epi-1α,25-$(OH)_2D_3$. (A) Ability of 20-epi-1α,25-$(OH)_2D_3$ to compete for C24-oxidation pathway enzymes. (B) VDR affinity of 20-epi-1α,25-$(OH)_2D_3$ compared to 1α,25-$(OH)_2D_3$. (C and D): Effect of fetal calf serum (containing DBP) on reporter gene induction by 20-epi-1α,25-$(OH)_2D_3$ in the COS-1 cell line. (Reproduced from ref. 65 with permission.)

of DBP, the curve for 1,25-$(OH)_2D_3$ is reduced by an order of magnitude, exaggerating the potency difference between the two molecules. Dilworth *et al.* [65] postulated that the remaining difference in transactivation activity is due in part to metabolic differences and in part to affinity differences at the transcriptional level (i.e., VDR; VDR-RXR; VDR-RXR-VDRE). However, it should be noted that the slower rate of metabolic inactivation of 20-epi-1α,25-$(OH)_2D_3$ has been challenged by some workers [66] and an alternative theory proposed. In this alternative hypothesis, it is proposed that 20-epi-1α,25-$(OH)_2D_3$ is metabolized at a similar rate to 1α,25-$(OH)_2D_3$ but that the metabolic intermediates, particularly the biologically active form 20-epi-24-oxo-1α,25-$(OH)_2D_3$, are not broken down as efficiently and thus accumulate [66].

It is interesting to note that Peleg *et al*. [67] (also see Chapter 83) have extended this work to study the precise details of the gene transactivation events invoved in 20-epi-1α,25-$(OH)_2D_3$ action. They have found that two 20-epi-compounds, 20-epi-1α,25-$(OH)_2D_3$ and KH1060, are unusual in that they are able to form highly stable, protease-resistant RXR-VDR-VDRE heterodimeric complexes *in vitro*. Whether the stability of these transactivating complexes is related to the increased transactivation ability in model reporter genes is still to be established. However the work shows promise. Also interesting is the apparent correlation between the stability of 20-epi-1α,25-$(OH)_2D_3$-containing transactivation complexes (observed by Peleg *et al*. in ref 67) and the metabolic stability of 20-epi-1α,25-$(OH)_2D_3$ to target cell cytochrome P450 hydroxylation (observed by Dilworth *et al*. in ref 65). Whether this correlation holds for other potent, metabolically-stable vitamin D analogs has yet to be investigated. The 20-epi-compound KH1060 will be discussed later in this chapter under Section G. "Oxa-group Containing Analogs."

20-Methyl vitamin D analogs are a group of biologically active compounds that have been synthesized mainly by the Schering company [118]. As with the 20-epi series, the presence of the 20-methyl group imposes a different conformation of the side chain and thus creates interesting problems for CYP24, as well as the VDR. Metabolic studies with 20-methyl-1, 25-$(OH)_2D_3$ and related compounds [68] suggest that the 20-methyl group makes 23-hydroxylation difficult and though 24-oxidation products can be detected by such techniques as LC/MS (Fig. 6; taken from M. Kaufmann and G. Jones, unpublished data), the 24-oxidation pathway intermediates build up in an analogous way to that proposed for 20-epi-1,25-$(OH)_2D_3$ [66]. In fact, another 20-methyl-1,25-$(OH)_2D_3$ analog with a 23-ene group is resistant to metabolism and is even more potent than 20-methyl-1,25-$(OH)_2D_3$ itself [69].

E. Homologated Analogs

This modification involves the insertion of carbon atoms into the vitamin D side chain. It can take two different forms: a) insertion of the carbon atoms in the main side chain between carbons 22–25 such that additional carbons are numbered 24a, 24b, 24c, etc.; b) insertion of extra carbon atoms on the terminal methyl groups making them into dimethyl (ethyl) groups (likened to lengthening the "claws" on a crab). These carbons are 26a, 27a, etc.

Homologated compounds were first developed in the early 1980s by Ikekawa and colleagues and initially tested for biological activity by DeLuca's group [70–72]. Stern's group showed that these analogs possess increased biological activity compared to 1α,25-$(OH)_2D_3$ when assayed in a cultured bone model *in vitro* [73]. Whether metabolism of these homologated analogs is different from that of 1α,25-$(OH)_2D_3$ or might play a role in the increased biological activity was initially unknown. The effect of lengthening the side chain poses interesting problems for the enzymes involved in hydroxylation of the side chain. The active site of the cytochrome P450 is forced to accommodate a longer side chain, which might alter the efficiency of hydroxylation and depending upon the way the side chain is anchored might change the site of hydroxylation (termed regioselectivity).

Dilworth *et al*. [74] examined this systematically by studying the effect on metabolism of adding one, two, or three carbons to the main vitamin D side chain. Homologs synthesized by the Leo chemist, Martin Calverley, and used by Dilworth *et al*. [74] with the 25-hydroxyl group already in place included:

MC 1127 (24a-homo-1α,25-$(OH)_2D_3$),
MC 1147 (24a,24b-dihomo-1α,25-$(OH)_2D_3$), and
MC 1179 (24a,24b,24c-trihomo-1α,25-$(OH)_2D_3$).

Only one compound was synthesized without the 25-hydroxyl group, and this was the 1α-OH-D_3 homolog with two extra carbon atoms:

MC 1281 (24a,24b-dihomo-1α-OH-D_3).

The results obtained from metabolic studies using HPK1A-ras keratinocytes as a source of target cell 23- and 24-hydroxylase enzymes (presumably CYP24) and using hepatoma cells as a source of 25-hydroxylase (presumably CYP27A) suggested that both cytochrome P450 isoforms continued to efficiently hydroxylate all homologs provided. Somewhat surprisingly, CYP24 maintained its hydroxylation sites at C-23 and C-24 despite the extension of the side chain by up to three carbons and seemingly preferring not to

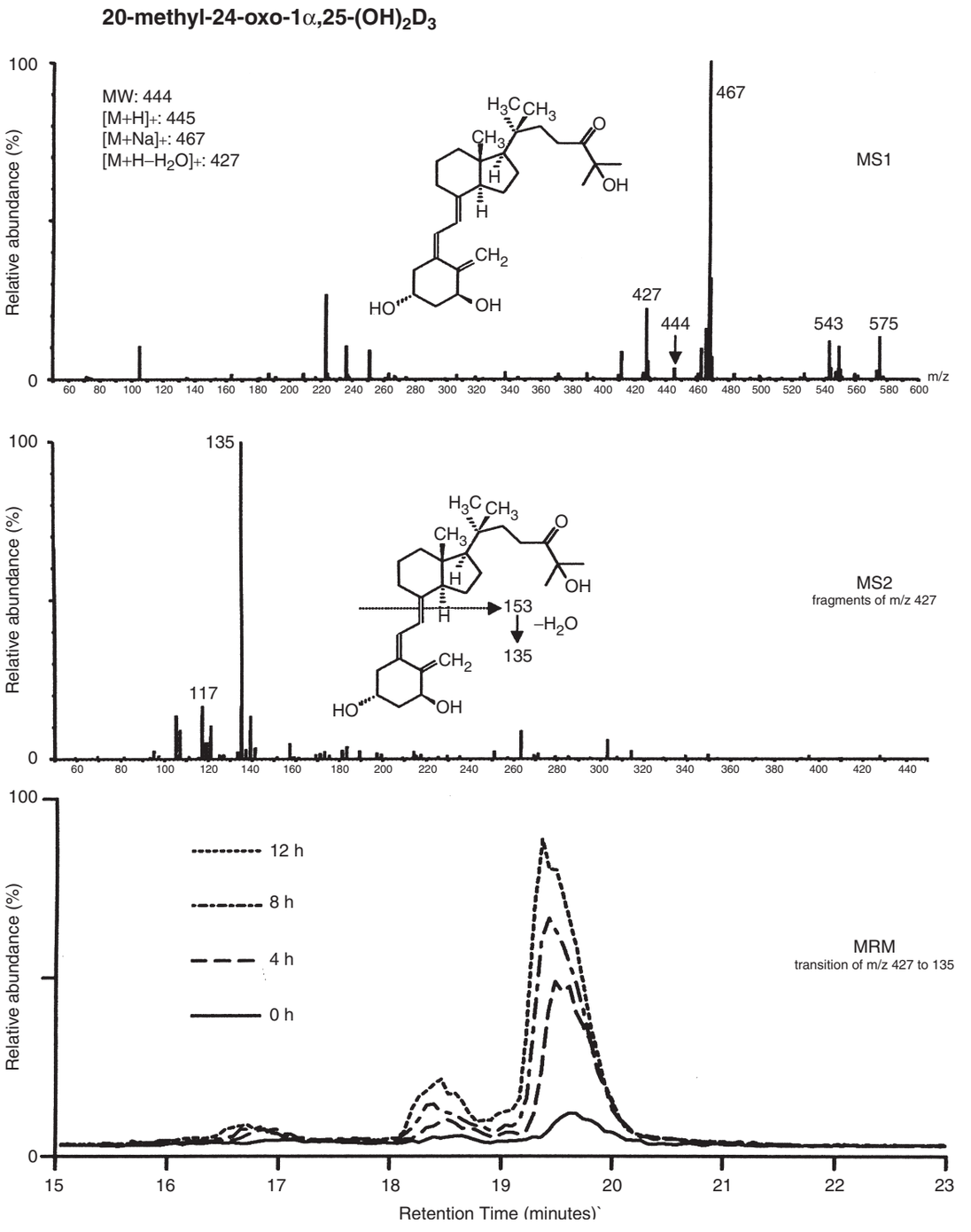


FIGURE 6 LC/MS of metabolites of 20-methyl-1α,25-$(OH)_2D_3$. Analysis of the purified metabolite by MS1 revealed several characteristic ions which helped putatively identify the metabolite 20-methyl-24-oxo-1α,25-$(OH)_2D_3$ based upon molecular weight. In MS2 mode, fragmentation of the dehydration product (m/z 427) produced a specific cis-triene cleavage fragment (m/z 135). In MRM mode, the transition from m/z 427 to 135 formed a sensitive and selective means to quantitate the production of this metabolite in HPK1A-*ras* cells when incubated with 20-methyl-1α,25-$(OH)_2D_3$ over a time course. (Kaufmann M. and Jones G., unpublished results.)

move down the side chain to C-24a, C-24b, or C-24c to be adjacent to the tertiary carbon, C-25. On the other hand, CYP27A hydroxylated MC1281 terminally at C-25 and C-27, appearing to ignore the longer internal side chain. These homologs therefore offer interesting insights into hydroxylation site selection by the vitamin D–specific cytochromes. Dilworth *et al*. [74] postulated that CYP24 must be directed to its hydroxylation site by the distance from the vitamin D ring structure, whereas CYP27 is directed by the distance of the hydroxylation site from the end of the side chain.

Dilworth *et al.* [75] have also examined the effect of introducing the terminal 26,27-dimethyl groups into the side chain of 1α,25-$(OH)_2D_3$ to make the analog 26,27-dimethyl-1α,25-$(OH)_2D_3$ (MC1548). They found that MC1548 was metabolized at the same rate as 1α,25-$(OH)_2D_3$ by the keratinocyte cell line HPK1A-ras. Products included metabolites of the C-24 oxidation pathway, and this was confirmed by the observation that MC1548 and 1α,25-$(OH)_2D_3$ were equally effective in blocking the metabolism of [1β-^{3}H]1α,25-$(OH)_2D_3$ to [1β-^{3}H] calcitroic acid. However, the products of MC1548 also included 26a-OH-MC1548, suggesting that the introduction of the dimethyl group into the side chain makes it susceptible to attack at a new terminal location by keratinocyte enzymes.

Although MC1548 is the simplest molecule in the dimethyl homologated series, it represents a valuable tool in understanding the relative importance of various modifications within complex homologated molecules such as EB1089 and KH1060 (see also Chapter 84). These latter molecules have greatly increased biological activity over 1α,25-$(OH)_2D_3$ in cell-differentiating systems *in vitro,* and elucidating the importance of metabolism to this increased potency *in vitro* should provide useful insights into vitamin D analog action [76]. The metabolism of EB1089 and KH1060 will be discussed in Sections: F. "Unsaturated Analogs" and G. "Oxa-group Containing Analogs," respectively.

F. Unsaturated Analogs

The idea of introducing double bond(s) into the side chain of vitamin D analogs arose from experience with vitamin D_2. Vitamin D_2 metabolites have similar biological activity to those of vitamin D_3 so that it appears that the introduction of the double bond is not deleterious. As mentioned already in Sections B. "Vitamin D_2 Derivatives" and C. "Cyclopropane-ring Containing Analogs of Vitamin D" in this chapter, the metabolism of the side chain is significantly altered by this relatively minor change.

The modification has not been confined to the introduction of a C22=C23 double bond. Roche has developed molecules with two novel modifications:

a) introduction of a C16-C17 double bond and
b) introduction of a C23-C24 triple bond

that when combined produce the highly successful 16-ene, 23-yne analog of 1α,25-$(OH)_2D_3$ [77] (see Table II for structure). As alluded to earlier, Leo Pharmaceuticals has introduced the promising unsaturated analog EB1089 that contains a conjugated double-bond system at C22-C23 and C24-C24a, in addition to both main side chain and terminal dimethyl types of homologation [78] (see Table II for structure). These two series of Roche and Leo compounds have shown strong antiproliferative activity both *in vitro* and *in vivo* [77,79,80].

The metabolism of the 16-ene compound by the perfused rat kidney has been studied by Reddy *et al*. [81]. Reddy *et al.* [81] found that the introduction of the C16-C17 double bond reduces 23-hydroxylation of the molecule, and the implication is that the D-ring modification must alter the conformation of the side chain sufficiently to subtly change the site of hydroxylation by CYP24, the cytochrome P450 thought to be responsible for 23- and 24-hydroxylation. It is worth noting that Dilworth *et al.* [65] also noted the absence of measurable 23-hydroxylation of the analog 20-epi-1α,25-$(OH)_2D_3$ in their studies, reinforcing the view that modifications around the C17-C20 bond profoundly influence the rate of 23-hydroxylation.

The metabolism of the 16-ene, 23-yne analog of 1α,25-$(OH)_2D_3$ by WEHI-3 myeloid leukemic cells has been studied by Satchell and Norman [82]. Though one might predict that because this molecule is blocked in the C-23 and C-24 positions that it must be stable to C-24 oxidation pathway enzyme(s), it was found experimentally that the 16-ene,23-yne analog has the same $t_{1/2}$ as 1α,25-$(OH)_2D_3$ when incubated with this cell line (6.8 hr). The main product of [25-^{14}C]1α,25-$(OH)_2$-16-ene-23-yne-D_3 was not identified by these workers, but appeared to be more polar than the starting material. It will be interesting to see in any possible follow-up work if the metabolite that they have isolated has lost its C23-C24 triple bond or is simply further hydroxylated at some alternative site in its side chain (e.g. at C-26). Based upon knowledge emerging from other analogs, this reviewer favors the latter.

Another unsaturated analog, which one might predict would be relatively metabolically stable, is EB1089 with its conjugated double-bond system. However, as pointed out earlier, EB1089 contains three structural modifications: the conjugated double-bond system is

accompanied by two types of side chain homologation. Nevertheless, as expected, the conjugated double-bond system dominates the metabolic fate of EB1089, there being no C-24 oxidation activity due to the blocking action of the conjugated diene system. When metabolism is studied with either *in vitro* liver cell systems or the cultured keratinocyte cell line, HPK1A-ras, disappearance of EB1089 is much slower than that of 1α,25-$(OH)_2D_3$ [83]. Such data are consistent with the fairly long $t_{1/2}$ in pharmacokinetic studies *in vivo* [84]. Since the conjugated system of EB1089 blocks C-24 oxidation reactions, it is not surprising that a different site in the molecule becomes the target for hydroxylation. Diode array spectrophotometry has allowed for the identification of the principal metabolic products of EB1089 as 26- and 26a-hydroxylated metabolites (Fig. 7) [85,86]. Note also that these metabolites of EB1089 have been chemically synthesized and shown to retain significant biological activity in cell differentiation and antiproliferative assays [86].

Again, it is interesting to note that with EB1089 and other molecules blocked in the C-23 and C-24 positions, such as 1α,24S-$(OH)_2D_2$ [48], the terminal carbons C-26 and C-26a become the alternative sites of further hydroxylation. However, it should also be noted that even in molecules not blocked in the C-23 and C-24 positions but containing the terminal 26- and 27-dimethyl homologation such as 26,27-dimethyl-1α,25-$(OH)_2D_3$ (MC1548) [75], there seems to be significant terminal 26a-hydroxylation occurring. Therefore, the hydroxylation of EB1089 at C-26 and C-26a may be in part a consequence of the introduction of the conjugated double-bond system and in part a consequence of the introduction of the terminal homologation.

When the C22=C23 double bond is present in the side chain in the absence of a C-24 methyl group, as in calcipotriol, the double bond appears vulnerable to reduction. As pointed out earlier, the principal metabolites of calcipotriol are reduced in the C22–C23 bond except for one, the C22 -C23 unsaturated, 24-ketone (MC1046) [19,28]. This suggests a C-24 ketone must be present to allow for this reduction to occur. Work of Wankadiya *et al.* [87] using the Roche compound, $^{22}\Delta$-1α,25-$(OH)_2D_3$, an analog that contains the C22=C23 double bond but lacks a C-24 subsituent, tends to indirectly support this theory. When incubated with the chronic myelogenous leukemic cell line, RWLeu-4, this molecule, like 1α,25-$(OH)_2D_3$, is converted, presumably via $^{22}\Delta$-1α,24,25-$(OH)_3D_3$ and $^{22}\Delta$-24-oxo-1α,25-$(OH)_2D_3$, metabolites analogous to intermediates in the

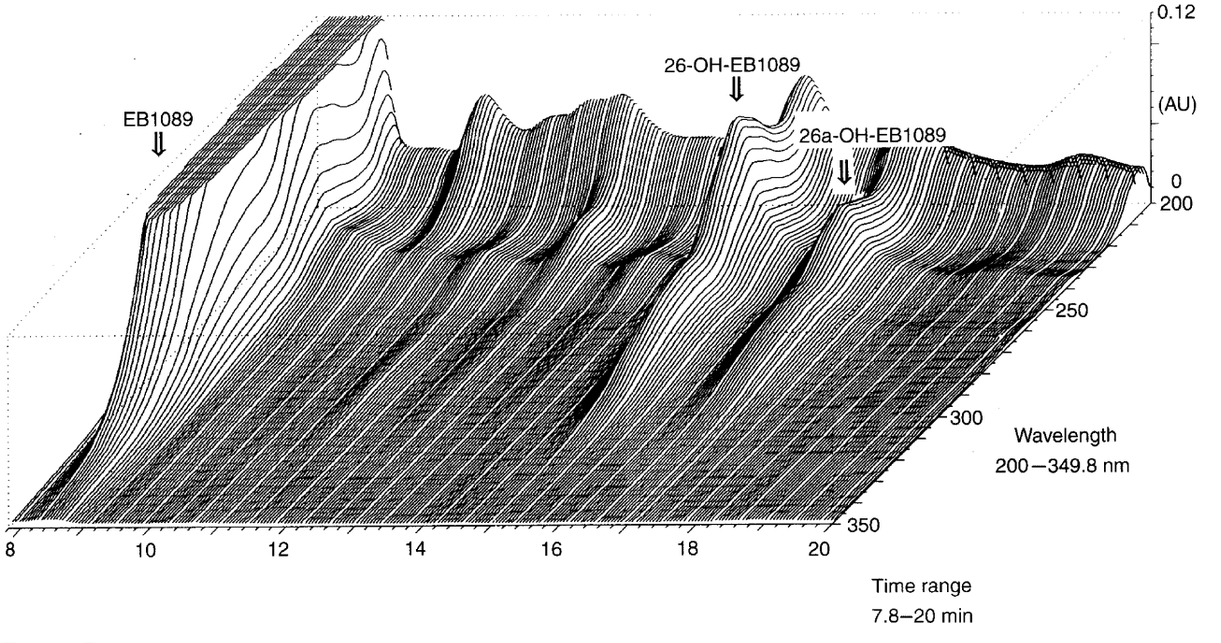


FIGURE 7 *In vitro* metabolism of EB1089 by HPK1A-ras cells. Diode array HPLC of lipid extracts following incubation of EB1089 (10 μM) with the human keratinocyte, HPK1A-ras for 72 hr. In addition to the substrate at 8.5 min, two metabolites showing the characteristic UV chromophore of EB1089 (λ_{max} 235 nm, shoulder 265 nm) are visible in the part of the HPLC profile reproduced here (8–20 min). Metabolite peaks at 15.03 min and 16.55 min were isolated by extensive HPLC and identified [68,69,71] by comparison to synthetic standards on HPLC, GC-MS, and NMR. The identifications are: Peak A at 15.03 min = 26-OH-EB1089; Peak B at 16.55 min = 26a-OH-EB1089. (Reproduced from Shankar *et al.* [85] with permission.)

C-24 oxidation pathway, to the side chain truncated product 24,25,26,27-tetranor-1,23-$(OH)_2D_3$, a molecule which lacks the C22=C23 double bond. However, Wankadiya *et al*. [87] did not identify intermediates in this process, and thus do not know at which stage C22=C23 reduction occurred.

G. Oxa-group Containing Analogs

These compounds involve the replacement of a carbon atom (usually in the side chain) with an oxygen atom. The best known of these are the 22-oxa analogs including 22-oxa-calcitriol (OCT) [88] and KH1060 [89]. Lesser known analogs include 23-oxa-series [90–92, 97] and 24-oxa-1α-hydroxyvitamin D_3 [93,94]. All of these molecules are metabolically fascinating to study because the oxa-atom makes the molecule inherently unstable should it be hydroxylated at the adjacent carbon atom. The hydroxylation at an adjacent carbon generates an unstable hemi-acetal, which spontaneously breaks down to eliminate the carbons distal to the oxa-group. In the case of: a) the 22-oxa compounds the expected product(s) would be C-20 alcohol/ketone; b) the 23-oxa compounds the expected product(s) would be C-22 alcohol/ketone; and c) the 24-oxa compounds the expected product(s) would be C-23 alcohol/ketone/acid.

The metabolism of OCT has been extensively studied in a number of different biological systems, including primary parathyroid [25] and keratinocyte cells [95], as well as cultured osteosarcoma, hepatoma, and keratinocyte cell lines [26] (see also Chapter 86). In all these systems, OCT is rapidly broken down. As outlined earlier, the use of two different radioactive labels in [26-^{3}H]OCT and [2β-^{3}H]OCT enabled Brown *et al*. [25] to suggest that the side chain was truncated, though definitive proof of the identity of the products was not immediately forthcoming. It was not until the work of Masuda *et al.* [26] that the principal metabolites were unequivocably identified by GC-MS as 24-OH-OCT, 26-OH-OCT, and hexanor-1α,20-dihydroxyvitamin D_3. In the case of the keratinocyte HPK1A-ras, an additional product, hexanor-20-oxo-1α-hydroxyvitamin D_3, is formed. These latter two truncated products are suggestive of hydroxylation of OCT at the C-23 position to give the theoretical unstable intermediate postulated at the beginning of the studies. Though all of these products were isolated from *in vitro* systems, there is evidence that the processes also occur *in vivo* because Kobayashi *et al*. [96] have generated data that suggests that the biliary excretory form of OCT in the rat is a glucuronide ester of the truncated 20-alcohol.

The 23-oxa derivative KH1650 (see Table II) is broken down rapidly to the expected 22-alcohol [97]. The metabolism of 24-oxa-1α-hydroxyvitamin D_3 (MC1090) has been studied at micromolar concentrations using the hepatoma, Hep3B [93]. As expected, 24-oxa-1α-OH-D_3 was found to be converted in high yield to two truncated products: tetranor-1,23-$(OH)_2D_3$ and calcitroic acid; again suggesting hydroxylation at the C-25 position adjacent to the 24-oxa group which results in an unstable intermediate. The products were again identified by GC-MS.

Both the above examples of simple oxa-analogs provide useful knowledge which can help in predicting the metabolic fate of a complex oxa-analog such as KH1060. This highly potent compound which possesses *in vitro* cell-differentiating activity exceeding that of any other analog synthesized to date, has four different modifications to the side chain of 1α,25-$(OH)_2D_3$ namely: 1) a 22-oxa group; 2) the 20-epi side chain stereochemistry; 3) 24a-homologation; and 4) 26- and 27-dimethyl homologation (see Table II for structure).

Since all of these changes are known to affect biological activity *in vitro* and *in vivo* as well as side chain metabolism, it comes as no surprise that the metabolism of KH1060 is extremely complex. KH1060 has a very short $t_{1/2}$ in pharmacokinetic studies *in vivo* [84], giving a metabolic profile with at least 16 unknown metabolites. Recently, Dilworth *et al*. [98] reported the first *in vitro* study using micromolar concentrations of KH1060 incubated with the keratinocyte cell line HPK1A-ras. Dilworth *et al.* [98] were able to discern 22 different metabolites after multiple HPLC steps and assigned structures to 12 of these metabolites (see Fig. 8). As would be expected from consideration of the studies of other oxa-compounds, two of these were truncated products identical to the molecules formed from another 22-oxa compound, OCT. As would be expected from consideration of the studies of other homologated compounds (see Section E. "Homologated Analogs"), other products are hydroxylated at specific carbons of the side chain including C-26 and C-26a. As with EB1089 and MC1548, the presence of dimethyl groups in the terminus of the side chain appears to attract hydroxylation to this site in KH1060. One novel metabolite found only for KH1060 is 24a-OH-KH1060, observed both in broken cell and intact cell models [99,100].

One important facet of this complex metabolic profile is that rather than simplifying our understanding of the mechanism of action of KH1060, these data complicate it. This is because biological assays of each of the metabolic products have shown that several of the principal and long-lived metabolic products of KH1060 retain significant vitamin D–dependent gene-inducing activity in reporter gene expression systems [100]. This point will be discussed further under Section III.B. Implications for Mechanism of Action of Vitamin D Analogs.

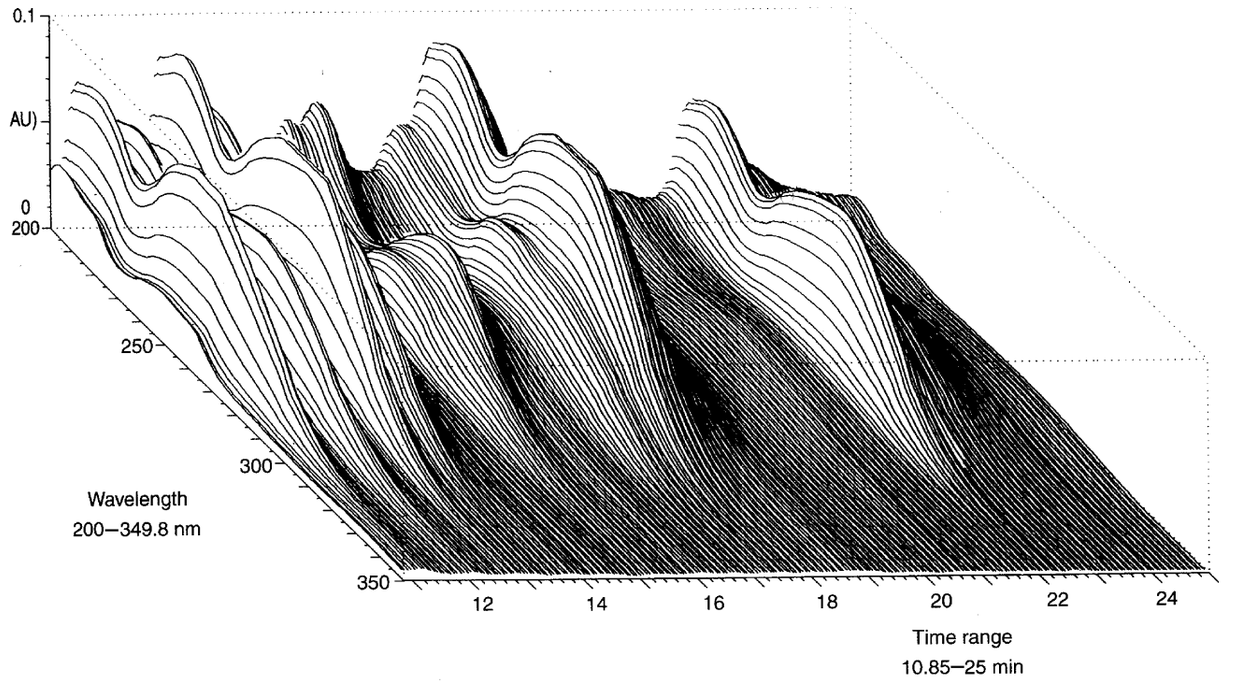


FIGURE 8 *In vitro* metabolism of KH1060 by HPK1A-ras cells. Diode array HPLC of lipid extracts following incubation of KH1060 (10 μM) with the human keratinocyte, HPK1A-ras for 72 hr. Nine peaks showing the characteristic UV chromophore of vitamin D (λ_{max} 265 nm, λ_{min} 228 nm) are visible in the part of the HPLC profile reproduced here (11–25 min). Rechromatography of these peaks on a second HPLC system resulted in the further resolution of these 9 peaks into 22 separate metabolites. Many of these metabolites were identified (ref. 82) by comparison to synthetic standards on HPLC and GC-MS. Identifications include: Peak at 13.39 min = 24a-OH-KH1060; Peak at 22.14 min = 26-OH-KH1060. (Reproduced from Dilworth *et al.* ref. 98 with permission.)

H. Other Analogs

There are several other analogs synthesized to date that do not fit readily into the groups discussed thus far and yet have been studied metabolically. These are listed briefly below.

1. 1α,24*R*-(*OH*)$_2$*D*$_3$

This compound is marketed as an antipsoriatic drug by Teijin, and its structure is shown in Table II. The metabolism of [1β-^{3}H]1α,24*R*-$(OH)_2D_3$ by the isolated perfused rat kidney was described by Reddy *et al*. [101]. Interestingly, Reddy *et al*. found two distinct pathways of metabolism, a major one involving direct C-24 oxidation (24-oxidation, 23-hydroxylation, side chain cleavage), resulting in calcitroic acid formation, and a second minor pathway, involving first 25-hydroxylation to 1α,24*R*,25-$(OH)_3D_3$, followed by the same C-24 oxidation steps occurring in the catabolism of 1α,25-$(OH)_2D_3$ (see Fig. 1). Since the authors used both pharmacological as well as "physiological" concentrations of the analog, they were able to generate sufficient quantities in order to identify their products by mass spectrometry. Their finding of 25-hydroxylase activity in the kidney is not a complete surprise since others [102] have detected the enzyme activity there, and CYP27A mRNA is detected in rat kidney tissue [103]. However, a follow-up study by the same researchers [104] suggests that the renal 25-hydroxylase which is able to hydroxylate 1α,24*R*-$(OH)_2D_3$ is unable to 25-hydroxylate the compounds vitamin D_3 and 1α-OH-D_3. The implication of this work is that 1α,24*R*-$(OH)_2D_3$ is metabolized by a nonspecific enzyme to intermediates of the C-24 oxidation pathway of 1α,25-$(OH)_2D_3$. If this is the case *in vivo*, one might anticipate the potential for the drug 1α,24*R*-$(OH)_2D_3$ to interfere with normal 1α,25-$(OH)_2D_3$ catabolism.

2. ED-71

The structure of this compound is also shown in Table II. It is unusual in that it represents one of the few A-ring modified analogs whose metabolism has been studied to date (see Chapter 86). It possesses a unique 2β-hydroxypropoxy group, in addition to the usual 1α-, 3β- and 25-hydroxy groups of 1α,25-$(OH)_2D_3$. The extra bulky group at the 2β-position has the effects of improving the affinity of ED-71 for the plasma-binding protein, DBP, but as a consequence may make

it more difficult for the analog to enter target cells. There is some optimism that these unique properties may make ED-71, a "long-lived vitamin D" and therefore show the necessary properties to be suitable as an anti-osteoporosis drug [105]. Since ED-71 has the normal side chain of 1α,25-$(OH)_2D_3$, it should be susceptible to the same C-24 oxidation sequence and other pathways as the natural hormone. Indeed, initial studies have provided evidence for the formation of several of the same 24- and 26-hydroxylated and 24-oxidized products as 1α,25-$(OH)_2D_3$, but at a much reduced rate [106]. There is some possibility for metabolism of 2β-group by nonspecific enzymes, though Masuda *et al*. [106] failed to observe such catabolites. Additional consideration of this compound can be found in Chapter 86.

3. 26,26,26,27,27,27-HEXAFLUORO-1α,25-$(OH)_2D_3$ (26,27-F_6-1α,25-$(OH)_2D_3$)

This analog was first synthesized in the early 1980s [107], along with a number of other side chain fluorinated analogs, to test the importance of certain key hydroxylation sites (e.g. C-23, C-24, C-25, C-26(27), C-1) to biological activity. It was noted immediately that 26,27-F_6-1α,25-$(OH)_2D_3$ was extremely potent (10-fold higher than 1α,25-$(OH)_2D_3$) in calcemia assays both *in vitro* and *in vivo* [108–110]. Lohnes and Jones [111] presented evidence using a bone cell line, UMR106, that 26,27-F_6-1α,25-$(OH)_2D_3$ had a longer $t_{1/2}$ inside target cells due to the apparent lack of 24-hydroxylation of 26,27-F_6-1α,25-$(OH)_2D_3$. At around the same time, Morii's group noted the appearance of a metabolite of 26,27-F_6-1α,25-$(OH)_2D_3$, which they have identified as 26,27-F_6-1α,23,25-$(OH)_3D_3$ [112]. This compound has excellent calcemic activity in its own right, but whether this derivative is in part responsible for the biological activity of 26,27-F_6-1α,25-$(OH)_2D_3$ is not conclusively proven. Nonetheless, 26,27-F_6-1α,25-$(OH)_2D_3$ has undergone clinical trials for hypocalcemia associated with hypoparathyroidism and uremia [113,114].

4. OTHER ANALOGS

As further generations of analogs emerge, they will likely be studied metabolically. Each major new modification must be tested for its impact upon the metabolic machinery, as well as on biological activity. There are some compounds listed in Table I that are currently untested or for which only preliminary results have been published. These include the aromatic analog KH1650 [97], A-ring modified analogs such as 2-methyl-1α,25-$(OH)_2D_3$ and 2-methylene-19-nor-20-epi-1α, 25-$(OH)_2D_3$ [64], as well as the side chain modified analogs 1α-OH-D_5 [57] and the sulphone family [115].

III. IMPORTANT IMPLICATIONS DERIVED FROM ANALOG METABOLISM STUDIES

A. Correlations with Pharmacokinetic Information

The susceptibility of a vitamin D analog to metabolism and excretion undoubtedly plays a significant role in determining the biological activity of that analog *in vivo*. The quickest and easiest way to acquire such knowledge is by pharmacokinetic analysis. From a classical vitamin D outlook that this author prefers to take, pharmacokinetic data reflect a few important parameters regarding each analog including:

a) The affinity of the vitamin D analog for DBP in the bloodstream.
b) The rate of target cell uptake and metabolism by target cell enzymes.
c) The rate of liver cell uptake, hepatic metabolism, and biliary clearance.
d) The rate of storage depot uptake and release.

Metabolism, whether target cell or liver, is reflected in only two components of this list of factors measured by pharmacokinetics. Therefore, it cannot be expected that *in vitro* metabolic parameters would exactly correlate with *in vivo* pharmacokinetic parameters. Nevertheless, a comparison of the two might be worthwhile. In the case of some of the analogs shown in Tables I and II, pharmacokinetic data [84,119,120] are available and can be compared to the data provided by *in vitro* metabolic studies.

In Table IV an attempt is made to compare pharmacokinetic data from these sources with DBP binding data and target cell metabolic data. As pointed out by Kissmeyer *et al*. [84], compounds segregate into at least two categories (perhaps more) on the basis of their pharmacokinetic parameters.

Calcemic Analogs (Strong or Weak) Those analogs with a long $t_{1/2}$ which is either a function of strong DBP binding *or* a reduced rate of metabolism (or both). The analog ED-71 has a strong DBP binding affinity. There appear to be a group of analogs in which a long $t_{1/2}$ is correlated with a slower rate of metabolism (e.g. EB1089, ED-71, 1α,24*S*-$(OH)_2D_2$). With the exception of ED-71, most of these active analogs bind DBP poorly.

Noncalcemic Analogs Those analogs with a short $t_{1/2}$, which is either a function of poor DBP binding *or* a rapid rate of metabolism (or both). Examples include calcipotriol, KH1060, and OCT.

TABLE IV Pharmacokinetic Data on Vitamin D Analogs

Compound[a]	Serum concentration at $t = 5$ min (ng/ml)	$t_{1/2}$ (hr)	AUC$^{\infty}$ (ng/ml × hr)	Serum clearance (ml/hr/kg)	Binding affinity for DBP (M)	Relative binding affinity for DBP[b]	Rate of metabolism[c]
$25OHD_3$	2040	> 2.8	9596	21	9×10^{-9}	33	Very slow
$1\alpha,25(OH)_2D_3$	2429	2.2	7355	27	$1.5–6.0 \times 10^{-7}$	1	Fast
$1\beta,25(OH)_2D_3$	2912	> 4	13,228	15	1.7×10^{-8}	17	–
Calcipotriol	121	0.2	27	7407	1.7×10^{-6}	0.1	Very fast
MC 1127	545	1.6	1216	167	5.2×10^{-6}	0.1	Fast
EB 1089	152	2.1	255	784	7.9×10^{-6}	0.03	Slow
CB 966	176	1.8	267	693	3.2×10^{-5}	0.02	–
KH 1139	154	0.7	142	1408	6.5×10^{-5}	0.007	–
KH 1060	103	0.4	46	4348	n.b.	0	Fast
KH 1049	104	0.5	40	5000	n.b.	0	–

Compound[d]	$t_{1/2}$ (hr)	Metabolic clearance rate (ml/min)	Relative binding affinity for DBP[e]	Rate of metabolism[f]
$1\alpha,25(OH)_2D_3$	7.0	5.0	1	Fast
OCT	2.5	48.2	266	Very fast

Compound[a]	Baseline (pg/ml)	AUC$^{\infty}$ $t_{1/2}$ (hr)	Relative binding (pg/ml × hr)	Rate of affinity for DBP[h]	Rate of metabolism[i]
$1\alpha,25(OH)_2D_3$	67.0	5.8	3690	1	Fast
$1\alpha,25(OH)_2D_2$	<10	5.1	2676	–	–
$1\alpha,24(OH)_2D_2$	<10	4.9	659	14	Slow

[a] Activity was measured following a single intravenous dose of 200 μg/kg to rats. From Kissmeyer *et al.* [84].
[b] Using human DBP, the relative numbers are compared to the $1\alpha,25(OH)_2D_3$ value. n.b., No binding. From Kissmeyer *et al*. [84].
[c] From Masuda *et al*. [19], Dilworth *et al.* [65, 98], and Shankar *et al.* [85].
[d] Following a single intravenous dose of 100 ng/animal to dogs. From Dusso *et al.* [120].
[e] Using rat DBP, the relative numbers are compared to the $1\alpha,25(OH)_2D_3$ value. From Dusso *et al.* [120].
[f] From Masuda *et al.* [26].
[g] Following a single oral dose of 0.39 ug/kg to rats. From Knutson *et al.* [146].
[h] Using rat DBP. From Strugnell *et al.* [47].
[i] From Jones *et al.* [48].

It should be noted that though these classifications are used in the vitamin D literature, they are somewhat artificial since **pure** "noncalcemic" analogs do not yet exist. All "noncalcemic" analogs will cause hypercalcemia if their concentration is raised sufficiently. The crucial issue is whether systemically administered, "weakly calcemic" or "noncalcemic" analogs can produce their anti-cell proliferation/pro-cell differentiation effects *in vivo* at concentrations lower than that required to produce calcemia. Various *in vivo* clinical trials currently in progress will be the acid test for this question.

B. Implications for Mechanism of Action of Vitamin D Analogs

There is currently tremendous interest in defining the mechanism of action of vitamin D analogs, particularly for clarifying the difference between "calcemic" or "noncalcemic" analogs. Chapters 82 and 83 will discuss other aspects of analog action in detail. It is obvious from the amount of space committed to this mechanism that the problem is not simple but multifactorial. Therefore, the next section of this chapter

will focus on the importance of metabolism to the complex picture.

Metabolism can have an impact on the mode of action in a few different ways:

1) Lack of high affinity binding to DBP in the blood can make the analog vulnerable to liver enzymes, which may lead to deactivation and excretion of the analog.
2) Target cell enzymes may *activate or deactivate* the administered analog:
   i) to metabolites which possess increased, equivalent, or slightly decreased biological activity at target genes, and which may have an extended $t_{1/2}$ inside the cell.
   ii) to metabolites which possess much reduced biological activity.
3) The rate of metabolism by target cell enzymes may be influenced by the rate of entry of "free" analog from the cell exterior *and* the association and dissociation rates of VDR-RXR-DNA complexes [121].

Chapter 82 will stress the importance of DBP binding and pharmacokinetics. It is clear that point 1) hepatic metabolism above relates mainly to this chapter. Chapter 83 will focus on the mechanisms by which analogs interact with VDR-RXR heterodimeric complexes and ultimately attract coactivators into the transcriptional machinery associated with the vitamin D–dependent gene in order to regulate gene expression. Points 2) and 3) target cell metabolism relate mainly to Chapters 82 and 83.

It should be noted with regard to molecular mechanisms of action at the target cell level that metabolism is often disregarded or given too little emphasis. Furthermore, certain metabolic assumptions are made when testing biological activity that are not always valid. These include:

i) the analog is biologically active as administered.
ii) the analog is stable in the *in vitro* target cell model used, whether *in vitro* organ culture, cultured target cell, or host cell/reporter gene construct.

The validity of this approach is made even more tenuous when data acquired with different *in vitro* models where metabolic considerations may or may not apply are compared to data acquired *in vivo* where metabolic considerations definitely apply. The reader is cautioned that invalid comparisons of *in vivo* and *in vitro* data abound in this field.

In the opinion of this reviewer, metabolism will turn out to be a key parameter, but not the *only* important parameter in vitamin D analog action. It is our view that only when we consider all of the parameters which can influence analog action within the overall equation will we be in a position to fully understand the molecular mechanisms underlying their "noncalcemic" or "calcemic" actions. From this it seems unlikely that there will be two sets of such parameters providing perfect "noncalcemic" and "calcemic" analogs, as is presented in Section III.A, but rather several different permutations of the same parameters giving rise to analogs with slightly different applications.

C. Implications for Future Drug Design

A case for the importance of metabolism within vitamin D analog action has been presented throughout this chapter. Much has been learned about those modifications to the vitamin D molecule, which change metabolism but in the process also improve biological activity. For some applications of vitamin D, this involves the concept of making "metabolism-resistant analogs" (e.g. those blocked in the C-23, C-24, C-26, or C-27 positions), which possess enhanced calcemic activity. For other applications of vitamin D, this involves the concept of making "metabolism-sensitive analogs" (e.g. those with oxa-groups at key side chain positions or a C22=C23 juxtaposed with a 24-hydroxyl) to localize the biological effect to the site of analog administration.

Over the immediate future we can anticipate:

1) A search for additional novel synthetic modifications to the vitamin D side chain and ring structure;
2) Continuation of the trend to combine proven modifications in order to synthesize newer generations of so-called "hybrid analogs";
3) Synthesis of "smart" molecules where metabolic and structure activity information gained from earlier generations of molecules is used to improve existing analogs.
4) A search for novel chemical entities included "nonsteroidal" vitamin D analogs that mimic certain actions of the vitamin D molecule (see Chapter 88).
5) Development of CYP24 inhibitors which mimic the action of 1,25-$(OH)_2D_3$ by blocking its catabolism [15,122].

One can envision that the VDR binding pocket studies [123,124] and cytochrome P450-substrate binding pocket studies [128] will provide particularly valuable information for the design of further generations of vitamin D analogs. The reader is referred to Chapter 15 for the latest information on VDR structure and modeling. The final section of this chapter outlines our

progress in the area of modeling of vitamin D related-cytochrome P450 isoforms.

D. Cytochrome P450 Isoform Modeling Studies

The cytochrome P450 superfamily constitute a group of over 100 proteins subdivided into microsomal and mitochondrial isoforms which are responsible for the metabolism (e.g. hydroxylation) of endogenous and exogenous (xenobiotic) compounds [125]. Their structure is well conserved across the superfamily with domains for heme-binding, ferredoxin-binding, O_2-binding, and substrate binding. These proteins are membrane-associated and are thus not easily studied by X-ray crystallographic means. For several mammalian steroidal cytochrome P450 isoforms (e.g. aromatase, cholesterol side-chain cleavage enzyme, 17-hydroxylase, rat 2B1, human 2D6) modeling studies (e.g. 126,127) have begun based upon information derived from crystal structures of soluble prokaryotic cytochromes P450 (CAM, BM-3, TERP, EryF). Thus, for the mammalian cytochromes P450, this work is in its infancy. Nevertheless, the approach appears highly promising. Such models, despite being crude first approximations, allow for identification of putative active-site residues suitable for site-directed mutagenesis studies. Refinements of the model derived from mutant proteins then follow.

In the case of CYP27A [128], CYP27B, and CYP24 [129] such an approach can now be undertaken not only using the information derived from the primary amino-acid sequence [130] and other modeled cytochrome P450s, but also using the information derived from natural mutations of CYP27A and CYP27B resulting in the human diseases, cerebrotendinous xanthomatosis and vitamin D–dependency rickets [131,132] (see Chapter 71). Note no human disease has currently been recognized involving mutations of CYP24. Modeling of the CYP proteins, substrate preferences (e.g. Ref 5) and mutated analyses of the CYPS should permit a much clearer picture of the analog/cytochrome P450 interactions occurring *in vivo.* It is expected that knowledge generated using this approach will eventually result in more rational vitamin D analog design in the future [133].

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References

1. DeLuca HF 1988 The vitamin D story: A collaborative effort of basic science and clinical medicine. FASEB J **2**:224–236.
2. Bouillon R, Okamura WH, Norman AW 1995 Structure-function relationships in the vitamin D endocrine system. Endocr Rev **16**:200–257.
3. Calverley MJ, Jones G 1992 Vitamin D. In: RT Blickenstaff (ed) Antitumor Steroids. Academic Press: San Diego, pp. 193–270.
4. Okuda KI, Usui E, Ohyama Y 1995 Recent progress in enzymology and molecular biology of enzymes involved in vitamin D metabolism. J Lipid Res **36**:1641–1652.
5. Guo Y-D, Strugnell S, Back DW, Jones G 1993 Transfected human liver cytochrome P-450 hydroxylates vitamin D analogs at different side-chain positions. Proc Natl Acad Sci USA **90**:8668–8672.
6. Jones G, Strugnell S, DeLuca HF 1998 Current understanding of the molecular actions of vitamin D. Physiological Reviews **78**:1193–1231.

6a. Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW 2003. De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxylase. J Biol Chem **278**:38084–38093.

7. Adams JS, Gacad MA 1985 Characterization of 1α-hydroxylation of vitamin D_3 sterols by cultured alveolar macrophages from patients with sarcoidosis. J Exp Med **161**:755–765.
8. Ohyama Y, Noshiro M, Okuda K 1991 Cloning and expression of cDNA encoding 25-hydroxyvitamin D_3 24-hydroxylase. FEBS Lett **278**:195–198.
9. Makin G, Lohnes D, Byford V, Ray R, Jones G 1989 Target cell metabolism of 1,25-dihydroxyvitamin D_3 to calcitroic acid. Evidence for a pathway in kidney and bone involving 24-oxidation. Biochem J **262**:173–180.
10. Reddy GS, Tserng K-Y 1989 Calcitroic acid, end product of renal metabolism of 1,25-dihydroxyvitamin D_3 through C-24 oxidation pathway. Biochemistry **28**:1763–1769.
11. St-Arnaud R, Arabian A, Travers R, Barletta F, Raval-Pandya M, Chapin K, Depovere J, Mathieu C, Christakos S, Demay MB, Glorieux FH 2000 Deficient mineralization of intramembranous bone in vitamin D-24-hydroxylase-ablated mice is due to elevated 1,25-dihydroxyvitamin D and not to the absence of 24,25-dihydroxyvitamin D. Endocrinology **141**:2658–2666.
12. Jones G, Byford V, Arabian A, St-Arnaud R 2000. Altered Pharmacokinetics of 1α,25-$(OH)_2D_3$ in Blood and Tissues of the CYP24-null Mouse. J Bone Miner Res **15**: abstract 1246, S199.

13. Akiyoshi-Shibata M, Sakaki T, Ohyama Y, Noshiro M, Okuda K, Yabusaki Y 1994 Further oxidation of hydroxycalcidiol by calcidiol 24-hydroxylase—A study with the mature enzyme expressed in *Escherichia coli.* Eur J Biochem **224**:335–343.
14. Beckman M, Prahl JM, DeLuca HF 1995 Side-chain metabolism of [26,27-^{3}H]-25-hydroxycholecalciferol (25-OH-D_3) by recombinant human 24-hydroxylase (p450cc24) expressed in baculovirus infected *Spodoptera frugiperda* (SF21) insect cells. J Bone Miner Res **10**:S395 (Abstract M573).
15. Masuda S, Gao M, Zhang A, Kaufmann, Jones G 2003 Importance of Cytochrome P450-mediated metabolism in the mechanism of action of vitamin D analogs. In: J Reichrath, M Friedrich, W Tilgen (eds) Vitamin D Analogs in Cancer Prevention and Therapy. Recent Results in Cancer Research Volume 164. Springer-Verlag: Germany, pp. 189–202.
16. Shinki T, Jin CH, Nishimuar A, Nagai Y, Ohyama Y, Noshiro M, Okuda K, Suda T 1992 Parathyroid hormone inhibits 25-hydroxyvitamin D_3-24-hydroxylase mRNA expression stimulated by 1α,25-dihydroxyvitamin D_3 in rat kidney but not in intestine. J Biol Chem **267**:13757–13762.
17. Tomon M, Tenenhouse HS, Jones G 1990 Expression of 25-hydroxyvitamin D_3-24-hydroxylase activity in CaCo-2 cells. An *in vitro* model of intestinal vitamin D catabolism. Endocrinology **126**:2868–2875.
18. Lohnes D, Jones G 1987 Side-chain metabolism of vitamin D in osteosarcoma cell line UMR-106: Characterization of products. J Biol Chem **262**:14394–14401.
19. Masuda S, Strugnell S, Calverley MJ, Makin HLJ, Kremer R, Jones G 1994 *In vitro* metabolism of the antipsoriatic vitamin D analog, calcipotriol, in two cultured human keratinocyte models. J Biol Chem **269**:4794–4803.
20. Yamada S, Nakayama K, Takayama H, Shinki T, Takasaki Y, Suda T 1984 Isolation, identification, and metabolism of (23S,25R)-25-hydroxyvitamin D_3-26,23-lactol: A biosynthetic precursor of (23S,25R)-25-hydroxyvitamin D_3-26,23-lactone. J Biol Chem **259**:884–889.
21. Sakaki T, Sawada N, Komai K, Shiozawa S, Yamada S, Yamamoto K, Ohyama Y, Inouye K 2000. Dual metabolic pathway of 25-hydroxyvitamin D_3 catalyzed by human CYP24. Eur J Biochem **267**:6158–6165
22. Horst RL, Wovkulich PM, Baggiolini EG, Uskokovic MR, Engstrom GW, Napoli JL 1984 (23S)-l,23,35-Trihydroxyvitamin D_3: Its biological activity and role in 1α,25-dihydroxyvitamin D_3-26,23-lactone biosynthesis. Biochemistry **23**: 3973–3976.
23. Jones G, Lohnes D, Strugnell S, Guo Y-D, Masuda S, Byford V, Makin HLJ, Calverley MJ 1994 Target Cell Metabolism of Vitamin D and Its Analogs. In: AW Norman, R Bouillon, M Thomasset (eds) Vitamin D. A Pluripotent Steroid Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications, de Gruyter: Berlin, pp. 161–169.
24. Bligh EG, Dyer WJ 1957 A rapid method of total lipid extraction and purification. Can J Biochem **37**:911–917.
25. Brown AJ, Berkoben M, Ritter C, Kubodera N, Nishii Y, Slatopolsky E 1992 Metabolism of 22-oxacalcitriol by a vitamin D–inducible pathway in cultured parathyroid cells. Biochem Biophys Res Commun **189**:759–764.
26. Masuda S, Byford V, Kremer R, Makin HLJ, Kubodera N, Nishii Y, Okazaki A, Okano T, Kobayashi T, Jones G 1996 *In vitro* metabolism of the vitamin D analog, 22-oxacalcitriol, using cultured osteosarcoma, hepatoma, and keratinocyte cell lines. J Biol Chem **271**:8700–8708.
27. Jones G 1986 Elucidation of a new pathway of 25-hydroxyvitamin D_3 metabolism. Meth Enzymol **123**:141–154.
28. Sorensen H, Binderup L, Calverley MJ, Hoffmeyer L, Rastrup Anderson N 1990. *In vitro* metabolism of calcipotriol (MC903), a vitamin D analog. Biochem Pharmacol **39**:391–393.
29. Suda T, Hallick RB, DeLuca HF, Schnoes HK 1970 25-Hydroxydihydrotachysterol$_3$ synthesis and biological activity. Biochemistry **9**:1651–1657.
30. Bosch R, Versluis C, Terlouw JK, Thijssen JHH, Duursma SA 1985 Isolation and identification of 25-hydroxydihydrotachysterol$_2$, 1α,25-dihydroxydihydrotachysterol$_2$ and lβ,25-dihydroxydihydrotachysterol$_2$. J Steroid Biochem **23**:223–229.
31. Jones G, Edwards N, Vriezen D, Porteous C, Tafford DJH, Cunningham J, Makin HLJ 1988 Isolation and identification of seven metabolites of 25-hydroxydihydrotachysterol$_3$ formed in the isolated perfused rat kidney: A model for the study of side-chain metabolism of vitamin D. Biochemistry **27**:7070–7079.
32. Qaw F, Calverley MJ, Schroeder NJ, Trafford DJH, Makin HLJ, Jones G 1993 *In vivo* metabolism of the vitamin D analog, dihydrotachysterol. Evidence for formation of 1α,25- and 1β,25-dihydroxydihydrotachysterol metabolites and studies of their biological activity. J Biol Chem **268**:282–292.
33. Bhattacharyya MH, DeLuca HF 1973 Comparative studies on the 25-hydroxylation of vitamin D_3 and dihydrotachysterol$_3$. J Biol Chem **248**:2974–2977.
34. Wing RM, Okamura WH, Pirio MP, Sine SM, Norman AW 1974 Vitamin D in solution: Conformations of vitamin D_3, 1,25-dihydroxyvitamin D_3 and dihydrotachysterol$_3$. Science **186**:939–941.
35. Qaw F, Schroeder NJ, Calverley MJ, Maestro M, Mourino A, Trafford DJH, Makin HLJ, Jones G 1992 *In vitro* synthesis of 1,25-dihydroxydihydrotachysterol in the myelomonocytic cell line, HD-11. J Bone Miner Res **7**:S161 (Abstract 274).
36. Shany S, Ren S-Y, Arbelle JE, Clemens TL, Adams JS 1993 Subcellular localization and partial purification of the 25-hydroxyvitamin D-1-hydroxylation reaction in the avian myelo-monocytic cell line HD-11. J Bone Miner **8**:269–276.
37. Qaw FS, Makin HLJ, Jones G 1992 Metabolism of 25-hydroxy-dihydrotachysterol$_3$ in bone cells *in vitro*. Steroids **57**:236–243.
38. Schroeder NJ, Qaw F, Calverley MJ, Trafford DJH, Jones G, Makin HLJ 1992 Polar metabolites of dihydrotachysterol$_3$ in the rat: Comparison with *in vitro* metabolites of 1α,25-dihydroxydihydrotachysterol$_3$. Biochem Pharmacol **43**: 1893–1905.
39. Suda T, DeLuca HF, Schnoes HK, Blunt JW 1969 Isolation and identification of 25-hydroxyergocalciferol. Biochemistry **8**:3515–3520.
40. Jones G, Schnoes HK, DeLuca HF 1975 Isolation and identification of 1,25-dihydroxyvitamin D_2. Biochemistry **14**:1250–1256.
41. Jones G, Rosenthal A, Segev D, Mazur Y, Frolow F, Halfon Y, Rabinovich D, Shakked Z 1979 Isolation and identification of 24,25-dihydroxyvitamin D_2 using the perfused rat kidney. Biochemistry **18**:1094–1101.
42. Jones G, Schnoes HK, Levan L, DeLuca HF 1980 Isolation and identification of 24-hydroxyvitamin D_2 and 24,25-dihydroxyvitamin D_2. Arch Biochem Biophys **202**:450–457.
43. Horst RL, Koszewski NJ, Reinhardt TA 1990 1a-Hydroxylation of 24-hydroxyvitamin D_2 represents a minor physiological pathway for the activation of vitamin D_2 in mammals. Biochemistry **29**:578–582.

44. Koszewski NJ, Reinhardt TA, Napoli JL, Beitz DC, Horst RL 1988 24,26-Dihydroxyvitamin D_2: A unique physiological metabolite of vitamin D_2. Biochemistry **27**:5785–5790.
45. Reddy GS, Tserng K-Y 1986 Isolation and identification of 1,24,25-trihydroxyvitamin D_2, 1,24,25,28-tetrahydroxyvitamin D_2, and 1,24,25,26-tetrahydroxyvitamin D_2: New metabolites of 1,25-dihydroxyvitamin D_2 produced in the rat kidney. Biochemistry **25**:5328–5336.
46. Lam HY, Schnoes HK, DeLuca HF 1974 1α-Hydroxyvitamin D_2: A potent synthetic analog of vitamin D_2. Science **186**:1038–1040.
47. Strugnell S, Byford V, Makin HLJ, Moriarty RM, Gilardi R, LeVan LW, Knutson JC, Bishop CW, Jones G 1995 1α,24(S)-dihydroxyvitamin D_2: A biologically active product of 1α-hydroxyvitamin D_2 made in the human hepatoma, Hep3B. Biochem J **310**:233–241.
48. Jones G, Byford V, Kremer R, Makin HLJ, Rice RH, deGraffenreid LA, Knutson JC, Bishop CA 1996 Antiproliferative activity and target cell catabolism of the vitamin D analog, 1α,24(S)-dihydroxyvitamin D_2 in normal and immortalized human epidermal cells. Biochem Pharmacol **52**:133–140.
49. Sjoden G, Smith C, Lindgren V, DeLuca HF 1985 1α-hydroxyvitamin D_2 is less toxic than 1α-hydroxyvitamin D_3 in the rat. Proc Soc Exp Biol Med **178**:432–436.
50. Horst RL, Koszewski NJ, Reinhardt TA 1988 Species variation of vitamin D metabolism and action: Lessons to be learned from farm animals. In: AW Norman, K Schaefer, H-G Grigoleit, D von Herrath (eds) Vitamin D: Molecular, Cellular, and Clinical Endocrinology, de Gruyter: Berlin, pp. 93–101.
51. Clark JW, Reddy GS, Santos-Moore A, Wankadiya KF, Reddy GP, Lasky S, Tserng K-Y, Uskokovic MR 1993 Metabolism and biological activity of 1,25-dihydroxyvitamin D_2 and its metabolites in a chronic myelogenous leukemia cell line, RWLEU-4. Bioorg Med Lett **3**:1873–1878.
52. Shankar VN, Propp AE, Schroeder NS, Surber BW, Makin HLJ, Jones G 2001 *In vitro* metabolism of 19-nor-1α,25-$(OH)_2D_2$ in cultured cell lines: inducible synthesis of lipid- and water-soluble metabolites. Arch Biochem Biophys **387**:297–306.

52a. Jones G, Byford V, Kremer R, Makin HLJ, Knutson JC, Bishop CW 1994. Novel cleavage of vitamin D_2 side chain during catabolism by keratinocyte cell line. Ninth Workshop on Vitamin D, Orlando, Florida, June 1994, abstract.

53. Zimmerman DR, Reinhardt TA, Kremer R, Beitz DC, Reddy GS, Horst RL 2001 Calcitroic acid is a major catabolic metabolite in the metabolism of 1α-dihydroxyvitamin D2. Arch Biochem Biophys **392**:14–22.
54. Horst RL, Omdahl JA, Reddy S 2003 Rat cytochrome P450C24 (CYP24) does not metabolize 1,25-dihydroxyvitamin D_2 to calcitroic acid. J Cell Biochem **88**:282–285.
55. Masuda S, Arabian A, McCaig J, Kaufmann M, Strugnell SA, Knutson JC, St-Arnaud R, Jones G 2002 CYP24-Null keratinocytes demonstrate that CYP24 is responsible for activation and inactivation of 1α$(OH)D_2$. J Bone Mineral Research **17**:S396, abstract SU459.
56. Byford V, Strugnell S, Coldwell R, Schroeder N, Makin HLJ, Knutson JC, Bishop CW, Jones G 2002. Use of vitamin D_4 analogs to investigate differences in hepatic and target cell metabolism of vitamins D_2 and D_3. Biochim Biophys Acta **1583**:151–166.
57. Mehta RG, Hussain EA, Mehta RR, Das Gupta TK 2003 Chemoprevention of mammary carcinogenesis by 1α-hydroxyvitamin D5, a synthetic analog of vitamin D. Mutat Res **523–524**:253–264.
58. Strugnell S, Calverley MJ, Jones G 1990 Metabolism of a cyclo-propane-ring-containing analog of 1α-hydroxyvitamin D_3 in a hepatocyte cell model: Identification of 24-oxidized metabolites. Biochem Pharmacol **40**:333–341.
59. Calverley MJ 1987 Synthesis of MC903, a biologically active vitamin D metabolite analog. Tetrahedron **43**:4609–4619.
60. Jones G, Calverley MJ 1993 A dialogue on analogs: Newer vitamin D drugs for use in bone disease, psoriasis, and cancer. Trends Endocrinol Metab **4**:297–303.
61. Binderup L 1988 MC903 A novel vitamin D analog with potent effects on cell proliferation and cell differentiation. In: AW Norman, K Schaefer, H-G Grigoleit, D von Herrath (eds) Vitamin D: Molecular: Cellular and Clinical Endocrinology de Gruyter: Berlin, pp. 300–309.
62. Calverley MJ, Binderup E, Binderup L 1991 The 20-epi modification in the vitamin D series: Selective enhancement of "nonclassical" receptor-mediated effects. In: AW Norman, R Bouillon, M Thomasset (eds) Vitamin D: Gene Regulation, Structure-Function Analysis and Clinical Application, de Gruyter: Berlin, pp. 163–164.
63. Binderup L, Latini S, Binderup E, Bretting C, Calverley M, Hansen K 1991 20-Epi-vitamin D_3 analogs: A novel class of potent regulators of cell growth and immune responses. Biochem Pharmacol **42**:1569–1575.
64. Shevde NK, Plum LA, Clagett-Dame M, Yamamoto H, Pike JW, DeLuca HF 2002 A potent analog of 1α,25-dihydroxyvitamin D_3 selectively induces bone formation**.** Proc Natl Acad Sci USA **99**:13487–13491.
65. Dilworth FJ, Calverley MJ, Makin HLJ, Jones G 1994 Increased biological activity of 20-epi-l,25-dihydroxyvitamin D_3 is due to reduced catabolism and altered protein binding. Biochem Pharmacol **47**:987–993.
66. Siu-Caldera ML, Sekimoto H, Peleg S, Nguyen C, Kissmeyer AM, Binderup L, Weiskopf A, Vouros P, Uskokovic MR, Reddy GS 1999 Enhanced biological activity of 1α,25-dihydroxy-20-epi-vitamin D_3, the C-20 epimer of 1α,25-dihydroxyvitamin D_3, is in part due to its metabolism into stable intermediary metabolites with significant biological activity. J Steroid Biochem Mol Biol **71**:111–121.
67. Peleg S, Sastry M, Collins ED, Bishop JE, Norman AW 1995 Distinct conformational changes induced by 20-epi analogs of 1α,25-dihydroxyvitamin D_3 are associated with enhanced activation of the vitamin D receptor. J Biol Chem **270**:10551–10558.
68. Shankar VN, Byford V, Prosser DE, Schroeder NJ, Makin HLJ, Wiesinger H, Neef G, Steinmeyer A, Jones G 2001 Metabolism of a 20-methyl substituted series of vitamin D analogs by cultured human cells: Apparent reduction of 23-hydroxylation of the side chain by 20-methyl group. Biochem Pharmacol **61**:893–902.
69. Danielsson C, Nayeri S, Wiesinger H, Thieroff-Ekerdt R, Carlberg C 1996. Potent gene regulatory and antiproliferative activities of 20-methyl analogs of 1,25 dihydroxyvitamin D_3. J Cell Biochem **63**:199–206.
70. Ostrem VK, Lau WF, Lee SH, Perlman K, Prahl J, Schnoes HK, DeLuca HF, Ikekawa N 1987 Induction of monocytic differentiation of HL-60 cells by 1,25-dihydroxyvitamin D analogs. J Biol Chem **262**:14164–14171.
71. Ostrem VK, Tanaka V, Prahl J, DeLuca HF, Ikekawa N 1987 24- and 26-homo-l,25-dihydroxyvitamin D_3: Preferential activity in inducing differentiation of human leukemic cells HL-60 *in vitro.* Proc Natl Acad Sci USA **84**:2610–2614.

72. Perlman K, Kutner A, Prahl J, Smith C, Inaba M, Schnoes HK, DeLuca HF 1990 24-homologated 1,25-dihydroxyvitamin D_3 compounds: Separation of calcium and cell differentiation activities. Biochemistry **29**:190–196.
73. Paulson SK, Perlman K, DeLuca H, Stern PH 1990 24- and 26-homo-l,25-dihydroxyvitamin D_3 analogs: Potencies on *in vitro* bone resorption differ from those reported for cell differentiation. J Bone Miner Res **5**:201–206.
74. Dilworth FJ, Scott I, Green A, Strugnell S, Guo Y-D, Roberts EA, Kremer R, Calverley MJ, Makin HLJ, Jones G 1995a Different mechanisms of hydroxylation site selection by liver and kidney cytochrome P450 species (CYP27 and CYP24) involved in vitamin D metabolism. J Biol Chem **270**:16766–16774.
75. Dilworth FJ, Scott I, Calverley MJ, Makin HLJ, Jones G 1995b Enzymes of side chain oxidation pathway not affected by addition of methyl groups to end of the vitamin D_3 side chain. J Bone Miner Res **10**:S388 (Abstract M546).
76. Binderup L, Carlberg C, Kissmeyer AM, Latini S, Mathiasen IS, Mork-Hansen C 1993 The need for new vitamin D analogs: Mechanisms of action and clinical applications. In: AW Norman, R Bouillon, M Thomasset (eds) Vitamin D. A Pluripotent Steroid Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications, de Gruyter: Berlin, pp. 55–63.
77. Zhou J-Y, Norman AW, Chen D-L, Sun G, Uskokovic M, Koeffler HP 1990 l,25-Dihydroxy-16-ene-23-yne-vitamin D_3 prolongs survival time of leukemic mice. Proc Natl Acad Sci USA **87**:3929–3932.
78. Binderup E, Calverley MJ, Binderup L 1991 Synthesis and biological activity of 1a-hydroxylated vitamin D analogues with poly-unsaturated side chains. In: AW Norman, R Bouillon, M Thomasset (eds) Vitamin D: Gene Regulation, Structure-Function Analysis and Clinical Application, de Gruyter: Berlin, pp. 192–193.
79. Colston KW, Mackay AG, James SY, Binderup L, Chandler S, Coombes RC 1992 EB1089: A new vitamin D analog that inhibits the growth of breast cancer cells *in vivo* and *in vitro.* Biochem Pharmacol **44**:2273–2280.
80. James SY, Mackay AG, Binderup L, Colston KW 1994 Effects of a new synthetic analog, EB1089, on the estrogen-responsive growth of human breast cancer cells. J Endocrinol **141**:555–563.
81. Reddy GS, Clark JW, Tserng K-Y, Uskokovic MR, McLane JA 1993 Metabolism of l,25-$(OH)_2$-16-ene D_3 in kidney: Influence of structural modification of D-ring on side chain metabolism. Bioorg Med Lett **3**:1879–1884.
82. Satchell DP, Norman AW 1996 Metabolism of the cell-differentiating agent l,25-$(OH)_2$-16-ene-23-yne vitamin D_3 by leukemic cells. J Steroid Biochem Mol Biol **57**:117–124.
83. Shankar VN, Makin HLJ, Schroeder NJ, Trafford DJH, Kissmeyer A-M, Calverley MJ, Binderup E, Jones G 1995 Metabolism of the antiproliferative vitamin D analog, EB1089, in a cultured human keratinocyte model. Bone **17**:326 (abstract).
84. Kissmeyer A-M, Mathiasen IS, Latini S, Binderup L 1995 Pharmacokinetic studies of vitamin D analogs: Relationship to vitamin D–binding protein (DBP). Endocrine **3**:263–266.
85. Shankar VN, Dilworth FJ, Makin HIJ, Schroeder NJ, Trafford DAJ, Kissmeyer A-M, Calverley MJ, Binderup E, Jones G 1997 Metabolism of the vitamin D analog EB1089 by cultured human cells: Redirection of hydroxylation site to distal carbons of the side chain. Biochem Pharmacol **53**: 783–793.
86. Kissmeyer A-M, Binderup E, Binderup L, Hansen CM, Andersen NR, Schroeder NJ, Makin HLJ, Shankar VN, Jones G 1997 The metabolism of the vitamin D analog EB1089: Identification of *in vivo* and *in vitro* metabolites and their biological activities. Biochem Pharmacol **53**:1087–1097.
87. Wankadiya KF, Uskokovic MR, Clark J, Tserng K-Y, Reddy GS 1992 Novel evidence for the reduction of the double bond in Δ^{22}-l,25-dihydroxyvitamin D_3. J Bone Miner Res **7**:S171 (Abstract 315).
88. Murayama E, Miyamoto K, Kubodera N, Mori T, Matsunaga I 1986 Synthetic studies of vitamin D analogs. VIII. Synthesis of 22-oxavitamin D_3 analogs. Chem Pharm Bull (Tokyo) **34**:4410–4413.
89. Hansen K, Calverley MJ, Binderup L 1991 Synthesis and biological activity of 22-oxavitamin D analogs. In: AW Norman, R Bouillon, M Thomasset (eds) Vitamin D: Gene Regulation, Structure-Function Analysis and Clinical Application, de Gruyter: Berlin, pp. 161–162.
90. Kubodera N, Miyamoto K, Akiyama M, Matsumoto M, Mori T 1991 Synthetic studies of vitamin D analogs. 9. Synthesis and differentiation-inducing activity of 1α,25-dihydroxy-23-oxa-vitamin D_3, thia-vitamin D_3 and aza-vitamin D_3. Chem Pharm Bull (Tokyo) **39**:3221–3224.
91. Baggiolini EG, Zacobelli JA, Hennessy BM, Batcho AD, Sereno JF, Uskokovic MR 1986 Stereocontrolled total synthesis of 1α,25-dihydroxycholecalciferol and 1α,25-dihydroxyergocalciferol. J Org Chem **51**:3098–3108.
92. Allewaert K, Van Baelen H, Bouillon R, Zhao X-Y, De Clercq P, Vanderwalle M 1993 Synthesis and biological evaluation of 23-oxa-, 23-thia- and 23-oxa-24-oxo-1α, 25-dihydroxyvitamin D_3. Bioorg Med Lett **3**:1859–1862.
93. Calverley MJ, Strugnell S, Jones G 1993 The seleno-acetal route to 1α-hydroxy-vitamin D analogs: Synthesis of 24-oxa-1α-hydroxyvitamin D_3, a useful vitamin D metabolism probe. Tetrahedron **49**:739–746.
94. Sarandeses LA, Valles MJ, Castedo L, Mourino A 1993 Synthesis of 24-oxa-vitamin D_3 and 1α-hydroxy-24-oxa-vitamin D_3. Tetrahedron **49**:731–738.
95. Bikle DD, Abe-Hashimoto J, Su MJ, Felt S, Gibson DFC, Pillai S 1995 22-Oxa calcitriol is a less potent regulator of keratinocyte proliferation and differentiation due to decreased cellular uptake and enhanced catabolism. J Invest Dermatol **105**:693–698.
96. Kobayashi T, Tsugawa N, Okano T, Masuda S, Takeuchi A, Kubodera N, Nishii Y 1994 The binding properties with blood proteins and tissue distribution of 22-oxa-1α,25-dihydroxyvitamin D_3, a noncalcemic analog of 1α,25-dihydroxyvitamin D_3 in rats. J Biochem (Tokyo) **115**:373–380.
97. Jones G, Byford V, Moore B, Logsted-Nielsen J, Calverley MJ, Hansen K, Kissmeyer A-M 2000. A novel aromatic vitamin D analog is rapidly metabolized in cultured cell lines in vitro. J Bone Mineral Research **15**: Abstract M507, S576.
98. Dilworth FJ, Calverley MJ, Kissmeyer A-M, Binderup E, Makin HLJ, Jones G 1996 KH1060, a potent vitamin D analog, is degraded in cultured keratinocytes via several different pathways. Bone Miner Res **11**:S424 (abstract T500).
99. Rastrup Anderson N, Buchwald FA, Grue-Sorensen G 1992 Identification and synthesis of a metabolite of KH1060, a new potent 1α,25-dihydroxyvitamin D_3 analog. Bioorg Med Chem Lett **2**:1713–1716.
100. Van Den Bemd G, J-C M, Dilworth FJ, Makin HLJ, Prahl JM, DeLuca HF, Jones G, Pols HAP, Van Leeuwen JPTM 2000 Contribution of several metabolites of the vitamin D analog 20-epi-22-oxa-24a,26a,27a-trihomo-1,

25-$(OH)_2$vitamin D_3 (KH1060) to the overall biological activity of KH1060 by a shared mechanism of action. Biochem Pharmacol **59**:621–27
101. Reddy GS, Ishizuka S, Wandkadiya KF, Tserng K-Y, Yeung B, Vouros P 1992 Metabolism of 1α,24(R)-dihydroxyvitamin D_3 into calcitroic acid in rat kidney through two different metabolic pathways. J Bone Miner Res **7**:S170 (abstract 312).
102. Tucker G, Gagnon RE, Haussler MR 1973 Vitamin D_3-25-hydroxylase: Tissue occurrence and lack of regulation. Arch Biochem Biophys **155**:47–57.
103. Axen E, Postlind H, Wikvall K 1995 Effects of CYP27 mRNA expression in rat kidney and liver by 1α,25-dihydroxyvitamin D_3, a suppressor of renal 25-hydroxyvitamin D_3-1α-hydroxylase activity. Biochem Biophys Res Commun **215**:136–141.
104. Weinstein EA, Siu-Caldera M-L, Ishizuka S, Reddy GS 1995 Evidence of 25-hydroxylation in the rat kidney for 1α,24*(R)*-dihydroxyvitamin D_3 only but not for vitamin D_3 or 1α-hydroxyvitamin D_3. J Bone Miner Res **10**:S497 (abstract T570).
105. Nishii Y, Sato K, Kobayashi T 1993 The development of vitamin D analogs for the treatment of osteoporosis. Osteoporosis Int **1** (Suppl):S190–193.
106. Masuda S, Makin HLJ, Kremer R, Okano T, Kobayashi T, Sato K, Nishii Y, Jones G 1994 Metabolism of 2β-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3 (ED-71) in cultured cell lines. J Bone Miner Res **9**:S289 (abstract B238).
107. Kobayashi Y, Taguchi T, Mitsuhashi S, Eguchi T, Ohshima E, Ikekawa N 1982 Studies on organic fluorine compounds. XXXIX. Studies on steroids. LXXIX. Synthesis of 1α,25-dihydroxy-26,26,26,27,27,27-hexafluorovitamin D_3. Chem Pharm Bull (Tokyo) **30**:4297–4303.
108. Koeffler HP, Armatruda T, Ikekawa N, Kobayashi Y, DeLuca HF 1984 Induction of macrophage differentiation of human normal and leukemic myeloid stem cells by 1α,25-dihydroxyvitamin D_3 and its fluorinated analogs. Cancer Res **44**:6524–6528.
109. Inaba M, Okuno S, Nishizawa Y, Yukioka K, Otani S, Matsui-Yuasa I, Morisawa S, DeLuca HF, Morii H 1987 Biological activity of fluorinated vitamin D analogs at C-26 and C-27 on human promyelocytic leukemia cells, HL-60. Arch Biochem Biophys **258**:421–425.
110. Kistler A, Galli B, Horst R, Truitt GA, Uskokovic MR 1989 Effects of vitamin D derivatives on soft tissue calcification in neonatal and calcium mobilization in adult rats. Arch Toxicol **63**:394–400.
111. Lohnes D, Jones G 1992 Further metabolism of 1α,25-dihydroxyvitamin D_3 in target cells. J Nutr Sci Vitaminol (Tokyo) Special Issue: 75–78.
112. Inaba M, Okuno S, Nishizawa Y, Imanishi Y, Katsumata T, Sugata I, Morii H 1993 Effect of substituting fluorine for hydrogen at C-26 and C-27 on the side chain of 1α,25-dihydroxyvitamin D_3. Biochem Pharmacol **45**:2331–2336.
113. Nakatsuka K, Imanishi Y, Morishima Y, Sekiya K, Sasao K, Miki T, Nishizawa Y, Katsumata T, Nagata A, Murakawa S 1992 Biological potency of a fluorinated vitamin D analog in hypoparathyroidism. Bone Miner **16**:73–81.
114. Nishizawa Y, Morii H, Ogura Y, DeLuca HF 1991 Clinical trial of 26,26,26,27,27,27-hexafluoro-1α,25-dihydroxyvitamin D_3 in uremic patients on hemodialysis: Preliminary report. Contrib Nephrol **90**:196–203.
115. Posner GH, Crawford K, Siu-Caldera ML, Reddy GS, Sarabia SF, Feldman D, van Etten E, Mathieu C, Gennaro L, Vouros P, Peleg S, Dolan PM, Kensler TW 2000 Conceptually new 20-epi-22-oxa sulfone analogs of the hormone 1α,25-dihydroxyvitamin D_3: synthesis and biological evaluation. J Med Chem **43**:3581–3586.
116. Perlman KL, Sicinski RR, Schnoes HK, DeLuca HF 1990 1α,25-Dihydroxy-19-nor-vitamin D_3, a novel vitamin D–related compound with potential therapeutic activity. Tetrahedron Lett **31**:1823–1824.
117. Posner GH, Dai H 1993 l-(Hydroxyalkyl)-25-hydroxyvitamin D_3 analogs of calcitriol-I. Synthesis. Bioorg Med Chem Lett **2**:1829–1834.
118. Neef G, Kirsch G, Schwarz K, Wiesinger H, Menrad A, Fahnrich M, Thieroff-Ekerdt, Steinmeyer A 1994 20-Methyl vitamin D analogs. In: AW Norman, R Bouillon, M Thomasset (eds) Vitamin D. A Pluripotent Steroid Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications, de Gruyter: Berlin, pp. 97–98.
119. Bouillon R, Allewaert K, Xiang DZ, Tan BK, Van Baelen H 1991 Vitamin D analogs with low affinity for the vitamin D–binding protein: Enhanced *in vitro* and decreased *in vivo* activity. J Bone Miner Res **6**:1051–1057.
120. Dusso AS, Negrea L, Gunawardhana S, Lopez-Hilker S, Finch J, Mori T, Nishii Y, Slatopolsky E, Brown AJ 1991 On the mechanisms for the selective action of vitamin D analogs. Endocrinology **128**:1687–1692.
121. Cheskis B, Lemon BD, Uskokovic MR, Lomedico PT, Freedman LP 1995 Vitamin D_3-retinoid X receptor dimerization. DNA binding, and transactivation are differentially affected by analogs of 1,25-dihydroxyvitamin D_3. Mol Endocrinol **9**:1814–1824.
122. Schuster I, Egger H, Astecker N, Herzig G, Schussler M, Vorisek G 2001 Selective inhibitors of CYP24: mechanistic tools to explore vitamin D metabolism in human keratinocytes. Steroids **66**:451–462.
123. Rochel N, Wurtz JM, Mitschler A, Klaholz B, Moras D 2001. The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. Mol Cell **5**:173–179.
124. Tocchini-Valentini G, Rochel N, Wurtz JM, Mitschler A, Moras D 2001 Crystal structures of the vitamin D receptor complexed to superagonist 20-epi ligands. Proc Natl Acad Sci USA **98**:5491–5496.
125. Guengerich FP 1991 Reactions and significance of cytochrome P-450 enzymes. J Biol Chem **266**:10019–10022.
126. Graham-Lorence S, Amarneh B, White RE, Peterson JA, Simpson ER 1995 A three-dimensional model of the aromatase cytochrome P450. Protein Sci **4**:1065–1080.
127. Vijayakumar S, Salerno JC 1992 Molecular modeling of the 3-D structure of cytochrome $P\text{-}450_{SCC}$. Biochim Biophys Acta **1160**:281–286.
128. Prosser DE, Dakin KA, Donini OAT, Weaver DF, Jia Z, Jones G 1996 A three-dimensional model of the cytochrome P450, CYP27A and its vitamin D binding site. J Bone Miner Res **11**:S313 (abstract M525).
129. Omdahl JL, Bobrovnikova EV, Annalora A, Chen P, Serda R 2003 Expression, structure-function, and molecular modeling of vitamin D P450s. J Cell Biochem **88**(2): 356–362.
130. Cali JJ, Russell DW 1991 Characterization of human sterol 27-hydroxylase. A mitochondrial cytochrome P450 that catalyzes multiple oxidation reactions in bile acid biosynthesis. J Biol Chem **266**:7774–7778.
131. Cali JJ, Hsieh C-L, Francke V, Russell DW 1991 Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. J Biol Chem **266**:7779–7783.

132. St-Arnaud R, Messerlian S, Moir JM, Omdahl JL, Glorieux FH. 1997 The 25-hydroxyvitamin D 1α-hydroxylase gene maps to the pseudovitamin D–deficiency rickets (PDDR) disease locus. J Bone Miner Res **12**:1552–1559.
133. Schuster I, Egger H, Nussbaumer P, Kroemer RT 2003 Inhibitors of vitamin D hydroxylases: Structure-activity relationships. J Cell Biochem **88**:372–380.
134. Barton DH, Hesse RH, Pechet MM, Rizzardo E 1973 A convenient synthesis of 1α-hydroxy-vitamin D_3. J Am Chem Soc **95**:2748–2749.
135. Paaren HE, Hamer DE, Schnoes HK, DeLuca HF 1978 Direct C-l hydroxylation of vitamin D compounds: Convenient preparation of lα-hydroxyvitamin D_3, lα,25-dihydroxyvitamin D_3 and 1α-hydroxyvitamin D_2. Proc Natl Acad Sci USA **75**:2080–2081.
136. Fraser D, Kooh SW, Kind P, Holick MF, Tanaka Y, DeLuca HF 1973 Pathogenesis of hereditary vitamin D–dependency rickets. N Engl J Med **289**:817–822.
137. Baggiolini EG, Wovkulich PM, Iacobelli JA, Hennessy BM, Uskokovic MR 1982 Preparation of 1-alpha hydroxylated vitamin D metabolites by total synthesis. In: AW Norman, K Schaefer, D von Herrath, H-G Grigoleit (eds) Vitamin D: Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism, de Gruyter: Berlin, pp. 1089–1100.
138. Baggiolini EG, Partridge JJ, Shiuey S-J, Truitt GA, Uskokovic MR 1989 Cholecalciferol 23-yne derivatives, their pharmaceutical compositions, their use in the treatment of calcium-related diseases, and their antitumor activity, US 4,804,502 [abstract]. Chem Abstr 111:58160d.
139. Morisaki M, Koizumi N, Ikekawa N, Takeshita T, Ishimoto S 1975 Synthesis of active forms of vitamin D. Part IX. Synthesis of 1α,24-dihydroxycholecalciferol. J Chem Soc Perkin Trans **1**(1):1421–1424.
140. Fukushima M, Suzuki Y, Tohira Y, Nishii Y, Suzuki M, Sasaki S, Suda T 1976 25-Hydroxylation of lα-hydroxyvitamin D_3 *in vivo* and in the perfused rat liver. FEBS Lett **65**:211–214.
141. Rosenthal AM, Jones G, Kooh SW, Fraser D 1980 25-Hydroxyvitamin D_3 metabolism by the isolated perfused rat kidney. Am J Physiol (Endocrinol Metab) **239**:E12–E20.
142. Reddy GS, Jones G, Kooh SW, Fraser D, DeLuca HF 1983 Effects of metabolites and analogs of vitamin D_3 on 24(R),25-dihydroxyvitamin D_3 synthesis. Am J Physiol **235**:E359–E364.
143. Henry HL 1979 Regulation of the hydroxylation of 25-hydroxyvitamin D_3 *in vivo* and in primary cultures of chick kidney cells. J Biol Chem **254**:2722–2729.
144. Chandler JS, Chandler SK, Pike JW, Haussler MR 1984 1,25-Dihydroxyvitamin D_3 induces 25-hydroxyvitamin D_3-24-hydroxylase in a cultured monkey kidney cell line (LLC-MK2) apparently deficient in the high affinity receptor for the hormone. J Biol Chem **259**:2214–2222.
145. Vieth R, Fraser D 1979 Kinetic behavior of 25-hydroxyvitamin D-1-hydroxylase and -24-hydroxylase in rat kidney mitochondria. J Biol Chem **254**:12455–12460.
146. Knutson JC, LeVan LW, Valliere CR, Bishop CW 1997 Pharmacokinetics and systemic effect on calcium homeostasis of 1α,24-dihydroxyvitamin D_2 in rats: Comparison with lα,25-dihydroxyvitamin D_2, calcitriol and calcipotriol. Biochem Pharmacol **53**:829–837.
147. Jones G 2002 Part III Pharmacological mechanisms of therapeutics: Vitamin D and analogs. In: J Bilezikian, L Raisz, G Rodan (eds) Principles of Bone Biology, Second Edition. Academic Press: San Diego, pp. 1407–1422.
148. Masuda S, Jones G 2003.Vitamin D Analogs: Drug design based upon proteins involved in vitamin D signal transduction. J Current Drug Targets: Immune, Endocrine and Metabolic Disorders **3**:43–67.
149. Jones G, Ramshaw H, Zhang A, Cook R, Byford V, White J, Petkovich M 1999 Expression and activity of vitamin D–metabolizing cytochrome P450s (CYP1alpha and CYP24) in human non small cell lung carcinomas. Endocrinology **140**:3303–3310.