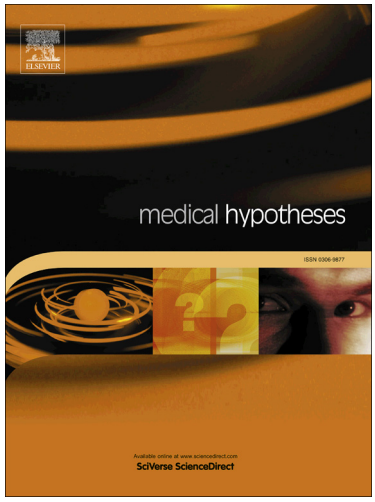
Accepted Manuscript



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PII: S0306-9877(17)31048-4
DOI: https://doi.org/10.1016/j.mehy.2017.12.017
Reference: YMEHY 8754

To appear in: *Medical Hypotheses*

Received Date: 10 October 2017
Accepted Date: 12 December 2017

Please cite this article as: R. Jorde, G. Grimnes, Serum cholecalciferol may be a better marker of vitamin D status than 25-hydroxyvitamin D, *Medical Hypotheses* (2017), doi: https://doi.org/10.1016/j.mehy.2017.12.017

Serum cholecalciferol may be a better marker of vitamin D status than 25-hydroxyvitamin D

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ABSTRACT

Vitamin D is produced in the skin upon sun-exposure or obtained through the diet. Vitamin D is hydroxylated to 25-hydroxyvitamin D (25(OH)D) in the liver and to the active form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) in the kidneys. To exert its effect $1,25(OH)_2D$ has to bind to the nuclear vitamin D receptor VDR. Lack of vitamin D leads to rickets in children and to osteomalacia in adults. 25(OH)D is used as a marker of a subject´s vitamin D status. Low serum 25(OH)D levels are associated with a number of diseases, risk factors for disease and increased mortality. However, intervention studies with vitamin D have generally been disappointing. Many, if not most cells have the hydroxylases necessary for intra-cellular activation of vitamin D. It is likely that more vitamin D diffuses or are transported into the cells than 25(OH)D and 1,25(OH)2D, and accordingly, most of the 1,25(OH)2D that bind to the VDR are derived from intra-cellular hydroxylation of vitamin D. Therefore, our hypothesis is that serum vitamin D is a better marker of a subject´s vitamin D status than 25(OH)D. Since the half-life in serum for vitamin D is approximately one day, giving vitamin D weekly or monthly will result in short-lived serum vitamin D peaks with periods of vitamin D deficiency in between. On the other hand, serum 25(OH)D, which has a half-life of weeks, will show high and stable serum levels throughout. Important vitamin D effects may have been missed in studies with intermittent dosing, and vitamin D in intervention trials should be given daily. Likewise, in epidemiological studies and clinical practice 25(OH)D has uniformly been used as marker. This may lead to gross misclassification of individuals that do not have a stable influx of vitamin D from sun-exposure or diet. In epidemiological studies serum vitamin D should be measured as well as 25(OH)D, and in clinical practice a 25(OH)D measurement should be interpreted in view of recent sun-exposure and diet history.

Introduction

Vitamin D is an ancient hormone essential for intestinal calcium absorption and therefore plays a central role in maintaining calcium homeostasis and skeletal integrity (1). The main source of vitamin D is solar UV-radiation, and except for fatty fish there are few dietary sources. Both dietary and solar vitamin D undergo a hydroxylation in the liver to 25-hydroxyvitamin D (25(OH)D) which traditionally has been considered the best biochemical marker of a subject´s vitamin D status (1). 25(OH)D serves as a substrate for 1-α-hydroxylase in the kidneys, which forms the active form of the vitamin, 1,25-dihydroxyvitamin D ($1,25(OH)_2$ D). This activation of vitamin D is strictly regulated by factors like parathyroid hormone (PTH) and FGF-23. While the first will increase $1,25(OH)_2$D production as a response to lower calcium levels, the latter is secreted as a response to increased $1,25(OH)_2$D levels (2). Thus, FGF-23 will inhibit vitamin D activation and rather stimulate the conversion from 25(OH)D to $24,25(OH)_2$D, which is an inactive metabolite ready to be excreted in the urine. The enzymes 25-hydroxylase and 1-α-hydroxylase have also been found in extra-renal tissues, and it is therefore assumed that activation of vitamin D can occur locally. The importance and regulation of this local activation is unknown (3).

In the circulation both 25(OH)D and $1,25(OH)_2$D are bound to plasma proteins, and less than 1% circulate in free form. The main binding protein is vitamin D binding protein (DBP), which accounts for more than 90 % of the vitamin D transportation, and a minor fraction binds to albumin (2). The active form of vitamin D binds to the nuclear vitamin D receptor (VDR). This receptor has been found not only in the intestines, but also in tissues throughout the body (3). It has recently been shown that the serum level of 25(OH)D is not only determined by intake of vitamin D and sun exposure, but also by body composition (4) and genetic factors (5).

Vitamin D deficiency in children may lead to rickets and in adults to osteomalacia and osteoporosis (6). There are also many indications for extra-skeletal effects of vitamin D, which are to be expected given the wide tissue distribution of the VDR and the vitamin D activating enzymes (3). This is supported by cross-sectional and longitudinal studies showing strong associations between vitamin D deficiency and cardiovascular disease, autoimmune diseases, infectious diseases, cancer and diabetes; as well as risk factors for these diseases (6). However, results from randomized controlled trials (RCTs) with vitamin D for prevention or treatment of these diseases/risk factors, have generally been inconclusive (7).

There could be several reasons for the discrepancy between the observational and interventional vitamin D studies. Most RCTs have included subjects with an already adequate vitamin D status and therefore no additional effect by vitamin D to be expected, and many studies have been underpowered and of short duration (8).

Lately, there has also been a focus on dosing regimens. Many studies have used weekly, monthly or annual doses which are easy to administer and result in high and (at least for weekly and monthly doses) stable serum 25(OH)D levels (9). Contrary to 25(OH)D, serum vitamin D has a short half-life of approximately one day (2), and intermittent dosing will result in short-lived peaks after each dose. There are strong indications that hydroxylations of vitamin D to $1,25(OH)_2D$ may occur within cells in peripheral tissues, and therefore may have biological importance by itself and not just act as a substrate for hepatic 25-hydroxylation. This was reviewed by Hollis and Wagner in 2013 who argued that RCTs with intermittent doses may have missed important vitamin D effects (10).

Our hypothesis

Our hypothesis is therefore: the serum vitamin D level is a better marker of a subject´s vitamin D status than 25(OH)D, and that this will have consequences not only for intervention trials but also for observational studies and clinical practice.

Arguments for biological importance of circulating vitamin D

The VDR is a nuclear receptor, and for the purpose of this paper/hypothesis, only genomic actions and $1,25(OH)_2D$ as ligand will be considered.

In order to bind to the VDR, circulating 1,25(OH)2D has to cross the cell membrane or be produced within the cell. Traditionally, it was considered that the 25-hydoxylation of vitamin D to 25(OH)D only occurred in the hepatocytes, and that the 1-α-hydroxylation of 25(OH)D to $1,25(OH)_2D$ only occurred in the kidneys (2). However, that appears not to be the case.

The main 25-hydroxylase is believed to be CYP2R1 which is able to hydoxylate both vitamin D3 and D2 (11). Mutations in the *CYP2R1* gene may affect the circulating serum 25(OH)D level (5) and even lead to severe rickets (12). With the use of real-time PCR the tissue distribution of *CYP2R1* has been examined and found expressed in the liver as expected (11) , but also in the testes (11), human brain pericytes (13), placenta (14), adipose tissue (15), and prostate (16). In *CYP2R1* knock-out mice, the reduction in serum 25(OH)D is approximately 50 %, and accordingly, other 25-hydroxylases must also exist (17). What regulates the activity of the *CYP2R1* (or other 25-hydroxylases) in peripheral tissues is not known, and may perhaps be substrate dependent as is the 25-hydoxylation in the liver (11).

While there are probably many 25-hydoxylases (17), there appears to be only one 1-α-hydoxylase of importance in humans, the CYP27B1 (18). Hydroxylation by CYP27B1 in the kidneys is the main source of circulating $1,25(OH)_2D$, but even in anephric humans $1,25(OH)_2D$ is detectable in serum (19).Thus, gene expression studies have demonstrated the

presence of *CYP27B1* in a number of tissues like bone cells, melanocytes, activated macrophages, keratinocytes, brain and endocrine glands (18). Contrary to *CYP2R1* in the liver, the activity of *CYP27B1* in the kidneys is under strict metabolic control by PTH and FGF-23 (2). In line with this, there are indications that extra-renal *CYP27B1* expression is also regulated, both by the end-product 1,25$(OH)_2$D in keratinocytes (20), by cytokines in pulmonary alveolar macrophages (21) and by FGF-23 and extracellular calcium in the parathyroid gland (22). Further progress in this area probably depends on identification of "missing" 25-hydroxylases as well as by development of tissue-specific knock-out models. However, taking the available data together, it seems plausible that many, if not most tissues are capable of producing the active vitamin D from its precursors, mainly for paracrine/local function.

How do vitamin D, 25(OH)D and 1,25(OH)2D enter the cells?

Both vitamin D that is obtained from the diet and vitamin D that is produced in the skin upon sun exposure, reach the general circulation where most of it is bound to DBP. Some also circulate in a free, unbound form. Vitamin D is taken up in the liver and hydroxylated to 25(OH)D which is released into the circulation where most of it is (as vitamin D) bound to DBP. 25(OH)D (both in the free and bound form) is taken up by the kidneys, further hydroxylated to the active form 1,25$(OH)_2$D, and released to the circulation where again most is bound to DBP. Thus, in the circulation six main forms (or complexes) are found:

- free vitamin D and DBP-bound vitamin D
- free 25(OH)D and DBP-bound 25(OH)D
- free 1,25$(OH)_2$D and DBP-bound 1,25$(OH)_2$D

The free forms are fat-soluble and presumably diffuse passively across the cell membranes (10). However, the dissociation constants for vitamin D, 25(OH)D and $1,25(OH)_2D$ with regard to DBP are quite different being 10^{-8} M, 10^{-9} M and 10^{-7} M, respectively (10). Therefore, much more vitamin D and $1,25(OH)_2D$ than 25(OH)D exist in the free form. Since the circulating concentrations of vitamin D is 100 – 1000 fold higher than that of $1,25(OH)_2D$, it is plausible that much more free vitamin D diffuse into the cells than free 25(OH)D and free $1,25(OH)_2D$.

However, there is another mechanism whereby vitamin D and its metabolites may enter cells, the megalin-cubilin endocytotic system (23). The endocytic receptors megalin and cubilin were first identified in the renal proximal tubule where they cause uptake of a number of proteins, including DBP (23). This is important for the body's vitamin D economy, and deletion of the kidney-specific megalin receptor in knock-out mice result in vitamin D deficiency, hypocalcaemia and osteopathy (24). Expression of megalin and/or cubilin has later been demonstrated in the lung alveoli, the eye, gall bladder, placenta, parathyroid and thyroid gland (23), mammary gland (25), muscle and in pre-adipocytes (26). However, the expression as evaluated by immunohistochemistry, is lacking, or very weak, in liver, spleen, testes, skin, adrenal gland and pancreas (27).

Whether the megalin-cubilin system has an equal affinity for the DBP-vitamin D, DBP-25(OH)D and DBP-$1,25(OH)_2D$ complexes are to our knowledge not known, nor what regulates the expression of this receptor, or if the affinity of the receptor for the DBP-vitamin D(metabolite) complexes is tissue specific. Accordingly, the importance of this system for vitamin D metabolism (with the possible exception for the reabsorption of DBP complexes in the proximal renal tubule) is not known.

It should be mentioned that vitamin D and its metabolites also bind to albumin in addition to DBP, but to a lesser extent (2). This vitamin D(metabolite)-albumin binding is less tight

than that to DBP, and it has therefore been speculated that this binding is disrupted in the microcirculation so that the albumin bound part can be considered as "free". If so, more "free" vitamin D, 25(OH)D and 1,25(OH)2D would be available for diffusion into the cells (28). Even if this should be true, our lines of arguments would still be the same. Furthermore, it is not known if the megalin-cubulin system also transport albumin-vitamin D complexes into the cells, but again, that would not change our hypothesis.

Thus, with the following assumption:

- large quantities of circulating unbound vitamin D easily diffuse across cell membranes
- *CYP2R1* and other yet unidentified 25-hydroxylases are widely distributed
- the activity of *CYP2R1* in peripheral tissues is likely to be unrestricted as in the liver
- the megalin/cubulin system will provide both vitamin D and 25(OH)D into the cells,

then it is reasonable to assume that most intracellular 25(OH)D is the result of intracellular hydroxylation of vitamin D. Furthermore, given the wide distribution of *CYP27B1* and the low concentration of free as well as DBP-bound $1,25(OH)_2D$ in the circulation, it is likely that most of the cellular $1,25(OH)_2D$ is derived from intra-cellular 1-α-hydroxylation of 25(OH)D. If so, then the circulating level of vitamin D better reflects intra-cellular vitamin D related activity/status than circulating levels of 25(OH)D.

Consequences for intervention studies with vitamin D

Since binding to DBP protects vitamin D and its metabolites from degradation, the differences in dissociation constants is also reflected in the circulating half-lives of these compounds, being approximately one day for vitamin D, weeks for 25(OH)D and a few hours for $1,25(OH)_2D$ (10). One would therefore assume that the serum profiles of vitamin D and 25(OH)D are highly affected by dosing regimens.

Thus, in a study by Heaney et al., who gave a bolus dose of 100 000 IU vitamin D to 30 healthy adults, mean serum vitamin D level peaked at 521 nmol/L after 6 – 8 hours and was almost eliminated from the circulation within a few days. On the other hand, serum levels of 25(OH)D peaked after one week and thereafter gradually disappeared after months (29).

In a study by Oberhelman et al. lactating women were randomized to 150 000 IU once or 5000 IU daily for 28 days. At the end of the trial both groups had mean serum 25(OH)D slightly above 100 nmol/L, whereas in the bolus dose group mean serum vitamin D was 17.5 nmol/L versus 45.8 nmol/L in the daily dose group (30). In an almost identical study in non-lactating women, the corresponding mean serum vitamin D levels in the two groups after 28 days were 7.1 nmol/l and 27.9 nmol/L, respectively (31). Of note was that in 17 of 20 subjects randomized to the bolus dose the serum vitamin D levels were undetectable after 28 days. However, the area under the curve for serum vitamin D was similar in the two groups.

Using a different design, Dimitris et al. gave vitamin D 35 000 IU weekly versus placebo to pregnant women who at baseline had a mean serum vitamin D level of 1.3 nmol/L. After at least eight weeks of treatment, the mean serum vitamin D level increased to a peak of 152 nmol/L one day after the weekly dose, and thereafter rapidly declined to a level of ~ 20 nmol/L the days before the next dose (32).

Accordingly, giving vitamin D weekly or monthly results in intermittent high serum vitamin D levels. Even after eight weeks of weekly doses, there is a five-fold difference in serum vitamin D levels between the first and the last days of the treatment week. Even if the area under the curve for serum vitamin D may be unaffected by the dosing regimens, these fluctuating levels are unlikely to be physiologically optimal. In our evolution as humans, we had hundred of thousands of years with a constant supply of vitamin D from skin production under the equatorial sun. It should be recalled that for sun-induced vitamin D synthesis in the skin there are protective mechanisms that block overproduction (1). One can therefore

speculate that the high peaks of serum vitamin D after bolus doses are more likely to be unhealthy than health promoting.

The consequence for clinical vitamin D trials is obvious; the doses should be given daily.

To prove this with certainty, one would have to perform large RCTs with daily versus intermittent dosing and with hard endpoints like cancer, cardio-vascular disease, fracture or mortality. Considering the difficulties there has been in demonstrating clinical effects of vitamin D even versus placebo (7), it is unlikely that such RCTs will ever be performed.

There are already some indications from RCTs that daily doses are preferable to intermittent doses regarding prevention of respiratory infections (33), whereas that has not been seen for cardiovascular diseases (34). Some short-term studies have shown effects by vitamin D itself where bolus doses of vitamin D caused rapid reduction in serum levels of hepcidin, an antibacterial protein that regulates the absorption, tissue distribution and extracellular iron by suppressing ferroportin-mediated export of iron (35). This rapid decline in serum hepcidin levels occurs when the serum vitamin D levels start to rise and long before the serum 25(OH)D levels increase.

On the other hand, some responses like vitamin D induced PTH suppression are obviously not dependent on the serum vitamin D level. Thus, in a study by Romagnoli et al. a 300 000 IU vitamin D bolus dose maintained the serum 25(OH)D to levels 75 nmol/L above baseline for 60 days with a sustained suppression of serum PTH throughout this period (36). This could not have been caused by serum vitamin D, which after 60 days should be very low or undetectable, but instead reflects the strong presence of the megalin/cubulin system in the parathyroid gland (23). Furthermore, in a study by Meyer et al. in 20 postmenopausal women randomized to 20 ug of calcifediol (25-hydroxylated vitamin D) versus 20 ug of vitamin D for four months, those given calcifediol had an 18 % higher increase in gait speed than those given vitamin D (37). Although the calcifediol treatment as expected (38) resulted in an

increase in serum 25(OH)D levels three times higher than the vitamin D treatment, it is reasonable to assume that for gait speed, which combines muscular strength and coordination, an increase in serum vitamin D was not necessary for improved function.

To demonstrate that serum vitamin D levels are (at least for some responses) more important than serum 25(OH), further short term experiments like those with hepcidin are needed where effects hours after a vitamin D dose are studied (35). Another approach could be to compare effects of a bolus dose versus daily dose after 3- 4 weeks, a time point were both regimens would have adequate serum 25(OH)D levels, but only those given daily doses would have adequate vitamin D levels. Potential surrogate measures for clinical effects could in addition to hepcidin be serum cathelicidin, an antimicrobial peptide that is part of the innate immune system and that correlates with serum 25(OH)D (39) and appears to increase after vitamin D supplementation (40); RNA expression of vitamin D sensitive genes in peripheral blood as well as tissue samples (41); telomerase activity that appears to increase with vitamin D supplementation (42); the ratio between 25(OH)D and 24,25$(OH)_2$D which may give an indication of the vitamin D status of the body (43); and inflammation (44) and bone turn-over markers (45).

Consequences for observational studies

Given a steady influx of vitamin D from food sources and/or from sun-exposed skin, there will be a very high correlation between the serum levels of vitamin D and 25(OH)D. However, in many settings a steady influx of vitamin D is not the case. Fatty fish, which has a high content of vitamin D, is for most persons not eaten at a regular basis, sun exposure can be very intermittent during a Norwegian summer and completely lacking during the winter if not on a sunny vacation (46). One may therefore assume a much greater variation both day-to-

day as well as between summer and winter for the serum vitamin D level than for the 25(OH)D level.

Accordingly, the serum vitamin D level may indicate the actual (present) intracellular vitamin D status, whereas the serum 25(OH)D level may be taken as an integrated measure of vitamin D intake and sun exposure over time. However, due to the short half-life of vitamin D (which we believe is the most important component) and the long half-life of 25(OH)D in serum, there will be numerous occasions where the serum 25(OH)D level apparently is "adequate", whereas the serum vitamin D level is low and the subject actually vitamin D deficient. Using serum 25(OH)D levels as a marker of an individual´s vitamin D status, which has been the routine so far (1, 6), may therefore in epidemiological studies create both type 1 and type 2 errors.

It should also be recalled that the serum 25(OH)D level is not only the result of vitamin D influx, but also subject to genetic effects (5), body composition (4) and use of hormonal contraceptives or estrogen substitution (47). There are several single nucleotide polymorphisms (SNPs) in the *CYP2R1* gene (25-hydroxylase gene) that affect the serum 25(OH)D level (5), but is unlikely to have any effect on serum vitamin D. Also SNPs in the *DBP/GC* gene (that affect transportation of vitamin D and metabolites) and the 24-hydroxylase gene *CYP24A1* (that cause degradation and removal of vitamin D metabolites from the circulation) affect the serum 25(OH)D level (5), whereas effects on serum vitamin D are not known. Perhaps due to sequestration in adipose tissue, or caused by simple distribution dilution, serum 25(OH)D levels are lower in obese than slim subjects (48). For hormonal contraceptives the mechanism for higher 25(OH)D levels are most likely due to increased levels of DBP and perhaps also a higher 25-hydroxylation in the liver (47). However, the effect of body composition and hormonal contraceptives on serum vitamin D is not known and might differ from 25(OH)D.

To our knowledge there has not been a single epidemiological study on serum vitamin D levels and health, probably due to the complex and time-consuming procedures necessary for vitamin D measurements. It is not unlikely that cross-sectional studies performed during the summer (where the correlation between serum vitamin D and 25(OH)D presumably will be very strong) will show similar relations for vitamin D and 25(OH)D regarding health and risk factors, whereas these relations during the winter may be completely different. A possible illustration of this was recently reported by Michaelsson et al. where summer concentrations of 25(OH)D predicted bone mass density in older women, whereas winter 25(OH)D levels was of limited value (49).

The obvious consequence for observational studies is therefore that both serum vitamin D and 25(OH)D should be measured until it is settled which one (or both combined) best reflects true vitamin D status.

Clinical consequences

Vitamin D deficiency is, based on measurements of 25(OH)D, considered to affect billons of people around the world (50). The importance of correct classification of vitamin D status, both from a health as well as an economical perspective, is obvious; adequate treatment should be given to those in need of supplementation and unnecessary medication avoided in those already vitamin D sufficient. The subjects at greatest risk for misclassification are those who recently have been on a sunny vacation, or consumed at lot of vitamin D containing food like a traditional Norwegian cod fish meal with liver and roe (51), or who take large vitamin D supplement doses every now and then, and thereafter have had little or no vitamin D supply. They may have very high serum 25(OH)D levels and thus be classified as vitamin D

sufficient, whereas their serum vitamin D levels may be very low and thus have intra-cellular vitamin D deficiency.

In a way, the relationship between vitamin D and 25(OH)D may have some analogy to blood glucose and HbA_{1c} (52) where serum 25(OH)D and HbA_{1c} gives an integrated measure, whereas serum vitamin D and blood glucose reflect the status at a particular time point. Severe hypoglycemia may be present in spite of elevated HbA_{1c} and both measures are needed for a proper clinical evaluation. Similarly, a subject may be severely vitamin D deficient in spite of an adequate serum 25(OH)D level. However, it should be stressed that in most instances there is a high and positive correlation between blood glucose and HbA_{1c} as well as between serum vitamin D and 25(OH)D.

At present serum vitamin D measurements are performed for research purposes only. If introduced as a routine test in clinical practice for evaluation of vitamin D status, that would add a considerable cost to an already expensive diagnostic procedure. Therefore, the consequence for clinical practice is that when measuring serum 25(OH)D, the result should be viewed in relation to recent activities and intakes.

Conclusions

We believe that serum vitamin D best reflects the intra-cellular vitamin D status. This is based on assumptions regarding diffusion of vitamin D metabolites into the cells, function of the megalin/cubulin system, and presence and activity of intra-cellular 25-hydroxylase and 1-α-hydroxylase. These uncertainties are not the only ones regarding vitamin D. At present we only know with certainty that vitamin D prevents rickets, but we do not know the importance of vitamin D for extra-skeletal health. Nor do we know with any degree of certainty how

much vitamin D we need, or if the importance of vitamin D is limited to certain periods of life. And last, but not least, there is a huge discrepancy between observational and interventional studies. Some of these uncertainties might be resolved if we not only focus on 25(OH)D, but include serum vitamin D in the evaluation of vitamin D status.

Conflict of interest

None declared

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