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Vitamin D in chronic liver disease

Caroline S. Stokes[1], Dietrich A. Volmer[2], Frank Grünhage[1], Frank Lammert[1]

[1]Department of Medicine II, Saarland University Medical Center, Homburg, Germany

[2]Institute of Bioanalytical Chemistry, University of Saarland, Saarbrücken, Germany

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Corresponding author

Prof. Dr. med. Frank Lammert

Department of Medicine II

Saarland University Medical Center

Kirrberger Str. 100

66421 Homburg

Germany

Phone: +49-6841-16-23201

Fax: +49-6841-16-23267

Email: frank.lammert@uks.eu

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Abbreviations cited: AGA, American Gastroenterology Association; AIH, autoimmune hepatitis; ALC, alcoholic liver cirrhosis; BMD, bone mineral density; BMI, body mass index; BSG, British Society of Gastroenterology; CCA, cholangiocarcinoma; CDAA, choline-deficient, L-amino acid-defined; CI, confidence interval; CLD, chronic liver disease; CYP2R1, cytochrome P450, family 2, subfamily R, polypeptide1; DBP, vitamin D binding protein; DHCR7, 7-dehydrocholesterol reductase; EASL, European Association for the Study of the Liver; Gc-globulin, group-specific component; GWAS, genome-wide association study; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IL-1, Interleukin-1; IL-6, Interleukin-6; IOM, Institute of Medicine; LC-MS, liquid chromatography/mass spectrometry; MMP, matrix metalloproteinase; MRM, multiple-reaction monitoring; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OR, odds ratio; PBC, primary biliary cirrhosis; PTH, parathyroid hormone; RCT, randomised controlled trial; RDA, recommended daily allowance; RXR, retinoid X receptor; SOC, standard of care; SVR, sustained viral response; TNF, tumor necrosis factor; UV, ultraviolet; VDR, vitamin D receptor; VDRE, vitamin D response element; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; $24,25(OH)_2D$, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D.

ABSTRACT

Background: Chronic liver disease (CLD) and several related extrahepatic manifestations such as hepatic osteodystrophy are associated with deficiency of vitamin D, which has therefore been suggested as therapeutic target. Vitamin D undergoes hepatic 25-hydroxylation, rendering the liver critical to the metabolic activation of this vitamin. Vitamin D deficiency is highly prevalent in CLD patients, and vitamin D levels are inversely related to the severity of CLD. Declining levels of carrier proteins such as albumin and vitamin D binding protein might also be critical in CLD. Intervention studies report improvements of CLD following supplementation, and benefits to health outcomes in particular with respect to hepatitis C virus infection have recently been documented. Content: We discuss vitamin D sources, functions and metabolism with a focus on the inherent complications of analytical measurements, such as the interference of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D C-3 epimers. Global discrepancies in the definition of optimal serum 25-hydroxyvitamin D levels are covered, and the prevalence of vitamin D deficiency in CLD is reviewed. We also address the functional mechanism underlying this deficiency, and refer to associations between genetic variation in vitamin D metabolism and CLD. Lastly, we focus on health implications of a vitamin D deficiency in CLD and consider therapeutic options. Summary:

Herein, we focus on the epidemiological and functional relationships between vitamin D deficiency and CLD, followed by a discussion of the potential implications for therapeutic interventions in clinical practice.

Key words: bioavailability; cholecalciferol; epimers; hepatic disease; hepatic osteodystrophy; 25-hydroxyvitamin D; vitamin D metabolites.

INTRODUCTION

Chronic liver disease (CLD) is defined as the process of long-term progressive destruction and regeneration of the liver, and with advancing disease, hepatic fibrosis (scarring) and cirrhosis frequently occur (1). Progression of CLD and deterioration of liver function is associated with various hepatic complications such as chronic liver failure, hepatocellular carcinoma (HCC), and infections. Hepatic osteodystrophy is an important extrahepatic manifestation of advanced liver disease mimicking features of classical osteoporosis with an increased risk for fractures (2). Recently the role of vitamin D in CLD has received much attention given its inherent activation process by the liver and the high prevalence of vitamin D deficiency in this patient group (3). Evidence is also beginning to unravel possible direct therapeutic benefits of vitamin D therapy. While clear evidence of an association between vitamin D and liver disease exists, it remains unknown whether vitamin D deficiency confers an enhanced risk to liver disease or whether liver disease causes vitamin D deficiency. This review summarizes the role of vitamin D in CLD, highlighting important functional aspects of the vitamin D / CLD relationship. Cholestatic liver diseases are however, not the focus of this review since they have been addressed in guidelines produced by the British Society of Gastroenterology (BSG) (4) and more recently by the European Association for the Study of

the Liver (EASL) (5). For a detailed review of cholestatic liver diseases which addresses vitamin D, please see Zollner et al (6).

VITAMIN D FUNCTIONS AND METABOLISM

The term vitamin D refers to a group of seco-steroid compounds, namely vitamin D_3 and vitamin D_2.[1] The former is principally obtained through skin synthesis, and the latter is mainly derived from mushrooms, yeast and chemical synthesis. Table 1 summarizes the various sources of this fat-soluble vitamin. Vitamin D functions intricately with parathyroid hormone (PTH) to maintain plasma calcium and phosphate concentrations and plays a crucial role in bone growth and remodeling (7). Hence, in a deficiency state, abnormal calcium, phosphate and bone metabolism consequently ensue. Vitamin D is biologically inert and must be endogenously activated via oxidative metabolic processes (Figures 1 and 2). The basic metabolism of vitamin D has been extensively reviewed by others e.g. (8) and will therefore not be described in detail here. In brief, vitamin D_3 is primarily acquired endogenously through the photochemical conversion of 7-dehydrocholesterol to previtamin D_3 in the skin - with sun exposure being paramount to this process. Previtamin D_3 has a limited capacity and excess (i.e. during high sun exposure) is converted to the inactive isomers, tachysterol (which also has a limited storage capacity) and lumisterol (with unlimited storage capacity). This process is reversible, and in times of previtamin D_3 depletion, the above process is reversed so that previtamin D_3 is formed and enters the metabolic pathway. Previtamin D_3 is then converted to vitamin D_3 through thermic isomerizaton which is transported to the liver

[1] Throughout this review, the generic form 'vitamin D' refers to the vitamin D_3 form.

mostly bound to vitamin D binding protein (DBP), also known as group-specific component (Gc-globulin).

Vitamin D_2 differs in that it is incorporated into micelles in the intestinal lumen following ingestion. It is then further amalgamated into chylomicrons via enterocyte absorption and reaches the liver from the venous circulation for hydroxylation. Vitamin D (D_3 and D_2) is hydroxylated in the liver by cytochrome P450 enzymes (e.g. cytochrome P450 family 2, subfamily R, polypeptide1 [CYP2R1]) to 25-hydroxyvitamin D (25(OH)D) and secreted in the circulation, again, mostly bound to DBP. Incidentally, since DBP is synthesized in liver, it represents a biomarker for advanced liver injury (9, 10). Moreover, the serum concentration of 25(OH)D is the most commonly used biomarker for vitamin D status, given its long half-life. Further hydroxylation to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) in the kidney converts the vitamin into its active form. Other extra-renal tissues containing 1α-hydroxylase enzymes also activate $1,25(OH)_2D$ to a certain extent, e.g. the central nervous system and immune cells such as macrophages and neutrophils (11). The complex metabolic process of vitamin D also contains a 'built-in' elimination system, whereby 25(OH)D and $1,25(OH)_2D$ are converted to 24,25-dihydroxyvitamin D_3 and 1,24,25-trihydroxyvitamin D_3, by CYP24A1 in the kidney, respectively (Figure 2).

$1,25(OH)_2D$ is secreted into the circulation bound to DBP, and once it has entered its target cells, it binds to a specific member of the nuclear receptor superfamily, the vitamin D receptor (VDR), which forms a heterodimer with retinoid X receptor (RXR) (12). Subsequently, this heterodimer associates with the vitamin D response element (VDRE) in the promoter of target genes and induces or represses gene transcription (13). VDR binding is associated with the differential expression of a plethora of genes, and through this vitamin D exerts specific effects, in particular the regulation of immune functions (14). It is currently estimated that $1,25(OH)_2D$ regulates approximately 2000 genes (15). Interestingly, VDR is expressed in multiple sites across the body such as, the parathyroid gland, pancreatic islet

cells, mammary glands, macrophages, promyelocytes, and keratinocytes. Consequently the identification of a multitude of vitamin D-related functions continues to emerge, including cell growth modulation, anti-inflammatory and neuromuscular effects (16).

OBSTACLES IN ANALYTICAL VITAMIN D MEASUREMENTS

In recent years, analytical assays based on liquid chromatography-mass spectrometry (LC-MS) have become the primary choice for high-end analyses of vitamin D and its metabolites in biological fluids. While very sensitive, precise and, if applied properly, accurate, this technique unfortunately, is not as straightforward to use; development of LC-MS methods for vitamin D thus require significant analytical expertise to overcome the various inherent limitations and potential for interference. Many of the limitations resulting in unsatisfactory accuracy and precision can be overcome by using stable isotope internal standards for calibration (17). For example, 25(OH)D and other vitamin D metabolites must be fully released from the transport proteins during the sample preparation step of the analytical determination, as this would otherwise lead to systematic errors and inaccurate results. LC-MS offers an inherent advantage over the established immunoassay techniques with respect to the protein binding as it allows the compensation of these detrimental effects by using isotope standards of the vitamin D metabolites. Careful incubation with the isotope standard assures that the isotope-labelled compound is bound to vitamin D-binding protein in the same manner as the endogenous target analyte (17). Other analytical issues, however, remain challenging. For example, detection sensitivity limitations only allow determination of the most abundant metabolite 25(OH)D on most modern LC-MS instruments unless derivatization reactions are performed to increase the ionization properties for the low abundant metabolites (18). Selectivity issues from improper choice of product ion transitions during multiple-reaction monitoring (MRM) LC-MS and isobaric contributions also remain a major problem in many published assays. The present authors hope that these issues will be overcome in the future with a more intelligent choice of

structure-specific product ions. A serious recent problem, however, is the occurrence of C-3 epimers of all vitamin D compounds. Figure 3 illustrates that C-3 epimers result from reversal of the stereochemical configuration of the C-3-bound hydroxyl group ($\alpha \rightarrow \beta$). The C-3 epimerization reaction was discovered (19, 20) as a separate pathway in addition to the oxidative transformations of vitamin D but their biological function remains unclear. Regardless of its biological significance, however, C-3 epimers are potential interferences in the analytical determination leading top systematic analytical errors if not properly accounted for (21, 22). Importantly, whether epimers are detected in vitamin D measurements may sometimes simply be a result of assay sensitivity. Of course, since one never knows in advance whether potential epimeric interferences are present, only proper chromatographic resolution to separate the epimers can currently give sample-independent reliable measurement of vitamin D status by LC-MS. If epimer-specific product ions can be produced by more sophisticated tandem mass spectrometry experiments remains to be seen but is likely to be very difficult.

Of note, analytical measurements of vitamin D metabolites have revealed considerable levels of the C-3 epimers of 25(OH)D and 1,25(OH)2D in humans and animal cell lines (23, 24). In particular, one study showed significant C-3 epimer concentrations of 25(OH)D in very young children but not in adults (23). Another study, however, reported considerable levels even in adults (24), and more recently, Lensmeyer et al. (25) detected C-3 epimers in 99% of samples, translating to 212 of 214 human samples where the age ranged from neonates to participants over 80 years. They also found a non-linear increase in epimer concentrations where the relative amounts of epimer to 25(OH)D ranged from 0% - 26%. It is important to remember that analytical assays will overestimate the vitamin D levels (e.g. by as much as 25% based on the findings above) if proper care is not taken to separate the different 3-OH epimers (and other isobaric interferences) to a point where measurement of vitamin D levels may appear as normal (24). This is important to keep in

mind when evaluating vitamin D status, especially in patients such as those with liver disease, who have a predisposition to vitamin D deficiency but which may not appear in the event of a large proportion of epimer interference.

Given its fat-soluble properties, non-hydroxylated vitamin D is mainly stored in adipose tissue (26). In fact, obesity has been associated with low vitamin D levels due its sequestration, and hence reduced bioavailability (27). Unfortunately, the extent of adipose storage remains unknown due to the lack of appropriate methodologies to measure vitamin D in adipose tissue.

The C-24-hydroxylation process is generally thought to be the first step in the degradation pathway of $1,25(OH)_2D$ and 25(OH)D. A comprehensive review of previous data suggests, however, that $24,25(OH)_2D$ is not merely a degradation product but has effects on its own (reviewed in (28)). In addition, the plasma concentration of $24,25(OH)_2D$, its ratio to other metabolites, and the rate of turnover of 25(OH)D have been proposed to have potential as alternative markers of vitamin D status (29).

DEFINING OPTIMAL VITAMIN D STATUS

Much controversy surrounds the definition of 'adequate' vitamin D status. On a biochemical level, abnormal calcium, phosphate and bone metabolism may develop rendering patients at greater risk of osteopenia, osteoporosis, and fractures (30). A deficiency of vitamin D has also been linked to extrahepatic diseases such as cancer (particularly of the colon), in addition to inflammatory, autoimmune, and metabolic diseases (31, 32). Optimal vitamin D status has often hinged on the inverse relationship between PTH and 25(OH)D and

discrepancies exist regarding the optimal level of 25(OH)D required for PTH levels to plateau, ranging from 18 ng/ml to 30 ng/ml (i.e. 45 nmol/l to 75 nmol/l) (33, 34). Specific levels have not been defined for patients with liver disease, however historically, vitamin D insufficiency was defined as serum 25(OH)D levels < 20 ng/ml (i.e. 50 nmol/l) for the general population (35). The Institute of Medicine (IOM) of the National Academies in the United States (36) also suggests a serum 25(OH)D concentration of 20 ng/ml to be adequate. In contrast, the Endocrine Society (Maryland, US) (30) recommends 25(OH)D levels of 30 ng/ml (i.e. 75 nmol/l) and even suggests that concentrations between 40 ng/ml - 60 ng/ml (i.e. 100 nmol/l - 150 nmol/l) may be advantageous as a precautionary measure due to the variability of assays in 25(OH)D determination (as highlighted in the previous section).

PREVALENCE OF VITAMIN D DEFICIENCY IN CLD

The global prevalence of vitamin D deficiency in the general population affects all age groups and ranges from 20% to 100% when referring to serum 25(OH)D concentrations < 20 ng/ml (30). The prevalence of vitamin D levels < 20 ng/ml in CLD has been reported to range from 64% to 92% and is commonly inversely related to disease progression (3, 37, 38). Some studies, however, have failed to find a difference in vitamin D status between patients with cirrhosis and those without (39), illustrating that the cause of this deficiency must be multifactorial.

Previously, vitamin D deficiency was thought to be predominantly found in cholestatic liver disorders due to impaired intestinal absorption commonly observed in such patients (4). Accumulating evidence however, supports its widespread presence in CLD, regardless of aetiology. Table 2 highlights examples of studies that report on vitamin D status in patients with CLD. For a detailed review of vitamin D specifically regarding the hepatitis C virus (HCV) please refer to Cholongitas et al. (40). Arteh et al. (3) found vitamin D < 32 ng/ml (i.e.

80 nmol/l) in 92% of 118 patients with CLD (HCV cirrhosis, n = 43; HCV without cirrhosis, n = 57; non HCV-related cirrhosis, n = 18). Likewise, Fisher et al. (37) found inadequate 25(OH)D levels (< 32 ng/ml) in 91% of patients with non-cholestatic CLD, and the majority (68%) were vitamin D deficient (< 20 ng/ml). Moreover, a significantly ($P < 0.0001$) higher prevalence of vitamin D deficiency in patients with cirrhosis (86%) compared to those without cirrhosis (49%) was observed, as illustrated by an inverse association between vitamin D status (assessed by 25(OH)D levels) and disease severity (assessed by the Child-Pugh score). Patients in Child-Pugh class C had significantly ($P < 0.001$) lower mean 25(OH)D concentrations than patients in class A (9.0 ± 4.0 vs. 18.3 ± 6.7 ng/ml). These findings are supported by Chen et al. (38), Miroliaee et al. (41) and Rode et al. (42), who found 75% of patients with liver cirrhosis to have 25(OH)D levels < 20 ng/ml. Most recently, Putz-Bankuti et al. (43) reported an inverse association ($r = -0.21$, $P = 0.08$) between serum 25(OH)D levels and the severity of liver disease in a cohort of 75 patients with cirrhosis. Interestingly, the authors also investigated survival in this cohort and 32% of patients had died during a median follow-up period of 3.6 years. Interestingly, Kaplan-Meier curves demonstrated a significantly ($P < 0.001$) increased mortality with low vitamin D concentrations. Figure 4 summarizes the differences in vitamin D status based on Child-Pugh scores from different studies.The data reflect that subjects in Child-Pugh class C have approximately half the 25(OH)D concentrations of class A, and in most cases (37, 38, 43, 44) the difference is statistically significant ($P < 0.01$).

A low vitamin D status is also evident in patients with other chronic liver diseases, particularly in the presence of cirrhosis. For instance, 50 patients with non-alcoholic steatohepatitis (NASH) and 10 patients with simple steatosis were observed by Targher et al. (45) to have lower 25(OH)D concentrations compared to 60 controls matched for age, sex and body mass index (BMI). Overall this Italian-based study found lower vitamin D status in patients with non-alcoholic fatty liver disease (NAFLD), which was also associated

with histopathological NAFLD features. These findings were recently corroborated by the same group (46) in a larger study (n = 262), in which low 25(OH)D levels were independently associated with NAFLD (n = 162) and were significantly ($P < 0.001$) lower than in subjects who were free from NAFLD and other liver diseases (15 ± 9 vs. 21 ± 9 ng/ml). Of note, when compared to the highest quartile, subjects in the lowest quartile of serum 25(OH)D levels displayed an odds ratio (OR) of 4.7 (95% confidence interval (CI), 2.2 - 10.3, $P < 0.001$) for NAFLD.

As would be expected, liver transplant patients exert a different pattern: Trautwein et al. (47) found 96% of these patients had inadequate vitamin D stores pre-transplant but post transplant, vitamin D deficiency was uncommon. Having normal vitamin D levels pre-transplant may however, have important implications for this patient group. A study by Bitetto et al. (48) found pre-transplant low serum 25(OH)D levels (< 12.5 ng/ml) to predispose to rejection episodes; the authors believe that supplementation with vitamin D might prevent acute cellular rejection via improved immune tolerance.

While the data summarized above clearly indicate that vitamin D deficiency is commonly found in CLD, most of the studies are cross-sectional – a study design which impedes on deciphering whether vitamin D deficiency affects the course of liver disease or whether liver disease has an influence on vitamin D status. Recently a longitudinal study observed no influence of vitamin D on the progression of CLD. Specifically, Corey et al. (49) evaluated the impact of vitamin D levels on the progression of CLD in a longitudinal nested case-control study as part of the HALT-C trial. The sample consisted of well-defined patients with HCV who had not achieved a sustained virological response (SVR). This study failed to find a difference in vitamin D levels in patients with and in those without progressive CLD during a four-year period. Both groups exhibited a decline in vitamin D levels, however, 75%

of subjects had normal vitamin D levels at baseline. The authors speculate that this specific observation may have resulted from supplementation, as detectable vitamin D_2 levels were identified in 55% of the cohort. This confounder unfortunately hampers the ability to evaluate the natural progression of CLD and vitamin D status in this cohort, and additional longitudinal studies are therefore duly warranted.

VITAMIN D DEFICIENCY IN CLD - MECHANISTIC RATIONAL

There are several probable causes for the observed inverse relationship between severity of liver disease and decreasing vitamin D status. The underlying mechanisms are almost certainly multifactorial in nature and likely to vary among different liver pathologies. Important potential mechanisms to consider are: (**1**) reduced exogenous exposure of patients to vitamin D sources (e.g. dietary, sunlight); (**2**) intestinal malabsorption of dietary vitamin D; (**3**) reduced endogenous production of vitamin DBP and albumin in the liver, which are impaired in the presence of cirrhosis; (**4**) impaired hepatic hydroxylation of vitamin D to 25(OH)D; and (**5**) increased catabolic removal of 25(OH)D.

Firstly, reduced external input of vitamin D from the diet and limited exposure to sunlight is common in patients with severe liver disease. As mentioned previously, the intestinal absorption of vitamin D could be affected in the presence of cholestasis since dietary vitamin D absorption depends on bile salts (41). In principle, malabsorption may play a causal role since fewer intestinal bile salts are present, which are required to absorb vitamin D in the terminal ileum.

Considering the essential role of the liver in the synthesis of vitamin D carrier proteins and its fundamental action in metabolising vitamin D, mechanistic effects and

biosyntheses in the liver are particularly interesting to discuss as possible causes for reduced vitamin D levels. Lower serum concentrations of vitamin D carrier proteins from reduced production in the liver affect 25(OH)D serum levels. Chen et al. (38) reported a positive correlation of serum 25(OH)D concentrations with albumin levels ($r = 0.655$, $P < 0.0001$). Rode et al. (42) found a correlation between 25(OH)D and albumin as well as bilirubin concentrations in serum ($P = 0.004$), and Fisher et al. (37) found serum 25(OH)D levels < 10 ng/ml to reliably predict lower serum albumin and platelet count in addition to higher international normalized ratio and serum bilirubin. In the same context, it is important to remember that for the analytical determination of vitamin D a significant portion of vitamin D is bound to the carrier proteins (DBP and albumin) and care must be taken that this fraction is analytically compensated for (see above). In addition, Bikle et al. (50) suggest low total vitamin D levels do not disrupt its biological activity when normal unbound vitamin D levels are maintained. Furthermore, DBP has a large excess of vitamin D binding capacity; for example, a 50% decline in DBP does not appear to result in reduced vitamin D levels (50). This may explain why declining serum 25(OH)D concentrations are more often observed in advanced liver disease progression, when this capacity is fully exhausted. Moreover, studies using DBP deficient mice have illustrated lower serum 25(OH)D and $1,25(OH)_2D$ levels compared to wild-type mice. Interestingly however, these mice maintained normal serum calcium and PTH levels (51). This could be because DBP does not influence the pool of biologically active $1,25(OH)_2D$ even though it is important for total circulating $1,25(OH)_2D$, as illustrated in a mouse model by Zella et al. (52).

Another mechanism rationalizes hepatic vitamin D metabolism as the cause for low 25(OH)D concentrations in liver disease patients. Impaired enzymatic conversion was related to the degree of hepatic dysfunction in patients with alcoholic cirrhosis (53). However, some studies report no correlations between lower vitamin D status and severity

of liver disease (54), or show disruption of 25(OH)D synthesis only in advanced stages of CLD (55).

VITAMIN D DEFICIENCY IN CLD - GENETIC ASSOCIATIONS

A variety of modifiable risk factors contributes to vitamin D deficiency in liver disease, in particular reduced ultraviolet (UV) light exposure (due to limited time spent outdoors), dietary insufficiency, or corticosteroid use (3, 4). Non-modifiable risk factors such as age, sex and gene variants could also be causally implicated. A genome-wide association study (GWAS) of serum 25(OH)D concentrations in 33,996 European individuals (56) identified genetic determinants of vitamin D status. Variants at three loci near genes involved in vitamin D synthesis, hydroxylation and transport (7-dehydrocholesterol reductase, *DHCR7*; *CYP2R1*; *DBP*) were shown to be associated with vitamin D status.

It remained unclear however, whether the impact of genetic variants in normal populations also affects vitamin D levels in CLD. We recently investigated the impact of the previously described polymorphisms on vitamin D levels and liver fibrosis in patients with CLD and replicated the association with vitamin D levels for two of the three loci (*DHCR7* and *CYP2R1*) (57). In this study we also found an association between the *DHCR7* variant and mildly elevated liver stiffness values in patients with CLD. This may indicate that these variants and vitamin D deficiency are associated with fibrosis initiation. The fact that we could not confirm this association in patients with advanced liver disease is likely to be due to other factors overriding the effect of vitamin D in later disease stages. This finding has also been reported recently by Baur et al. (58) who found rapid fibrosis progression in HCV patients with low vitamin D status along with a variant in the *NR1I1* gene encoding VDR (specifically, the bAt [C-C-A] haplotype).

The genomic effects of vitamin D (1α,25-hydroxyvitamin D) have often been studied through its relationship with VDR, and numerous studies have investigated the role of genetic variants in the *NR1I1* gene. Interestingly different groups independently detected association signals between polymorphisms and primary biliary cirrhosis (PBC) as well as autoimmune hepatitis (AIH), which have been replicated in different ethnic groups (59, 60) (61). Moreover, *NR1I1* polymorphisms have been implicated in the development of HCC and hepatic bone disease. For instance, Falleti et al. (62) found significant ($P < 0.05$) associations between carriage of the *b/b* genotype of *Bsm*I as well as the [TT] genotype of *Taq*I and HCC. In contrast, the abscence of haplotype *BAT* [A-T-C] was significantly ($P < 0.05$) associated with HCC.

HEALTH IMPLICATIONS OF VITAMIN D DEFICIENCY IN CLD

DIRECT IMPACT ON THE LIVER

As mentioned above, a deficiency of vitamin D in CLD is reported to exacerbate the progression of liver fibrosis (3). In a population with genotype 1 chronic hepatitis C, a significant ($P < 0.0001$) inverse correlation between 25(OH)D serum concentrations and stages of fibrosis was reported (63). Moreover, multivariate logistic regression analysis showed an independent association between low 25(OH)D and increased necroinflammatory activity (OR 2.23, 95% CI 1.01 - 4.93, $P = 0.04$), and more severe fibrosis (Scheuer score ≥ 3) (OR 0.94, 95% CI 0.89 - 0.99, $P = 0.02$), thus the lower the vitamin D the higher the grade of inflammation and the stage of fibrosis. This finding was corroborated in a retrospective study by Bitetto et al. (64), though the association was not independent. A similar finding was previously reported in patients with NAFLD where lower 25(OH)D concentrations were associated with hepatic steatosis, inflammation and fibrosis

($P < 0.001$). This alludes to the possibility that vitamin D deficiency might be causally related to the development and progression of NAFLD (45).

A recent study in rats administered 1α-hydroxycholecalciferol orally for six weeks in a choline-deficient and iron-supplemented L-amino-acid-defined (CDAA) diet-induced NASH model (65). The authors observed a dose-dependent amelioration of NASH progression and concluded that the active from of vitamin D may benefit as an adjunctive therapy for NASH. Randomised controlled trials (RCTs) investigating the potential effects of vitamin D versus placebo have been initiated (http://clinicaltrials.gov/). Other possibilities for beneficial mechanistic effects have also been proposed. Vitamin D plays a role in the inhibition of zinc-dependent endoproteinases, specifically matrix metalloproteinases (MMP), which degrade extracellular matrix components. Hence, a reduced concentration of vitamin D is associated with an increased circulation of MMPs, and liver fibrosis is associated with the over-accumulation of extracellular matrix components (66).

HEPATIC OSTEODYSTROPHY refers to the specific CLD-associated bone disease and related metabolism abnormalities with both osteopenia and osteoporosis commonly occurring as extrahepatic manifestations of CLD. Likewise, hepatic osteodystrophy can pursue long after liver transplantation and is therefore considered a long-term complication of CLD (47). The international incidence of bone disease is reported to vary from 11% to 48% in patients with CLD (67). They are regarded as a 'high risk' group, since the osteoporotic fracture rate is double that of age-matched controls and the prevalence of osteoporosis is between 20% and 60% - with the variance dependent on diagnostic criteria and patient selection (2).

The pathogenesis of hepatic osteodystrophy is multifactorial and only partially understood. It is mainly characterized by reduced markers of bone formation and high bone

turnover (44) resulting from impaired osteoblast function as well as reduced concentrations of insulin-like growth factor 1, whose role is to maintain bone mass and which is a key player in the bone remodelling process (68). Interestingly, a recent study in jaundiced patients found elevated bilirubin to negatively affect osteoblast differentiation and mineralization and may thus contribute to osteoporisis in these patients (69). Other potential mechanisms include increased cytokine concentrations such as Interleukin (IL) 1, IL-6 and tumor necrosis factor (TNF) (70).

Numerous risk factors for hepatic osteodystrophy exist and include increased alcohol intake, low BMI, hypogonadism and corticosteroid use – all of which are frequently encountered in CLD (71) (72). The most prominent association of hepatic osteodystrophy with a specific CLD is reported for PBC and other cholestatic conditions given the inherent intestinal malabsorption of vitamin D accompanying these conditions. Nevertheless, it cannot be determined whether cholestasis is an independent risk factor as the majority of patients with PBC are post-menopausal women, which may confound this association (72). Conflicting findings in observation studies exist with regards to correlations between vitamin D status and measurements of bone mineral density (BMD) in CLD patients. For instance, Chen et al. (38) found a significant correlation between lumbar spine BMD and 25(OH)D levels in patients with cirrhosis compared to controls. Moreover, both serum 25(OH)D and $1,25(OH)_2D$ concentrations were considered to be strong independent predictors of hip bone density in patients with cirrhosis (44). Conversely, others have failed to find evidence to support such correlations (73).

Intervention studies in which patients with CLD receive vitamin D and/or calcium have also yielded conflicting results. Several studies focused on patients with PBC and are reviewed by Collier et al. (4). In some of these studies, vitamin D supplementation did not

delay the progression of osteoporosis, nor increase BMD (74). In contrast, Shiomi et al. (75) observed BMD to increase after 12 months of vitamin D supplementation (1 μg calcitriol/day) in patients with cirrhosis. One reason for these conflicting results may be attributed to the multifactorial nature of osteodystrophy in CLD. Alternatively, one might argue that the heterogeneity amongst many studies hinders between-study comparability, since bone measurements are carried out in various locations, e.g. lumbar spine or hip, and different methods of BMD assessment are employed, in addition to issues such as small sample sizes and low statistical power of studies. In addition, differences in vitamin D dosage and treatment duration hamper between-study comparisons, and combination therapy is a potential confounder as very few studies evaluated the benefits of vitamin D therapy alone, but rather combined it with calcium and other medications known to benefit bone health. In line with the above, EASL (5) published guidelines for the management of cholestatic diseases in 2009, which recommend that vitamin D (400-800 IU/day) supplementation be considered in all patients with cholestatic liver diseases, despite the lack of firm evidence base.

HEPATOCELLULAR AND INTRAHEPATIC CHOLANGIOARCINOMA often develop in patients with liver cirrhosis (76), and an inverse association between HCC and vitamin D has been reported (62). Vitamin D might mediate positive effects through its anti-apoptotic activity on hepatocytes (77). Calcitriol has proven anti-inflammatory and anti-angiogenic properties and not only promotes cell differentiation, but also inhibits cancer cell proliferation. For instance, *in vivo* and *in vitro* studies report inhibitory effects of 1α,25-dihydroxyvitamin D on HCC cell lines (78). Interestingly, this finding was replicated for cholangiocarcinoma (CCA) cell lines by Seubwai et al. (79), who also observed increased VDR expression in the tumor tissues of patients with CCA. Overall, however, evidence for a beneficial effect of vitamin D from prospective human intervention stuTHERAPEUTIC STRATEGIES dies is still lacking.

THERAPEUTIC STRATEGIES

A recent intervention study in Australia investigated the effects of oral vitamin D replacement in 158 outpatients with CLD (42). This cohort included patients with all types of liver diseases and 64% (n = 101) with 25(OH)D levels < 20 ng/ml received either 1000 IU ergocalciferol or 2000 IU cholecalciferol supplements daily for an average of four months. During this time period, Rode et al. (42) found the serum 25(OH)D to increase by 60% (from 12.8 to 20.4 ng/ml). On the other hand, a recent study by Malham et al. (80) found no differences in vitamin D status between alcoholic liver cirrhosis (ALC) and PBC patients receiving vitamin D supplements compared to those who did not, however this was not a deliberate intervention study and the dosage was unspecified. Based on the previously mentioned studies though, it appears that vitamin substitution benefits vitamin D deficient CLD patients and is able to raise 25(OH)D concentrations. These findings illustrate that inadequate 25-hydroxylation of vitamin D may not be the issue in these patients, but rather inadequate exogenous vitamin D supply.

Recently vitamin D deficiency has been linked to a low rate of sustained virological response (SVR) in HCV patients undergoing interferon-based therapy, and to more severe liver fibrosis (63, 64, 81). Conversely, some studies suggest vitamin D supplements improve treatment response in patients with chronic hepatitis C and increase the likelihood of achieving SVR (82, 83). For example, Abu Mouch et al. (83) conducted a RCT in which 27 of 58 HCV (genotype 1) infected patients received peginterferon/ribavirin plus 1000 - 4000 IU vitamin D per day (dosage based on aim of increasing serum 25(OH)D to > 32 ng/ml). The standard of care (SOC) group received peginterferon/ribavirin only. At 24 weeks post treatment, the percentage of patients who were HCV-RNA negative differed significantly ($P < 0.001$) between the vitamin D + SOC (86%) and SOC (41%), indicating that vitamin D might improve SVR when given as an adjunct to SOC. Vitamin D might exert its beneficial effects by increasing T cell activity (84). The anti-inflammatory properties of vitamin D could also assist through the reduction of inflammatory mediators such as IL-6 and TNF (85).

CURRENT RECOMMENDATIONS FOR VITAMIN D DEFICIENCY TREATMENT IN CLD

Many of the aforementioned studies recommend the inclusion of vitamin D assessment and replacement where necessary in the management of CLD. The risk of bone disease in CLD patients, particularly those with cirrhosis, warrants the routine use of vitamin D therapy, and though studies on the benefits of vitamin D supplementation are conflicting, the importance of adequate vitamin D concentrations remains from a preventative perspective. What has yet to be determined, however, is the optimal therapeutic dose, which may turn out to be individual-specific. Exogenous sources depend on dietary habits, supply of food fortification and the use of supplements containing vitamin D (e.g. fish oils) and the extent to which an individual spends time outdoors, in addition to type of clothing, use of sun protection, and geographical location. Ideally, all these factors should be considered on a case by case basis.

The general advice from the IOM and Endocrine Society Clinical Practice Guidelines is a recommended daily allowance (RDA) of 600 IU for adults aged 19 - 70 years, though this intake can be increased to 1,500 - 2,000 IU for patients at risk of a vitamin D deficiency (30, 36). The American Gastroenterology Association (AGA) and the Endocrine Society Clinical Practice guidelines specifically recommend adults with vitamin D deficiency be treated with 50,000 IU of vitamin D once weekly for a period of 8 weeks, in order to reach serum 25(OH)D levels > 30 ng/ml (2, 30). These guidelines unfortunately do not specifically refer to CLD but rather to the general population. Regarding specific guidelines for patients with CLD, BSG recommends supplementation of 1g calcium and 800 IU vitamin D per day as a general measure (4) and as previously mentioned, EASL recommends 400 - 800 IU vitamin D supplementation/day be considered in those with cholestatic liver diseases (5).

CONCLUSIONS

Vitamin D deficiency is highly prevalent in patients with CLD and inversely correlated with disease severity. Vitamin D deficiency or CLD may represent cause or effect, since multiple endogenous and environmental factors affect vitamin D metabolism. Benefits of normalized vitamin D status are emerging in patients with CLD, for example when vitamin D supplementation improves response to antiviral therapy and reduces rejection rates after liver transplantation. These benefits however, need to be substantiated in further RCTs. The majority of studies in patients with CLD have been cross-sectional, thus impeding the opportunity to study changes in vitamin D temporally with CLD progression. Many studies are based on small sample sizes or vary in their dosage and duration of vitamin D substitution. Unfortunately, given the inherent difficulties in precise analytical measurement techniques, we currently do not have an accurate assessment of vitamin D status in CLD patients. The observed widespread deficiency might in fact be more severe then previously thought, if we consider the overestimation in serum 25(OH)D levels owing to the presence of C-3 epimers. Whether intra- and extrahepatic vitamin D levels in patients with advanced liver diseases can be normalized by supplementation with 25(OH)D should be validated further, with optimal dose and duration for vitamin D replacement being determined. Thus, the road ahead must continue to be travelled. Until then, monitoring of serum 25(OH)D is reasonable in CLD, and lifestyle advice should form part of the crux of clinical practice in patients with vitamin D insufficiency. Finally, in patients with CLD, vitamin D might represent an affordable adjunct to SOC with limited side effects, having the potential to improve the patient's quality of life.

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Table 1. Sources of vitamin D

Food (naturally present)	Vitamin D_3: oily fish (e.g. salmon, mackerel, tuna, sardines), egg yolk Vitamin D_2: mushrooms
Food (fortified)	e.g. margarine, breakfast cereals, milk (global variation in fortified foods) May contain vitamin D_2 or vitamin D_3
Oral supplements	May contain vitamin D_2 or vitamin D_3
Sun exposure*	Photochemical conversion of 7-dehydrocholesterol to previtamin D_3

*Daily sunlight exposure for 5 -15 minutes (between 10am - 3pm) at latitudes above 37^{o} during spring, summer and autumn is suggested as adequate for individuals with lighter skin (86).

Table 2. Examples of serum 25-hydroxyvitamin D deficiency levels in patients with chronic liver diseases

Author, date, place	Study population characteristics	Sample size and type of CLD	25(OH)D cut off (ng/ml)	Time of year 25(OH)D measured	Main outcomes
Putz-Bankuti et al. Austria (43)	68% Male Mean age 58 +/- 11 years	**N = 75** Alcoholic cirrhosis (46) Chronic HCV cirrhosis (14) NAFLD/cirrhosis (10) Genetic CLD/cirrhosis (5)	**< 20 = deficiency**	July - December	Mean 25(OH)D 16.0 +/- 9.2ng/ml 25(OH)D < 20 ng/ml in 71% No significant difference in 25(OH)D between patients with alcoholic cirrhosis and cirrhosis of non-alcoholic origin (17.2 +/- 9.3 vs. 15.4 +/- 9.4 ng/ml, P = 0.480). 25(OH)D levels inversely correlated with Child-Pugh score (r = -0.21, P = 0.080)
Barchetta et al. (46) Italy	**NAFLD:** 55% Male Mean age 52 +/- 8 years **non-NAFLD:** 51% Male;Mean age 50 +/- 8 years	**N = 262** NAFLD (162) non-NAFLD (100)	**> 20 = normal**	NA	25(OH)D levels < 20 ng/ml independently associated with NAFLD & significantly lower than in non-NAFLD (14.8 ± 9 vs. 20.5 ± 9 ng/ml, P < 0.001, OR 0.95, 95% CI 0.92-0.98)
Bitetto et al. (64) Italy	52% Male Mean age 47 (range: 18 - 77)	**N = 211** ***Chronic HCV***	**≥ 20 = normal** 20 - 10 = deficiency < 10 = severe	All seasons	Median 25(OH)D 20.7 ng/ml 25(OH)D > 20 ng/ml in 54%; < 20 ng/ml in 46% (of which 16% ≤ 10 ng/ml)
Lange et al. (81) Germany	52% Male Mean age 45 (range: 22 - 72)	**N = 468** ***Genotype 1 chronic HCV*** **(+ 6000 age matched healthy controls)**	**≥ 20 = normal** 20 - 10 = deficiency < 10 = severe	NA	Mean 25(OH)D 17 ng/ml (range: 3 - 80) 25(OH)D > 30 ng/ml in 8% of HCV patients vs. 29% of controls 25(OH)D < 20 ng/ml in 66% of HCV patients vs. 41% of controls (P < 0.00001) 25(OH)D < 10 ng/ml in 25% of HCV patients vs. 12% of controls (P < 0.00001)
Malham et al. (80) Denmark	Gender, age - unspecified	**N = 123** PBC (34) ALC (89) - 4 patients with ALC & 13 PBC received vitamin D at time of blood sampling, however no significant 25(OH)D levels reported.	**≥ 20 = normal** 20 - 10 = insufficient 10 - 5 = deficiency < 5 = severe	All seasons	25(OH)D < 5 ng/ml in 18% of ALC patients, compared to 0% in PBC group 25(OH)D 10-5 ng/ml = 37% in ALC & 16% in PBC patients, respectively 25(OH)D 20-10 ng/ml = 30% in ALC & 41% in PBC patients, respectively Median 25(OH)D in ALC was 53% lower than PBC (9.6 ng/ml vs. 18 ng/ml, P < 0.001) Inverse association between severity of cirrhosis (Child-Pugh score) and vitamin D deficiency (contingency coefficient C = 0.29, P < 0.05, Chi^2 test)

Arteh et al. (3) USA	Caucasians (55%) African Americans (45%) 50% Male Mean age 53 +/- 9 years	**N = 118** HCV cirrhosis (43) HCV no cirrhosis (57) non HCV-related cirrhosis (18)	**≥ 32 = normal** 20 - 32 = insufficient 19 - 7 = deficiency < 7 = severe	NA	92% had 25(OH)D < 32ng/ml Logistic regression model showed independent risk factors for vitamin D <7 ng/ml: - female gender (OR 3.8, 95% CI 1.23-11.7, P = 0.002) - African American race (OR 8.43, 95% CI 2.5-27.8, P = 0.0001) - cirrhosis (OR 4.7, 95% CI 1.5-15.53, P = 0.01)

Author, date, place	Study population characteristics	Sample size and type of CLD	25(OH)D Cut off (ng/ml)	Time of year 25(OH)D measured	Main outcomes
Miroliaee et al. (41) Iran	**CLD group** 56% Male Mean age 42.3 +/- 12.2 years **Control group** 60% Male Mean age 40.98 +/- 9.29 years	**N = 90** ***non-cholestatic CLD*** *cirrhosis (51)* *no cirrhosis (39)* HCV (28) HBV (26) AIH (19) Cryptogenic (17) **(+40 healthy controls)**	**≥ 32 = normal** 31 - 20 = insufficient < 20 = deficiency	NA	Significantly higher prevalence of vitamin D deficiency in cirrhotic versus nonchirrhotic patients (76.5 vs. 17.9%, P < 0.001) Child-Pugh class B and C had significantly lower vitamin D levels than class A (P < 0.001)
Rode et al. (42) Australia	European, Anglo-Saxon, Asian descent (% unspecified) 52% Male Mean age 54 +/- 15.6 years	**N = 158** *cirrhosis* (65) *no cirrhosis* (93) Viral (60) NASH (23) Alcoholic (22) Autoimmune (12) Hemochroma (9) Cholestatic (5) Wilson's (2) Other (25)	**≥ 22 = normal** 22 - 10 = deficiency < 10 = severe	November - July	49% had 25(OH)D 22-10 ng/ml 15% had 25(OH)D < 10 ng/ml Patients with cirrhosis were more likely to be deficient in 25(OH)D (75%, P = 0.028)
Petta et al. (63) Italy	53% Male Mean age 52 +/- 12 years	**N = 197** ***G1 chronic HCV*** **(+ 49 matched healthy controls)**	**> 30 = normal**	All seasons	73% of G1HCV patients had 25(OH)D < 30 ng/ml vs. 6% in controls (P < 0.001) 25(OH)D significantly lower in G1HCV patients vs. controls (25.1 ± 9.9 vs. 43.1 ± 10.2 ng/ml, P < 0.0001)
Fisher et al. (87) Australia	50% Male Mean age 65.5 +/- 17.7 years	**N = 90** ***Hepatobiliary (and pancreatic) disorders*** undergoing ERCP	**≥ 32 = normal** 32 - 20 = insufficient 19 - 10 = deficiency < 10 = severe	NA	25(OH)D < 32 ng/ml in 80%, of which: 19-10 ng/ml in 46%; <10 ng/ml in 12%
Fisher et al. (37) Australia	63% Male Mean age 49 +/- 12.1 years	**N = 100** ***noncholestatic CLD*** *cirrhosis* (51) Alcohol (40) HCV (38)	**≥ 32 = normal** 32 - 20 = insufficient 19 - 10 = deficiency < 10 = severe	NA	25(OH)D < 32ng/m in 91%; 32-20 ng/m in 23%; < 19ng/ml in 68% Higher prevalence of vitamin D deficiency in presences of cirrhosis vs. no cirrhosis (86 vs. 49%, P = 0.0001) Correlation of vitamin D deficiency with severity of CLD (Child-Pugh class C vs. A (9 +/- 4 vs. 18.3 +/- 6.7, P < 0.001)

		HBV (12) AIH (4) Hemochromatosis (4) NASH (2)			
Author, date, place	**Study population characteristics**	**Sample size and type of CLD**	**25(OH)D cut off (ng/ml)**	**Time of year 25(OH)D measured**	**Main outcomes**
Targher et al. (45) Italy	67% Male Mean age 47 +/- 3 years	**N = 120** **NAFLD (60)** **matched controls (60)** (excluded advanced CLD patients)	NA	November - March	Low 25(OH)D levels independently predicted NAFLD with logistical regression analysis (OR 2.3, 95% CI 1.4-5.1, P < 0.001) Mean 25(OH)D 20.4 +/- 9 ng/ml (NAFLD) vs. 29.8 +/-6 ng/ml (controls) , P < 0.001. Control group (29.8 +/-6 ng/ml) vs. Simple Steatosis group (n=10) (23.8 +/-8 ng/ml) vs. NASH group, (n=50) (14.8 +/-9 ng/ml) P < 0.001
Crawford et al. (44) Australia	72% Male Mean age 50.3 +/- 0.9 years	**N = 113** *Cirrhosis* HCV (30) HBV (20) ALC (19) PSC (8) PBC (7) CAH (5) Other (24)	**48 - 16 = normal**	NA	Significant decrease in 25(OH)D with increaseing severity of cirrhosis (r= -0.38, P < 0.001) Two thirds of patients with Child-Pugh classes B & C had 25(OH)D < 16 ng/ml
Monegal et al. (39) Spain	67% Male Mean age 50 +/- 7.6 years	**N = 58** *HCV/HBV had cirrhosis* HCV (35) ALC (14) PBC (6) HBV (3) **(+ 29 healthy controls)**	**> 20 = normal**	All seasons	64% of CLD patients had 25(OH)D < 20 ng/ml 25(OH)D significantly lower in cirrhotic patients vs. controls (10.3 ± 9.1 vs. 23.1 ± 8.8 ng/ml, P = 0.000)
Chen et al. (38) Taiwan	**CLD Group** 100% Male Mean age 64.1 +/- 9.9 years **Control group** 100% Male Mean age 63.6 +/- 9.0 years	**N = 74** *Cirrhosis* HBV (39) HCV (15) Cryptogenic (10) ALC (9) Metrotrexate-induced (1) **(+ 16 healthy controls)**	NA	NA	Non significant difference in 25(OH)D between patients vs. controls (23.3 +/- 13.2 vs. 28.1 +/- 8.3, P = 0.09) Signficant difference in 25(OH)D in Child-Pugh classes B & C vs. controls (P < 0.05 & P < 0.0001 respectively)

Abbreviations used: ALC: alcoholic liver cirrhosis; AIH: autoimmune hepatitis; CAH: chronic autoimmune hepatitis; CI: Confidence Inverval; CLD: chronic liver disease; ERCP: endoscopic retrograde cholangiopancreatography; G1HCV: genotype 1 hepatitis C virus; HBV: hepatitis B virus; HCV: hepatitis C virus; NA: not available; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; OR: odds ratio; PSC: primary sclerosing cholangitis; PBC: primary biliary cirrhosis; 25(OH)D: 25-hydroxyvitamin D

Figure Legends

Figure 1. Metabolism of vitamin D.

UVB, ultraviolet B light; DBP: vitamin D binding protein; 25(OH)D: 25-hydroxyvitamin D; $1,25(OH)_2D$: 1,25-hydroxyvitamin D; $24,25(OH)_2D$: 24,25-dihydroxyvitamin D; $1,24,25(OH)_3D$: 1,24,25-trihydroxyvitamin D; 25-OHase: 25-hydroxylase; 24-OHase: 24-hydroxylase

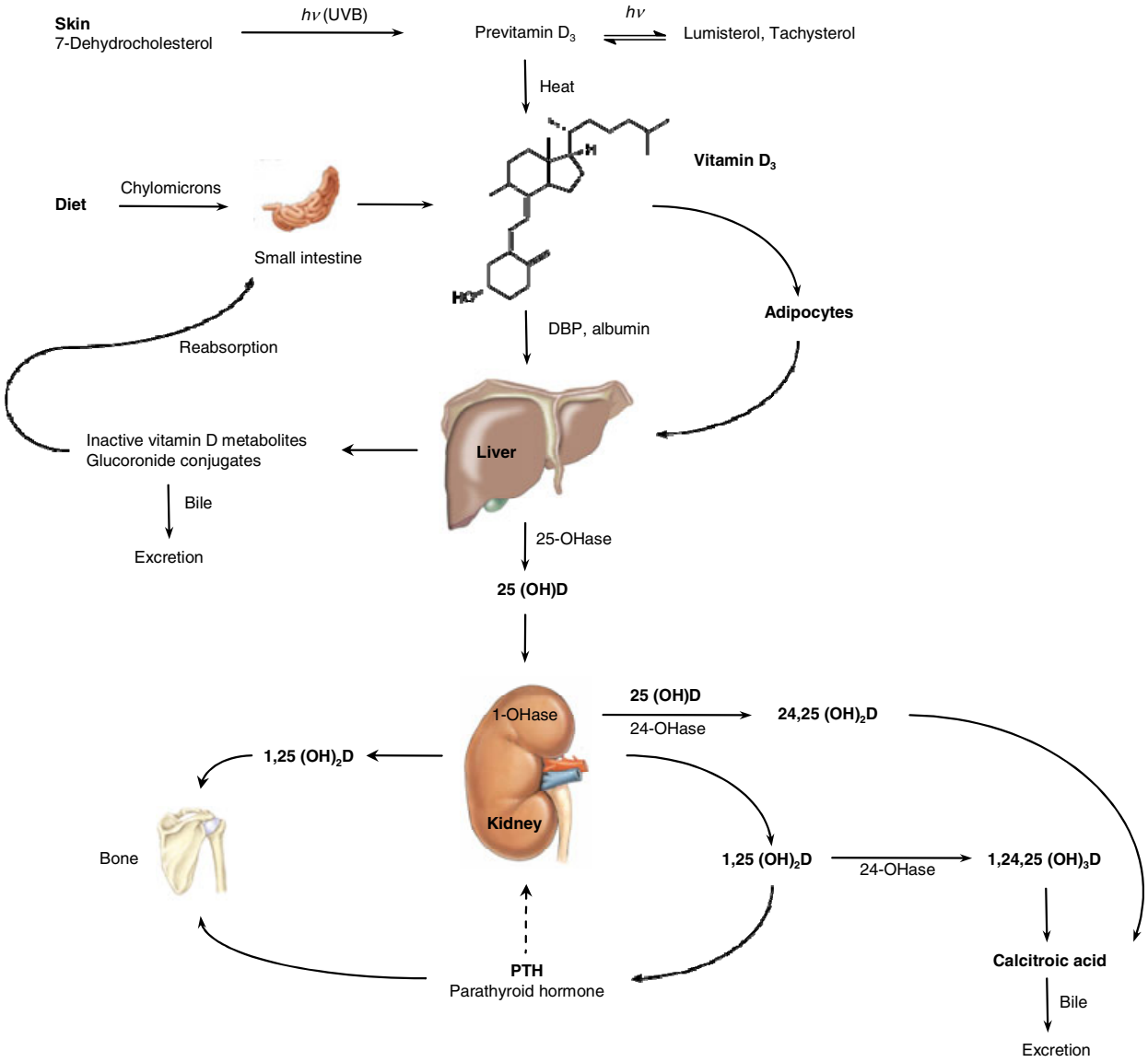
Figure 2. Metabolic pathways of vitamin D.

CYP2R1: cytochrome P450 family 2, subfamily R, polypeptide1; CYP2D11: cytochrome P450, family 2, subfamily d, polypeptide 11; CYP2D25: cytochrome P450, family 2, subfamily d, polypeptide 25; CYP27B1: cytochrome P450, family 27, subfamily b, polypeptide 1; CYP24A1: cytochrome P450, family 24, sufamily a, polypeptide 1.

Figure 3. Epimerization of 25-hydroxyvitamin D. An equivalent reaction takes place for 1,25-dihydroxyvitamin D.

Figure 4. Serum 25-hydroxyvitamin D concentrations (ng/ml) in patients with cirrhosis, stratified by Child-Pugh score.

The studies by Crawford et al. (44), Fisher et al. (37), Chen et al. (38) and Putz-Bankuti et al. (43) show that patients with severe cirrhosis (Child-Pugh class C) have approximately half the amount of serum 25-hydroxyvitamin D concentrations than patients with less severe cirrhosis (Child-Pugh class A). Due to different data presentation methods, only four of the studies mentioned in this review were compared.



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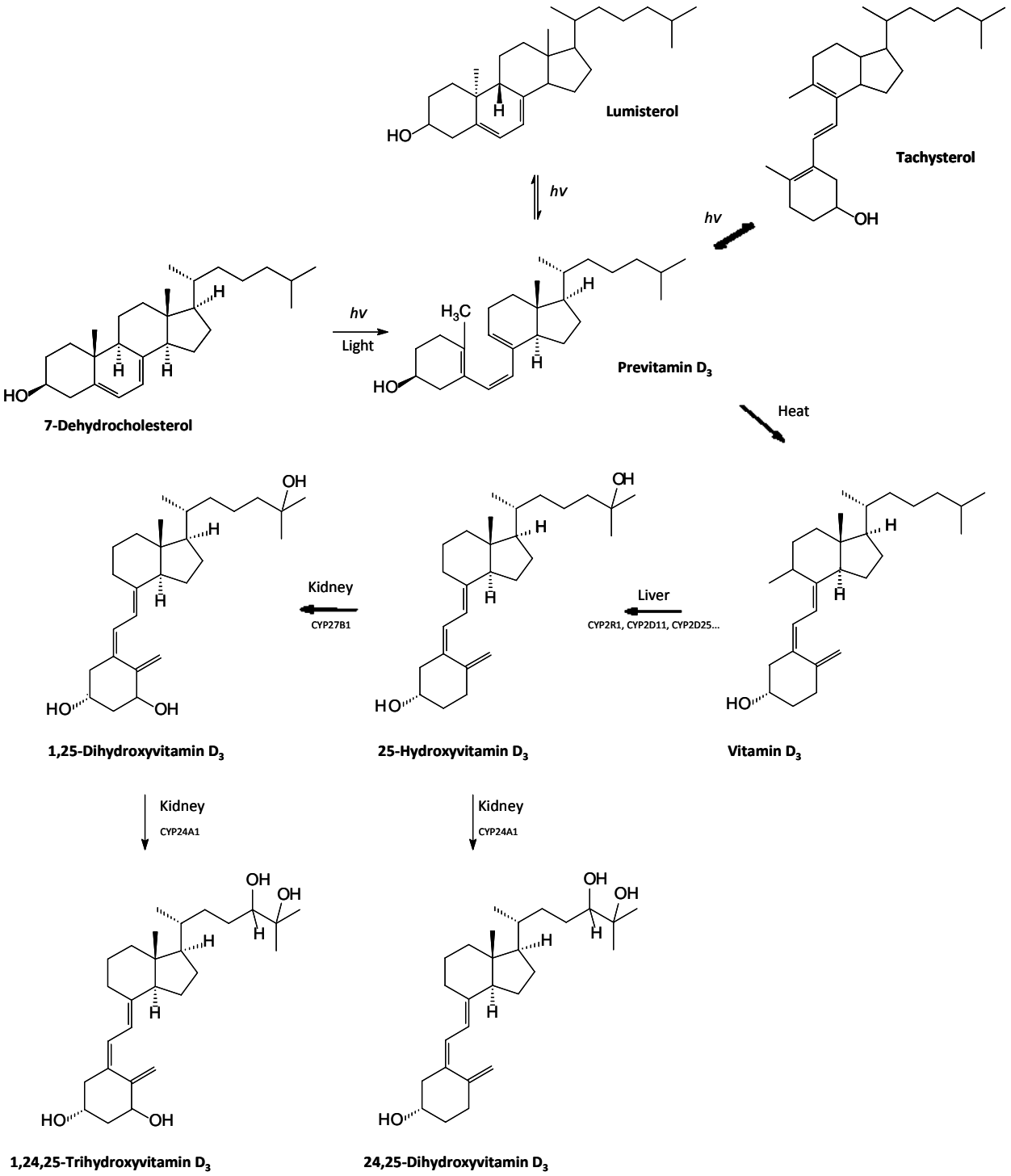
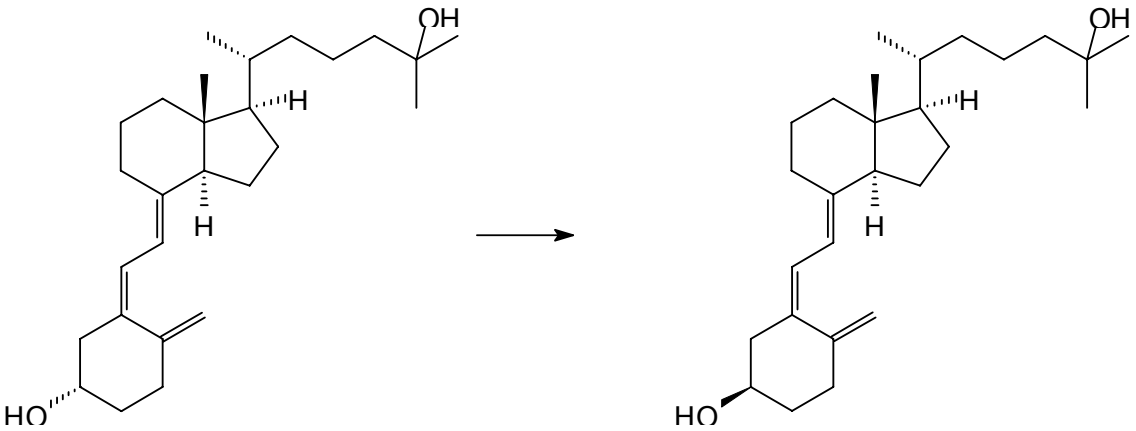


Figure 2. Metabolic pathways of vitamin D.



25-Hydroxyvitamin D **3-epi-25-Hydroxyvitamin D**

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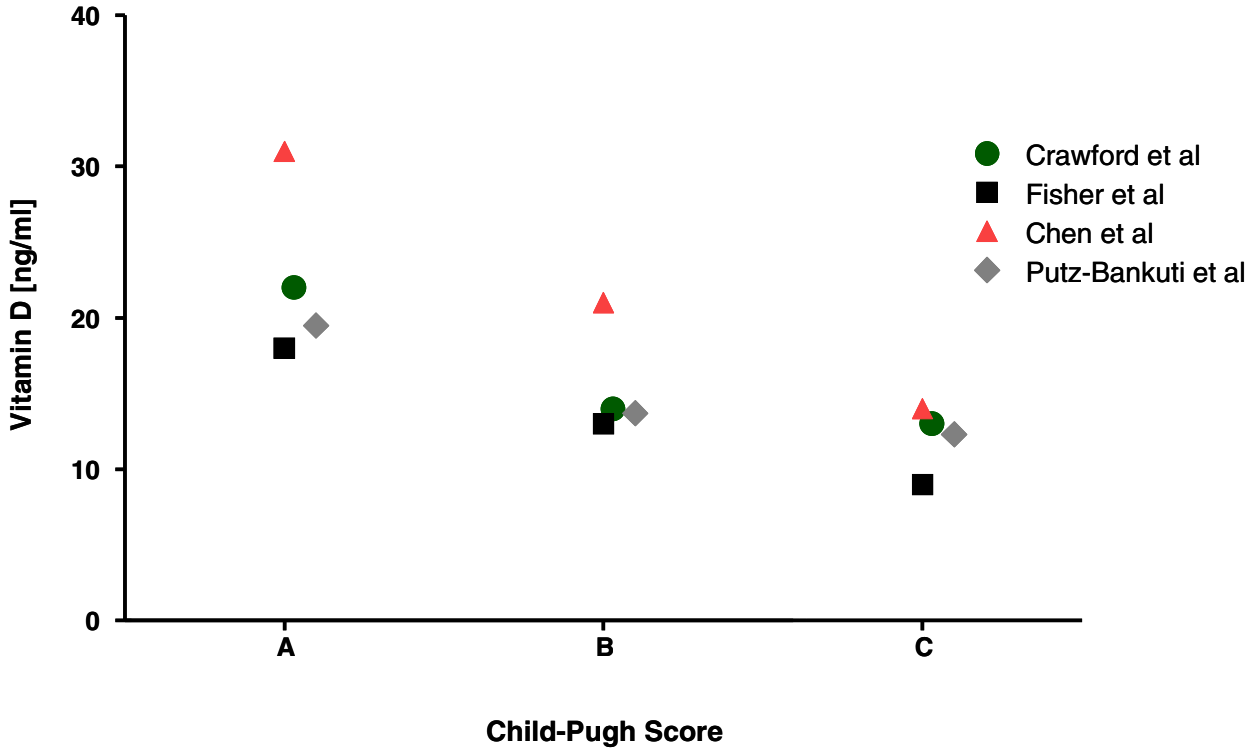

Figure 4. Serum 25-hydroxyvitamin D concentrations (ng/ml) in patients with cirrhosis, stratified by Child-Pugh score.