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Low 25-Hydroxyvitamin D Level is Independently Associated with Non-Alcoholic Fatty Liver Disease

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Abstract

Background and Aims—We sought to explore associations between serum 25-hydroxyvitamin D [25(OH)D] levels and non-alcoholic fatty liver disease [NAFLD] in an integrated healthcare delivery system in the U.S.

Methods and Results—607 NAFLD cases were randomly matched 1:1 with controls for age, sex, race and season of measurement. Conditional logistic regression was used to evaluate if serum 25(OH)D levels were associated with increased odds of NAFLD (diagnosed by ultrasound) after adjusting for body mass index and history of diabetes, renal, peripheral vascular and liver diseases (model 1) and also for hypertension (model 2). Mean (SD) serum 25(OH)D level was significantly lower in the group with NAFLD as compared with that in the matched control group (75±17 vs. 85±20 nmol/L [30±7 vs. 34±8 ng/mL], $P<0.001$). Inadequate 25(OH)D status progressively increased the odds of NAFLD when classified categorically as sufficient (25(OH)D 75 nmol/L [>30 ng/mL], reference group), insufficient (37–75 nmol/L [15–30 ng/mL]; adjusted odds ratio [OR]: 2.40, 95% confidence interval [CI]: 0.90–6.34) or deficient (<37 nmol/L [<15 ng/mL]; adjusted OR: 2.56, 95% CI: 1.27–5.19). When modeled as a continuous variable, increased $\log_{10}$ 25(OH)D was inversely associated with the risk of prevalent NAFLD (adjusted OR: 0.25, 95% CI: 0.064–0.96, $P=0.02$).

Conclusion—Compared with matched controls, patients with NAFLD have significantly decreased serum 25(OH)D levels, suggesting that low 25(OH)D status might play a role in the development and progression of NAFLD.

Keywords

inflammation; fatty liver; NAFLD; risk factors; vitamin D; 25(OH)D

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INTRODUCTION

Non-alcoholic fatty liver disease [NAFLD] has reached epidemic proportions in Western societies such as the United States, affecting approximately 20 to 30% of adults [1–3], with frequency increasing to ~70–80% in type 2 diabetes [4] and ~90% in severe obesity [5]. As these frequencies are largely based upon diagnosis by liver ultrasound, which is not (totally) sensitive when liver fat infiltration is <30% [6], the actual prevalence of NAFLD is likely even greater. NAFLD encompasses a spectrum of liver diseases, ranging from pure steatosis (fatty liver) to non-alcoholic steatohepatitis (NASH; characterized by liver inflammation and fibrosis) and cirrhosis, and represents the most common cause of chronic liver disease in adults in Western societies [6].

In parallel, vitamin D₃ insufficiency or deficiency, as indicated by low circulating levels of serum 25-hydroxyvitamin D [25(OH)D], is a highly prevalent pathologic condition across various populations, and is associated with increased systemic markers of inflammation [7–10], which may be at least partially reduced by vitamin D₃ supplementation [11–14]. Systemic, chronic inflammation appears also to be central in the pathogenesis of NAFLD, as it may promote the development of intra-hepatic necro-inflammation [5]. NAFLD may, in turn, exacerbate the systemic, chronic inflammatory state by releasing several pro-inflammatory factors from the steatotic and inflamed liver, thus perpetuating a vicious cycle [15]. Thus, it is also plausible to speculate that circulating serum 25(OH)D levels might play a role in the development and/or progression of NAFLD via its potentially anti-inflammatory properties. Indeed, rodent models support that 25(OH)D deficiency plays a role in the progression of NAFLD [16, 17]. There is, however, limited quantitative evidence regarding the relation between low serum 25(OH)D levels and NAFLD (as diagnosed by either ultrasound or biopsy) in large samples of individuals, including U.S. cohorts, as the few existing studies have been conducted in limited Italian patient populations [18, 19].

The aim of this study was to examine the association between serum 25(OH)D levels and NAFLD as diagnosed by liver ultrasound (the most widely used imaging test for detecting hepatic steatosis in clinical practice) using a matched cohort for age, sex, race and season of blood draw. We hypothesized that inadequate 25(OH)D status would be associated with increased odds of NAFLD, after adjustment for important clinical risk factors.

METHODS

Data source

A retrospective cohort study was performed using the Intermountain Healthcare Enterprise Data Warehouse, which incorporates comprehensive electronic health and administrative data. Intermountain Healthcare is a non-profit organization with 23 hospitals and over 150 outpatient clinics and averages 130,000 admissions annually. Its facilities range from major adult tertiary-level care centers to small clinics and hospitals that are the only source of care in rural communities.

Study Population

The study sample included all adult patients who had a serum 25(OH)D measurement and a diagnosis of NAFLD between January 1, 1999 to December 31, 2009. We matched 607 patients with NAFLD (cases) 1:1 with healthy controls for age, sex, race and season of blood draw. Of note, the 607 control participants were chosen from 62,757 adults randomly selected without a diagnosis of NAFLD by ICD9 code, but did not have a liver ultrasound examination to exclude hepatic steatosis. All participants (controls and cases) were required to have administrative and clinical data in the Intermountain Healthcare system at least 3 months prior to the measurement of serum 25(OH)D level. We excluded patients who were

<18 years old, pregnant, end-stage renal disease patients on dialysis, and individuals with an outpatient or inpatient diagnosis by ICD9 code of liver disease secondary to alcohol abuse that included: alcoholic liver disease, alcoholic cirrhosis and unspecified alcoholic liver damage (571.0; 571.2; 571.3). The Institutional Review Board at Intermountain Healthcare System and University of Colorado approved the project.

Primary predictor and outcome

The primary predictor was serum 25(OH)D level, which was measured in all participants using an INCSTAR 25(OH)D two step assay procedure with a coefficient of variation of less than 10%. The same methodology for the measurement of serum 25(OH)D was employed during the entire 10-year period (1999–2009). The first step in the procedure involves the rapid extraction of 25(OH)D from the serum using acetonitrile. Following extraction, the treated sample is assayed by using an equilibrium radioimmunoassay procedure. This method is based on an antibody with specificity to 25(OH)D. The sample, antibody, and tracer are incubated at 20–25°C for ninety minutes. A second antibody-precipitating complex is used to achieve phase separation. The radioimmunoassay method tends to overestimate the level of 25(OH)D because the antibody recognizes all forms of dihydroxy-vitamin D and D steroids.

The outcome of interest was the presence of NAFLD. All cases of NAFLD were identified by having an ICD9 code for NAFLD (571.8 and 571.9) in an outpatient or inpatient claim and by having hepatic steatosis on ultrasound within 3–15 months of the assessment of subject characteristics and measurement of serum 25(OH)D levels. Liver ultrasonography was performed through longitudinal and transverse scanning of the right upper quadrant with the patient in both the supine and left lateral decubitus positions. A single expert radiologist reviewed the video recordings of these ultrasounds evaluating the liver imaging for evidence of hepatic steatosis based on standard criteria (i.e., liver parenchymal brightness, liver-to-kidney contrast, deep beam attenuation, brightness of vessel walls, and definition of the gallbladder wall) [22–24]. The intra-observer variability for the ultrasound diagnosis of steatosis was within 3% [25].

Statistical analyses

All statistical analyses were performed with SAS software, version 9.13 (SAS Institute, Cary, NC). Cases were matched 1:1 with controls on age, sex, race and season of blood drawn. Chi Square test of independence was used to test the distribution of categorical variables between cases and controls. Wilcoxon Rank Sum test was used to test for differences in age, body mass index (BMI), serum 25(OH)D level and serum aminotransferases. Race was reduced to non-Hispanic white, Hispanic, and other due to low frequencies in some of the categories. Season was classified as winter, spring, summer and fall. Serum 25(OH)D levels were logarithmically transformed to improve the normality of the distribution. Serum 25(OH)D levels were classified as <37, 37–75 and >75 nmol/L [<15 , 15–30 and >30 ng/mL]. These thresholds were chosen in keeping with the definition of 25(OH) D deficiency and insufficiency published in previous reports [26]. Serum 25(OH)D levels were also evaluated continuously as $\log_{10}$ 25(OH)D.

Two conditional logistic regression models were used to evaluate if serum 25(OH)D levels, which were included as either a categorical or continuous variable, were associated with increased odds of NAFLD. Model 1 was adjusted for BMI, history of diabetes mellitus, renal disease, peripheral vascular disease and liver diseases. Since cases were matched to controls for age, sex, race and season of blood drawn, it was unnecessary to adjust for these variables. Since the presence or absence of hypertension was available only in a sub-sample of subjects ($n=390$ cases and $n=258$ controls), a second model was also performed including

hypertension as a covariate (model 2; all covariates above plus hypertension). Of note, all confounding variables were part of the Charlson Comorbidity Index score [27]. All *P* values presented are two-sided.

RESULTS

Clinical characteristics

The clinical characteristics of NAFLD cases and control subjects are shown in Table 1. Since the experimental design matched cases and controls for age, sex, race and season, these variables were almost identical between the groups. BMI and the prevalences of diabetes mellitus, peripheral vascular disease and liver diseases were significantly higher in cases than controls ($P < 0.005$ for all), whereas the frequency of renal disease did not significantly differ between the groups. Compared with controls, cases also had significantly higher serum aminotransferase levels ($P < 0.0001$ for both). Hypertension diagnosis was not different between cases and controls in the sub-group of patients ($n=390$ cases and $n=258$ controls) in which these data were available.

Notably, serum 25(OH)D levels were significantly lower in the group of NAFLD patients as compared to controls (Figure 1; $P = 0.0003$). When serum 25(OH)D level was characterized as either <75 or <37 nmol/L [<30 ng/mL or <15 ng/mL], the prevalence of vitamin D insufficiency or deficiency was greater in cases than in controls ($P = 0.001$ and 0.02 , respectively).

Relation between low vitamin D and NAFLD

Serum 25(OH)D level was categorized as deficient (<37 nmol/L [<15 ng/mL]), insufficient ($37-75$ nmol/L [$15-30$ ng/mL]) or sufficient (>75 nmol/L [>30 ng/mL]). As shown in Figure 2, insufficient ($37-75$ nmol/L [$15-30$ ng/mL]) or deficient (<37 nmol/L [<15 ng/mL]) serum 25(OH)D levels were associated with a significantly increased odds of NAFLD in the unadjusted regression model compared to the reference group of those with a serum 25(OH)D level >75 nmol/L [>30 ng/mL]. After adjusting for BMI, history of diabetes, renal disease, peripheral vascular disease and liver diseases (model 1), the lowest two serum 25(OH)D groups still were associated with an increased odds of NAFLD compared to the reference (vitamin D sufficient) group. Almost identical results were obtained even when hypertension was added to the multivariate regression model (model 2).

As shown in Table 2, when serum 25(OH)D level was evaluated linearly there was a significant, inverse relation between serum 25(OH)D level and the odds of NAFLD [OR: 0.35; 95% CI: 0.21–0.60, $P = 0.0001$ for every $\log_{10}$ increase in 25(OH)D]. This relation remained essentially unchanged after adjustment for BMI, history of diabetes, renal disease, peripheral vascular disease and liver diseases [adjusted OR: 0.43; 95% CI: 0.20–0.93; $P = 0.03$ for every $\log_{10}$ increase in 25(OH)D] and even after additional adjustment for hypertension (adjusted OR: 0.25; 95% CI: 0.10–0.96; $P = 0.02$). Results were similar when we excluded patients with liver diseases [model 2 adjusted OR: 0.21; 95% CI: 0.04–0.96, $P = 0.04$ for every $\log_{10}$ increase in 25(OH)D]. In addition, there was no interaction effect of 25(OH)D * renal disease on NAFLD ($p=0.24$ [model 1]), indicating that patients with renal disease did not respond differently to 25(OH)D with regard to NAFLD risk compared to the overall cohort.

DISCUSSION

In this case-control study, we have demonstrated for the first time that a lower serum 25(OH)D level is associated with the presence of NAFLD on ultrasound, independently of age, sex, race, season, BMI, history of diabetes mellitus, renal disease, peripheral vascular

disease, liver diseases and hypertension in a large sample of U.S. adults. Whether evaluated as either a continuous variable or categorized in clinically significant cut-offs, lower serum 25(OH)D level was independently associated with an increased odds of NAFLD. While the present study cannot draw any firm conclusions regarding causality, the association of lower serum 25(OH)D levels with NAFLD independent of numerous confounding factors suggests that inadequate 25(OH)D status might play a role in the development and/or progression of NAFLD.

Our results are consistent with recent observations of some smaller studies performed in Italy demonstrating that patients with NAFLD (as diagnosed by either ultrasound or biopsy) have significantly lower serum 25(OH)D levels than control subjects without steatosis [18] [19]. In fact, serum 25(OH)D level was inversely associated with NAFLD on ultrasound in 262 consecutive patients referred to the Diabetes and Metabolic Diseases clinics for metabolic evaluation, independent of metabolic syndrome features [18]. Furthermore, in 60 consecutive patients with biopsy-proven NAFLD (the majority of them with NASH) serum 25(OH)D level was significantly lower than in healthy controls matched for age, sex and BMI, and the histological severity of NAFLD was inversely associated with serum 25(OH)D levels, independent of potential confounding factors [19]. Our results extend these previous findings by demonstrating a strong, inverse relation between NAFLD and serum 25(OH)D levels: a) in a U.S. population derived from an integrated healthcare delivery system; b) in patients not being evaluated for the metabolic syndrome; c) in patients with less severe NAFLD, and d) in the largest sample size to date with NAFLD diagnosis by ICD9 code and liver ultrasound.

The underlying mechanisms accounting for the relation between low serum 25(OH)D levels and NAFLD have still not been fully elucidated. A plausible explanation for our findings is that the low levels of 25(OH)D found in NAFLD may simply reflect an 'unhealthy' lifestyle (inadequate diet or poor sunlight exposure), which itself promotes the development and progression of NAFLD, or, alternatively, the close association of NAFLD with age, obesity and diabetes, which also relate to severity of hypovitaminosis D. However, since serum 25(OH)D level was inversely associated with NAFLD, independently of these shared risk factors, it could also be hypothesized that inadequate vitamin D status might, at least in part, contribute to the development and progression of NAFLD.

Preliminary experimental evidence in rodents suggests that vitamin D deficiency may promote the development of NAFLD through increased inflammation. In fact, vitamin D deficiency in obese rats fed a Western diet exacerbates the development of NAFLD in part by increasing inflammation [28]. In contrast, when serum 25(OH)D levels are increased using UV light therapy, the progression of NAFLD in rats is slowed, as indicated by reduced histological evidence of inflammation [17]. Thus, experimental animal data are consistent with the possibility that a low serum 25(OH)D level might play a role in NAFLD pathogenesis, possibly via suppression of its anti-inflammatory properties. Indeed, chronic inflammation is considered a key factor in NAFLD progression [22, 24], with the severity of the hepatic disease relating to both circulating and intra-hepatic mRNA expression of several pro-inflammatory factors [5]. It has been hypothesized that increased visceral adiposity promotes the release of both free fatty acids and pro-inflammatory cytokines [15]. In turn, this causes activation of inflammation within the liver, possibly via increased nuclear factor κ B (NF κ B) activity, thus promoting downstream transcription of pro-inflammatory cytokines and ultimately perpetuating a vicious cycle [2]. Increased levels of serum 25(OH)D might act to reduce the development/progression of NAFLD by countering these inflammatory processes.

Across various adult populations, lower serum 25(OH)D levels are associated with higher circulating markers of inflammation [7–10, 29], and vitamin D₃ supplementation can effectively reduce these inflammatory markers, including C-reactive protein [9, 13, 30, 31], tumor necrosis factor- α [12] and interleukin-6 [31], and increase some anti-inflammatory cytokines, such as interleukin-10 [12]. In-vitro studies support the possibility that the anti-inflammatory effect of vitamin D₃ may be mainly mediated by inhibition of the pro-inflammatory transcription factor NF κ B [32–34]. This is consistent with the finding that inadequate 25(OH)D status in healthy adults is associated with increases in both vascular endothelial cell NF κ B protein expression and vascular NF κ B signaling [35]. Hence, low serum 25(OH)D levels may promote these inflammatory processes that are also involved in the development and progression of NAFLD.

An alternative explanation for our findings is that serum 25(OH)D level is possibly low in the presence of NAFLD due to impaired liver hydroxylation of vitamin D₃ to 25(OH)D. However, recent findings support that CYP27A1 and CYP2R1 enzyme expression, as well as the ability to hydroxylize vitamin D₃, are well persevered in NASH and do not contribute to lowered serum 25(OH)D levels [36]. Thus, it is unlikely that abnormal hepatic metabolism of vitamin D has any major contribution to our findings.

The present study has several strengths and limitations. While the results cannot show causality, they demonstrate a strong relation between lower serum 25(OH)D levels and the presence NAFLD diagnosed by ICD9 code and confirmed by liver ultrasound. These results are strengthened by the facts that the sample size was relatively large, cases and controls were well matched for age, sex, race and season of blood drawn (which are among the strongest determinants of serum 25(OH)D levels), and additional important confounding factors were included in our modeling. We recognize that we do not have complete data for the diagnosis of the metabolic syndrome in our multivariate regression models. However, the inclusion of BMI, diabetes and hypertension as covariates largely reflects the major components of the syndrome. While BMI and the prevalence of diabetes were significantly greater in NAFLD cases than matched controls, the prevalence of hypertension did not differ from cases. This is likely due to the high prevalence of hypertension in the controls, which would make it difficult to detect a difference from cases, who had a similar prevalence of hypertension to reports in other studies [37, 38].

Last, while liver biopsy is the gold standard for diagnosing NAFLD, it is not feasible to perform regularly in large epidemiological studies. As was the case in this study, liver ultrasound is most commonly used in clinical practice and has good sensitivity and specificity for detecting hepatic steatosis in individuals with moderate to severe NAFLD (>30% liver fat infiltration). Thus, some cases of NAFLD may have been misclassified as controls. In addition, since controls were selected on the basis of not having a diagnosis of NAFLD by ICD9 code and liver ultrasound was not performed in these patients to completely exclude hepatic steatosis, some additional cases of NAFLD may have been misclassified as controls. However, in both scenarios, the possible inclusion of some controls with NAFLD might have led to underestimation of the differences in serum 25(OH)D levels between cases and controls rather than the opposite, and therefore, we believe that it does not detract from the significance of our findings. We also acknowledge that previously recorded liver ultrasounds were analyzed by a single expert radiologist, rather than the original operator, which could potentially have limited accuracy. We do not feel that this is a significant limitation as this methodology is frequently employed in large population studies such as NHANES [39, 40], and ICD9 codes were used in addition to the diagnosis of NAFLD by liver ultrasound.

In conclusion, our results indicate that low serum 25(OH)D levels are associated with NAFLD, independent of age, sex, race, season of measurement, BMI, history of diabetes, renal disease, peripheral vascular disease, liver diseases and hypertension. Future studies are needed to prospectively determine if vitamin D supplementation reduces the development and progression of NAFLD, as well as the physiological mechanisms mediating any effects.

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Figure 1. Circulating 25(OH)D Levels in Cases vs. Controls

Box-plots of serum 25-hydroxyvitamin D [25(OH)D] levels in non-alcoholic fatty liver disease (NAFLD) cases vs. controls. Middle line indicates median, borders of the box indicate the 25th to 75th percentile, whiskers indicate the 10th and 90th percentile, and dots indicate 5th and 95th percentile. * $P < 0.0005$ vs. control.

Figure 2. Odds Ratios of NAFLD

Logistic regression odds ratios (OR; 95% confidence interval [CI]) of non-alcoholic fatty liver disease [NAFLD] according to serum 25-hydroxyvitamin D [25(OH)D] level compared to sufficient (>75 nmol/L [>30 ng/mL]) reference group (gray bars: insufficient (37–75 nmol/L [15–30 ng/mL]); white bars: deficient (<37 nmol/L [<15 ng/mL])). Regression model 1 is adjusted for BMI and history of diabetes, renal disease, peripheral vascular disease and liver disease. Regression model 2 is further adjusted for hypertension.

Table 1

Clinical Characteristics

Variable	N (case/ctrl)	Cases	Controls	P-value
Age (years)	607/607	55.75 ± 4.19	55.75 ± 14.27	0.99
Sex (% Female)	607/607	450 (74.14%)	450 (74.14%)	1.0000
Race (% Hispanic)	607/607	47 (7.86%)	47 (7.86%)	1.0000
Race (% Other)	607/607	12 (2.01%)	12 (2.01%)	1.0000
Race (% White)	607/607	539 (90.13%)	539 (90.13%)	1.0000
BMI (kg·m ⁻²)	406/478	33.4 ± 8.3	29.6 ± 7.3	< 0.0001
Diabetes (%)	607/607	216 (35.58%)	151 (24.88%)	< 0.0001
Renal Disease (%)	607/607	65 (10.71%)	61 (10.05%)	0.70
Peripheral Vascular Disease (%)	607/607	74 (12.19%)	43 (7.08%)	0.0029
Liver Diseases (%)	607/607	97 (15.98%)	49 (8.07%)	< 0.0001
Hypertension (%)	390/258	296 (75.90%)	214 (82.95%)	0.1613
ALT (U·L ⁻¹)	381/411	33 (24–50)	29 (22–37)	< 0.0001
AST (U·L ⁻¹)	382/413	33 (26–44)	28 (24–35)	< 0.0001
25(OH)D (nmol·L ⁻¹)	607/607	65 (45–88)	73 (53–95)	0.0003
25(OH)D < 75 nmol·L ⁻¹ (%)	607/607	369 (60.79%)	326 (53.71%)	0.0097
25(OH)D < 37 nmol·L ⁻¹ (%)	607/607	95 (15.65%)	65 (10.71%)	0.015

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; 25(OH)D, 25-hydroxyvitamin D. Data are mean (SD) or mean (% of total), except ALT, AST and 25(OH)D, which are median (interquartile range).

Table 2

Logistic Regression

Model	OR	95% CI	P-value
Unadjusted	0.35	0.21–0.60	0.0001
Model 1: adjusted for BMI, history of diabetes, renal disease, peripheral vascular disease and liver diseases	0.43	0.20–0.93	0.031
Model 2: Model 1 + adjustment for hypertension	0.25	0.10–0.96	0.022

BMI, body mass index; CI, confidence interval; OR, odds ratio.