Published in final edited form as:

Am J Gastroenterol. 2016 June ; 111(6): 852–863. doi:10.1038/ajg.2016.51.

Vitamin D Deficiency is Associated with Increased Risk of Nonalcoholic Steatohepatitis in Adults with Nonalcoholic Fatty Liver Disease: possible role for MAPK and NF- κ B?

James E. Nelson¹, Christian L. Roth², Laura Wilson³, Kathie Yates³, Bradley Aouizerat^{4,5}, Vicki Morgan–Stevenson⁸, Elizabeth Whalen¹, Andrew Hoofnagle⁶, Michael Mason¹, Vivian Gersuk¹, Matthew M. Yeh⁷, and Kris V. Kowdley^{1,8} for the NASH Clinical Research Network

¹Benaroya Research Institute at Virginia Mason Medical Center, Seattle WA

²Seattle Children's Research Institute, Seattle WA

³Johns Hopkins Bloomberg School of Public Health, Dept of Epidemiology, Baltimore, MD

⁴Department of Physiological Nursing, University of California at San Francisco, San Francisco, CA

⁵Institute for Human Genetics, University of California at San Francisco, San Francisco, CA

⁶Departments of Laboratory Medicine and Medicine, University of Washington, Seattle, WA

⁷Department of Pathology, University of Washington School of Medicine, Seattle WA

⁸Liver Care Network, Swedish Medical Center, Seattle, WA

Abstract

Objective—To determine the relationship of serum vitamin D deficiency to histologic features of NAFLD, and associated demographic, clinical, laboratory, and transcriptomic data in the well characterized NASH CRN cohort.

Methods—Serum vitamin D 25(OH)D (VD) was quantified by liquid chromatography-tandem mass spectrometry in 190 adults (>18 yrs) with biopsy-proven NAFLD. Subjects were categorized according to their level of VD as either sufficient (>30ng/ml), insufficient (20–30ng/ml), or deficient (VDD; <20 ng/ml). Multivariable logistic regression was used to investigate the association of VDD and the presence of definite nonalcoholic steatohepatitis (NASH) and individual histological features of NAFLD after adjusting for age, sex, race, BMI, ALT, and diabetes status. Hepatic transcriptomic data was compared between VDD and non-VDD subjects.

Results—VDD was present in 55% of subjects and was independently associated with definitive NASH (OR 3.15, 95% CI 1.62–6.15, $p=0.001$), increased lobular inflammation (OR=1.98, 95% CI,

Corresponding Author: Kris V. Kowdley, MD, 1101 Madison Street, Suite 200, Seattle, WA, (p) (206) 386-3606, (f) (206) 320-7431, kris.kowdley@swedish.org.

Disclosures: No conflicts of interest exist

Author contributions: study concept and design (JEN, CLR, KVK); acquisition of data (JEN, BA, LW, KY, AH, VG, MMY); analysis and interpretation of data (JEN, CLR, EW, MM, MMY, KVK); drafting of the manuscript (JEN); critical revision of the manuscript for important intellectual content (JEN, CLR, KVK); statistical analysis; (JEN, EW); obtained funding (JEN, BA, KVK).

1.08–3.61, $p=0.026$), more ballooning (OR=2.38, 95%CI, 1.32–4.30, $p=0.004$), and the presence of fibrosis (OR=2.32, 95%CI, 1.13–4.77, $p=0.022$). There was a significant inverse relationship between lower levels of serum resistin and increased VD level category ($p=0.013$). The *KRT10*, *SEMA3B*, *SNORD3C*, *ARSD*, and *IGKV4-1* genes were differentially expressed ($FDR<0.05$) between VDD and non-VDD subjects. Gene ontology and pathway analysis suggest activation of the MAPK and NF- κ B pathways in VDD NAFLD subjects.

Conclusions—VDD is prevalent among U.S. adult NAFLD patients and is independently associated with a definitive diagnosis of NASH and increased histological severity. Novel associations in pro-inflammatory pathways were identified that suggest the mechanism for VDD in the pathogenesis of NASH and support dietary and/or lifestyle modifications to increase vitamin D levels in these patients.

INTRODUCTION

Vitamin D, specifically the biologically active vitamin D metabolite 1,25-dihydroxyvitamin D [calcitriol: 1,25(OH) $_2$ D], is known to exert important physiological effects in addition to well-known effects on calcium metabolism. Vitamin D receptors (VDRs) are ubiquitously expressed in several tissues, including gut, liver, adipose tissues, cardiac and skeletal muscles, β -cells, and immune cells such as lymphocytes, dendritic cells, and monocytes/macrophages(1). Vitamin D3 (VD3) is inversely associated with obesity and insulin resistance (IR), and there is increasing evidence that vitamin D deficiency (VDD) may contribute to the development of diabetes mellitus, and the metabolic syndrome (MetS) (2, 3). VDD may also have a potential role in autoimmune and inflammatory processes through production of pro-inflammatory cytokines (2–4). VDD continues to be largely undertreated in children and adults worldwide (4). Recently, in adolescents in the U.S., 14% to 55% were reported to be vitamin D deficient with 25-hydroxy vitamin D (25(OH)D) concentrations < 20 ng/mL(5, 6). In the US, obese Black Americans are at particularly high risk for VDD; higher rates of VDD, obesity and T2DM were found in African Americans as compared to Caucasians (7, 8). Recent studies of VDD in humans and animal models indicate that VDD also contributes to increased oxidative stress, systemic inflammation, decreased adiponectin levels, toll-like receptor activation and non-alcoholic fatty liver disease (NAFLD)(2, 9–13).

NAFLD is the most common liver disease in developed countries, affecting one of every three adults (14) and about 10% of children (15). NAFLD has emerged as a major risk factor for diabetes and cardiovascular disease, independent of obesity (16, 17). Given the potential for NAFLD to progress to NASH, the more severe form of the disease, with the risk of further progression to cirrhosis and hepatocellular cancer, it is imperative that metabolic risk factors and pathological mechanisms are identified. VDD has emerged as a risk factor for NAFLD; several large epidemiologic studies using surrogate markers of NAFLD such as alanine aminotransferase (ALT) levels or abdominal imaging studies, suggest VDD is prevalent among cases of suspected NAFLD compared to controls without evidence of liver disease (18–20). However, this association failed to persist upon multivariate analysis controlling for covariates in some studies (19–20). A recent meta-analysis concluded that NAFLD patients have decreased 25(OH)D levels compared to controls (21). VDD has also been associated with an increased likelihood of steatosis, fibrosis, and necroinflammation in

both children and adults in biopsy-proven NAFLD (2, 22–25), but other studies have failed to find an association (26, 27). Two recent rat studies by our group (9) and Nakano et al (28) showed that VDD leads to insulin resistance, increased inflammation and exacerbates the severity of NAFLD. Nakano et al also showed that phototherapy leading to increased VD levels can improve hepatocyte apoptosis, inflammation, fibrosis, and IR (28). In our previous rodent study we found activation of the NF- κ B pathway and increased hepatic expression of Toll-like receptor (TLR) genes TLR-2, -4 and -9 as well as resistin, hemeoxygenase, and interleukins (IL)-1 β , -4, -6, suggesting that VDD may contribute to NASH pathogenesis through TLR activation and stimulation of a downstream inflammatory response (9).

The goal of this study was to determine the relationship of serum vitamin D levels to histologic features of NAFLD, and associated demographic, clinical, transcriptomic and laboratory data in the well characterized multi-center NASH CRN cohort.

METHODS

Study population

This study was approved as an ancillary study of the NASH CRN. All subjects gave written informed consent and the study was approved by the institutional review board at each local site of the NASH CRN. The inclusion and exclusion criteria for patients enrolled in NASH CRN studies are published (29, 30). For this study a total of 190 NASH CRN adult subjects (age $\geq$ 18 years) with biopsy-proven NAFLD (defined as $>5\%$ steatosis) and available serum for evaluation of serum vitamin D levels. Subjects were randomly chosen to approximate equivalent representation of each clinical center, while maximizing the number of subjects with available transcriptomic and cytokine level data. Histological features of fatty liver disease were assessed by the pathology committee of NASH CRN in a centralized consensus review format using criteria previously described (31). The quality of liver biopsy specimens was similar to previous reports (29–31). Subjects were categorized according to their level of serum vitamin D as either vitamin D sufficient (vitamin D >30 ng/ml), vitamin D insufficient (vitamin D 20 – 30 ng/ml) or vitamin D deficient (<20 ng/ml). Demographic information such as age, gender, ethnicity, race, household income, and medical history to identify co-morbidities and medications were obtained from patient interviews during screening. A physical exam including body weight and height measures was performed following the NASH CRN protocol (29, 30). Laboratory data including hepatic, hematologic, metabolic, lipid, and serum iron assessments were analyzed for subjects with values collected within 6 months of the liver biopsy. Total dietary consumption of and supplementation of vitamin D and calcium were determined from the Block 98 food frequency questionnaire (NutritionQuest, Berkeley, CA); alcohol consumption was determined from the AUDIT-C questionnaires completed during study visits closest to the time of biopsy (available in 73% of subjects). Exercise habits were determined from a Physical Activity Questionnaire (available in 68% of subjects). The prevalence of the metabolic syndrome (MetS) in this cohort was defined using the World Health Organization criteria.

Serologic data

Serum samples were collected in the morning following a 12 h fast and stored at -80°C until use. Serum TNF- α , IL-6 IL-1 β IL-8, resistin and adiponectin levels, determined using Luminex technology and the human cytokine LINCOplex kit (Catalog number HCYTO-60K, Millipore, St. Charles, MO), were available in 68%, 67%, 58%, 67%, 67% and 66% of subjects, respectively. The lower limit of detection for these assays was 0.66, 0.79 and 0.19 pg/mL, respectively.

Total serum vitamin D 25(OH)D (25(OH)D₂ + 25(OH)D₃) was measured in 190 adults (>18 yrs) by liquid-liquid extraction using heptane, derivatization with 4-phenyl-1,2,4-triazoline-3,5-dione, and liquid chromatography-tandem mass spectrometry [Xevo TQ-MS mass spectrometer (Waters, Milford, Connecticut)] at the University of Washington Clinical Mass Spectrometry Facility (a CLIA certified laboratory) supervised by A.N.H (32). Serum 25(OH)D measurements were calibrated against SRM 972 from the National Institute of Standards and Technology. The limit of quantification is 1 ng/mL and the inter-assay imprecision is 8.6–9.2% CV.

Determination of average total monthly hours of sunlight by clinical center

Using the “Comparative Climatic Data For the United States Through 2010” published by the National Climatic Data Center (<http://www.ncdc.noaa.gov/>) we determined the mean hours of sunshine for each NASH CRN enrolling center city at the time of sample collection for each subject. Monthly data for each NASH CRN site are presented in supplementary tables 1–12. This data is the average of 50.25 years (range 30–68 years). The following formula was used:

$$\% \text{SUNSHINE} \times [(\# \text{CLEAR DAYS} \times 0.85) + (\# \text{PARTLY CLOUDY DAYS} \times 0.45) + (\# \text{CLOUDY DAYS} \times 0.10)] \times 24 = \text{Mean hours of sunlight}$$

- SUNSHINE - average percentage of possible (percentage of the day which is daylight hours for that location during that month)
- CLEAR DAYS - defined as 70% - 100% sunshine; mean value of 85% was used.
- PARTLY CLOUDY DAYS - defined as 30% – 60% sunshine; mean value of 45% was used.
- CLOUDY DAYS - defined as 0% – 20% sunshine; mean value of 10% was used.

Determination of metabolic equivalents (METs)

Since physical activity (and socio-economic status) have been defined as risk factors for VDD (33), we extrapolated data from the Physical Activity and Modified (PA) Questionnaires used in the NASH CRN Database 1 and PIVENS Studies and convert to Metabolic Equivalents (METs) minutes per week as previously described (34).

RNA isolation and RNA sequencing library construction

RNA was isolated from flash frozen liver tissue using either the RNAqueous-Micro Kit (Life Technologies, Grand Island, NY) or the miRNeasy kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. Quality of the isolated RNAs was assessed using an Agilent Bioanalyzer to determine an RNA Integrity Number (RIN) value. Only RNAs with RIN ≥ 7.0 were used for sequencing. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer. RNA-seq libraries were constructed from 100 ng of RNA using the TruSeq Stranded Total RNA Sample Prep Kit (Illumina), with ribosomal depletion. Libraries were clustered on a flowcell using the TruSeq Paired-end Cluster Kit, v3 using a cBot clustering instrument (Illumina), followed by paired-end sequencing on a HiScanSQ (Illumina) for 50 cycles in either direction. After the run was completed, the reads were demultiplexed and FASTQ files were generated using Illumina's Casava software for each sample output. Read quality was analyzed by generating QC plots.

Sequence alignment and gene counts

Libraries were processed via a custom Galaxy workflow created for paired end, stranded data (35–37). Read alignment to Ensembl's GRCh37.66 gtf (38) was performed using TopHat (v1.4.1) (39). The single-paired flag was set to "paired," library type was set to "FR First Strand," and mean inner distance between pairs was set to 170. All other TopHat parameters were set to defaults. HTSeq-count was used to generate gene counts following conversion of BAM files into SAM files with mode set to "Intersection (nonempty)," stranded set to "Reverse," and minimum alignment quality set to 0 and all other set to default parameters (40).

Normalization and differential expression

The R Bioconductor package edgeR was used to determine differential expression between VDD (n=31) or not VDD (i.e., 25(OH)D3 levels ≥ 20 ng/ml; n=38) (41). A standard edgeR workflow was utilized (i.e., normalize the data, filter the genes, build a model matrix for the comparison, estimate dispersions/coefficient of variation, run the negative binomial generalized linear model, test for significance including multiple testing correction). For normalization, edgeR calculates TMM (trimmed mean of M-values) between each pair of samples that corrects for both sequencing depth variation between samples and possible under-sampling of genes due to highly expressed genes in a sample. TMM calculates a set of scaling factors for the library sizes to minimize these two technical factors of RNAseq data. Count data was filtered to remove genes which had a TMM normalized count of <1 in $>90\%$ of the libraries. This resulted in 19,057 genes being included in the linear model fit. Then a model matrix was built to perform the 2 group comparison. The biological coefficient of variation (CV) was 29%. Finally, a negative binomial general linear model fit was run for VDD yes vs. no, with adjustment for multiple comparisons using a FDR < 0.05 .

Pathway and functional annotation analysis

Enrichment for Gene Ontology terms was performed using AmiGO 2 v2.2.3 (42). Pathway analysis was performed by searching the Reactome pathway database v50 (43). Gene-

annotation enrichment analysis and functional annotation clustering was performed using DAVID v6.7 (44).

Statistical analysis

Baseline demographic, clinical, and laboratory characteristics were recorded as numbers and percentage for categorical data and means and standard deviation for continuous data. Differences between vitamin D level categories were assessed using the Kruskal Wallis test for continuous variables and Fisher's Exact test for categorical data. Trends across categories were analyzed using non-parametric trend test (STATA command *nptrend*) for continuous variables and Cochran-Mantel-Haenszel Stratified Test of Association for categorical data (STATA command *emh*). Binary and ordinal markers such as the presence of definitive NASH and NAS were analyzed using logistic regression and ordinal logistic regression, respectively using VDD as a nominal variable (i.e., patients with sufficient and insufficient vitamin D levels were merged into the control group). Models were adjusted for covariates defined *a priori*, including age, sex, race, BMI, ALT, season and the presence of metabolic syndrome and diabetes type 2. Factors thought to contribute to vitamin D levels, measured on a continuous scale, were analyzed using univariate and stepwise multivariable linear regression. These included age, sex, race, ethnicity, BMI, household income level, mean amount of sunlight during the month of sample collection, mean weekly metabolic equivalents, dietary and supplemental vitamin D (mg/day). A p value of <0.10 was used as a cutoff for incorporation into the model. Statistical analysis was performed using STATA version 12.1 (StataCorp LP, College Station, TX) or R version 3.1.3 (45). Nominal, two-sided p-values were used and were considered to be statistically significant if $p < 0.05$.

RESULTS

Patient characteristics

One hundred ninety subjects with biopsy-proven NAFLD were evaluated in the present study. VDD was present in 55% (105 subjects) of this cohort, with 26% and 19% classified as vitamin D insufficient and sufficient, respectively. Overall the majority of subjects in this study cohort were non-Hispanic whites (77%), obese (71%) and female (53%). Patient characteristics including clinical, demographic, racial, and specific dietary/behavioral factors thought to effect vitamin D levels, such as dietary and supplemental vitamin D and calcium consumption, household income, weekly metabolic equivalent expenditure and compiled average sunlight for each site during the month of sample collection are summarized in Table 1. There was a significant positive trend for increased age with higher serum vitamin D levels. There were significant differences between groups for supplemental calcium consumption as well as for average sunlight at the time of serum collection. There was a positive trend of increased dietary vitamin D consumption across groups, which failed to attain statistical significance ($p=0.059$). No other differences between groups were observed including for BMI, race, ethnicity or the presences of obesity, diabetes, or the metabolic syndrome.

Association of VDD and nonalcoholic steatohepatitis in NAFLD subjects

There was a significant difference in the proportion of subjects given the diagnoses of “not NASH/borderline NASH compared to definitive NASH” according to their serum vitamin D level category. Subjects with VDD were more likely to be diagnosed as “definitive NASH” (67%), compared to subjects in either the vitamin D insufficient (47%) or sufficient (53%) categories ($p=0.045$, Fisher's exact). VDD was associated with the presence of definitive NASH (univariate logistic regression, OR 2.01, 95% CI 1.13–3.69, $p=0.017$). The estimated magnitude of the odds ratio increased adjusting for covariates age, sex, BMI, race, ALT level, and the presence of diabetes mellitus, (stepwise multivariable logistic regression; OR 3.15, 95% CI 1.62–6.15, $p=0.001$; Fig 1A). There was no significant difference in the absolute VD3 levels between patients with and without NASH (median 17.85, range (4.9–54.5); median 20.7, range (2.3–45.3), $p=0.16$, Wilcoxon rank sum test, respectively). It is important to note that this study was not powered to detect a difference if VD3 levels between subjects with or without NASH.

Relationship of VDD and histologic severity of NAFLD

We analyzed the relationship of VDD and histologic features of NAFLD using stepwise ordinal and logistic regression after adjusting for covariates defined *a priori*, including age, sex, BMI, race, ALT, and diabetes status. Increased histologic severity of NAFLD, as evidenced by a higher NAFLD Activity Score (NAS), was associated with VDD (OR=1.90, 95%CI, 1.13–3.23, $p=0.016$; Fig. 1B). In agreement with an association with NAS, VDD was also associated with more ballooning (OR=2.38, 95%CI, 1.32–4.30, $p=0.004$; Fig. 1C) and a higher lobular inflammation grade (OR=1.98, 95%CI, 1.08–3.61, $p=0.026$; Fig. 1D). Lastly, VDD was associated with the presence of any fibrosis (OR=2.32, 95%CI, 1.13–4.77, $p=0.022$; Fig. 1E). VDD was not associated with grade of steatosis or other histologic features of NAFLD such as Mallory bodies, acidophils or megamitochondria. Furthermore, we found that neither mean sunlight or dietary vitamin D levels were associated with any of the histological features of NASH shown in Fig 1. This suggests that the *in vivo* physiological state of VDD *per se* was associated with the observed worsened histological features of NASH rather than the main contributory factors of VDD alone.

Differences in laboratory tests between VD level category groups

Mean levels of routine clinical laboratory assessments and pro-inflammatory cytokines/adipokines IL-1 β , IL-6, IL-8, TNF- α , resistin and adiponectin are shown in Table 2. There was a significant difference between the VD groups in the level of resistin and number of leukocytes, but no differences in aminotransferases or metabolic abnormalities including fasting insulin and glucose levels, HOMA-IR and plasma lipid levels.

Factors affecting serum vitamin D 25(OH)D3 levels

In this study we collected data on several variables thought to influence serum vitamin D levels, as shown in Table 3 (2, 3, 26, 32, 46, 47). Linear regression analysis was performed to investigate the relationship of these factors to vitamin D 25(OH)D3 levels in this population. In univariate analysis Hispanic ethnicity was associated with decreased serum vitamin D 25(OH)D3 levels ($\beta=-0.16$, $p=0.029$). Increased BMI ($\beta=-0.14$, $p=0.052$) and

non-white race ($\beta = -0.14$, $p = 0.054$) were also associated with decreased serum vitamin D 25(OH)D3 levels, but failed to reach statistical significance. Mean sunlight at the time of sample collection ($\beta = 0.31$, $p < 0.001$), increased age ($\beta = 0.17$, $p = 0.018$) and dietary vitamin D consumption ($\beta = 0.17$, $p = 0.047$) were associated with higher vitamin D 25(OH)D3 levels. To investigate the relationship between the combined effects of these factors on 25(OH)D3 levels, forward stepwise multivariable linear regression modeling was performed; a p-value of < 0.10 was used as a cutoff for incorporation into the model. Overall, Hispanic ethnicity ($\beta = -0.18$, $p = 0.038$) and BMI ($\beta = -0.20$, $p = 0.018$), were inversely associated with serum vitamin D 25(OH)D3 levels, while mean sunlight at the time of sample collection ($\beta = 0.27$, $p = 0.002$), was positively associated with serum vitamin D 25(OH)D3 levels.

Hepatic transcriptomic profiling of VDD subjects

To investigate if VDD is associated with changes in hepatic gene expression in NAFLD, we compared available hepatic transcriptomic profiling data obtained by RNA sequencing in a subset of our cohort; 31 VDD subjects as compared to 38 subjects with vitamin D 25(OH)D3 levels ≥ 20 ng/ml. Four protein coding genes and the gene for the small nucleolar RNA (snoRNA), C/D box 3C (*SNORD3C*), were differentially expressed between these groups at $FDR < 0.05$. As shown in Table 4, the *KRT10* (Keratin 10), *ARSD* (Arylsulfatase D), and *SNORD3C* genes were down-regulated, while the *SEMA3B* (Sema Domain, Immunoglobulin Domain (Ig), Short Basic Domain, Secreted), *IGKV4-1* (Immunoglobulin Kappa Variable 4-1) were up-regulated in the VDD subjects. An additional four protein encoding genes and another snoRNA gene (*SNORD3D*), were differentially expressed between these groups at $0.05 < FDR < 0.1$. These included the upregulated *IGLV8-61* (Immunoglobulin Lambda Variable 8-61), *FOS* (v-Fos FBJ Murine Osteosarcoma Viral Oncogene Homolog) and *SLC51B* (Solute Carrier Family 51, Beta Subunit) genes and the *SNORD3D* and *TFF3* (Trefoil Factor 3) genes, which were down regulated. The potential relationship to NASH pathogenesis, shown in Table 4, was identified for each gene by screening the DAVID Bioinformatics Database (44) and through a review of the literature. Higher order functional analysis was investigated for the top 10 differentially expressed genes shown in Table 4 by searching for enrichment of Gene Ontology (GO) terms using AMIGO2 (43) (supplemental table 13) and pathway analysis was performed by searching the Reactome pathway database (44) (supplemental table 14). Similar results were obtained in both analyses and included many immune-related GO terms and Reactome pathways. The DAVID Bioinformatics Database also identified a cluster of annotations (Enrichment Score: 2.01) related to Immunoglobulin-like domains, which identified the *IGLV8-6*, *IGKV4-1* and *SEMA3B* genes (supplemental table 15).

DISCUSSION

In this study, we report an inverse association between worsened histologic features of NAFLD and vitamin D 25(OH)D3 levels after adjusting for potential confounding variables. VDD was also associated with a definitive diagnosis of NASH. While a number of studies have investigated the association of VDD and NAFLD compared to non-disease controls using surrogate diagnostic measures such as liver enzyme levels or ultrasonography, relatively few studies have attempted to investigate whether VDD would be associated with

biopsy-proven NASH and histologic disease severity in adults (2). Our findings are in agreement with the majority of these studies (22, 25, 26). The role of VDD in studies performed in children with biopsy-proven NAFLD have also shown conflicting results (23, 24, 27). There were several similarities between our findings and those of Bril et al (26), despite the fact these studies were discrepant in regard to an association of VDD with histological severity. For example, both studies found that VDD subjects were significantly younger than subjects with sufficient or insufficient VD levels and that a lower proportion of Caucasians compared to non-Caucasians and a higher proportion of Hispanics compared to non-Hispanics were VDD compared to the other two categories. Moreover, neither study found differences in liver enzymes, plasma lipids, or fasting glucose and insulin levels between the groups. We did however find significantly higher leukocyte counts in VDD subjects which likely reflects the overall induction of inflammation in these subjects.

The association of VDD with worsened NASH pathology including increased NAS and lobular inflammation is in agreement with our recent study in a rat model of NAFLD and VDD (9). The observed increased levels of serum resistin in this study is also consistent with the increased in hepatic resistin gene expression seen in our rat model. However, we did not see decreased serum adiponectin or an increase in other proinflammatory cytokines levels in our study. Resistin has been shown to be elevated in NAFLD subjects and is associated with increased insulin resistance and NF- κ B activation (48, 49). Moreover, our novel hepatic transcriptome profiling results implicate activation of the MAPK and NF- κ B inflammatory pathways. This study clearly demonstrates the relationship between NAFLD activity/severity and VDD based on liver biopsy results. However, as in previous reports, this relationship could not be found between serum markers of disease severity and VDD (2, 22, 25, 26). It should also be noted that since VDD is also common in patients with non-cholestatic chronic liver diseases it cannot be ruled out that parenchymal damage may have a causative role in the development of VDD (50, 51). Moreover, the lack of beneficial effects of vitamin D supplementation in a number of different disease treatment trials questions the etiological role of VDD (52, 53).

We also sought to investigate potential factors which could contribute to VDD in this cohort. Using the “Comparative Climatic Data For the United States Through 2010” published by the National Climatic Data Center, we compiled sunlight data for each month at each of the eight NASH CRN clinical sites. As expected, we found that the mean hours of sunlight during the month of sample collection was an independent predictor of higher VD3 levels. We also found that a higher BMI and Hispanic ethnicity was inversely associated with VD3 levels. BMI has previously been associated with VDD which may be due to sequestration of VD3 within adipose tissue, although this remains unclear (46). It is likely that the association of lower VD3 levels and Hispanic ethnicity we observed is due to decreased formation of pre-VD3, from pro-VD3 due to absorption of UV radiation by melanin as a result of the darker skin pigmentation in these subjects (47).

Recent studies have provided insight into new physiologic functions of VD3 (i.e., a protective anti-inflammatory role), in addition to the classic action of VD3 in bone mineral metabolism, corresponding with the identification of widespread tissue distribution of the vitamin D receptor (2). Recent studies have shown that VD3 suppresses several pathways

involved in inflammation and cytokine production including MAPK and NF- κ B (54–56). In cultured human adipocytes, VD3 protects against macrophage-induced activation of NF- κ B and MAPK signaling and subsequent release of several cytokines and chemokines, via up-regulation of I κ B α expression and reduced NF- κ B p65 phosphorylation and down-regulation of phosphorylated p38 MAPK and phosphorylated Erk1/2, respectively (54). In a similar study, Mutt et al have shown that VD3 inhibits I κ B phosphorylation-mediated NF- κ B translocation into the nucleus in LPS-induced primary adipocytes and bone marrow-derived human mesenchymal stromal cells differentiated into adipocytes, resulting in decreased IL-6 secretion and transcription (55). Since increased accumulation of macrophages in adipose tissue is a hallmark of obesity and NAFLD, these studies provide a mechanistic link between VDD and the inflammatory response which is a key pathogenic event in NAFLD. In addition, dietary vitamin D supplementation decreased MAPK and NF- κ B activation in a murine model of colon cancer (56).

The results of our hepatic transcriptome profiling are in agreement with the above studies implicating up-regulation of the MAPK and NF- κ B inflammatory pathways in our VDD NAFLD subjects. For example, Reactome pathway analysis revealed several common Reactome pathways for 5 of the top 12 DE genes, which includes four immunoglobulin genes (i.e., *IGKV4-1*, *IGLV8-61*, *IGHG1*, *IGKC*) and the transcription factor *FOS*. The common Reactome pathways for these genes include: Fc epsilon receptor (Fc ϵ RI) signaling, Fc ϵ RI mediated MAPK activation, Fc ϵ RI mediated NF- κ B activation, Classical antibody-mediated complement activation, Fc γ R activation, Creation of C4 and C2 activators and initial triggering of complement. Fc ϵ RI is the high affinity IgE receptor which is present on mast cells, and other antigen presenting cells including macrophages and dendritic cells. Binding of IgE to Fc ϵ RI induces a number inflammatory autocrine and paracrine effects including activation of the MAPK, NF- κ B, TGF- β and Toll-like receptor (TLR) signaling pathways resulting in production of cytokines and chemokines such as TNF- α , IL-1, IL-8, MCP-1 and other inflammatory mediators, thus facilitating amplification of both innate and acquired immune responses (57, 58). Binding of IgE to Fc ϵ RI also strongly activates the transcription factor *FOS* (57, 58). *FOS* is then phosphorylated and stabilized by ERK through activation of the MAPK pathway (59). Together with JUN, *FOS* dimerizes to form the transcription factor AP-1, which is a major regulator of inflammation, apoptosis, differentiation and transformation (59). AP-1 synergizes with SMAD3 and SMAD4 at TRE binding sites to activate genes in response to induction of TGF- β signaling (60). Interestingly, a recent study showed that VD3 ameliorated TGF- β -induced fibrogenesis in primary hepatic stellate cells and that this response was in part dependent upon certain SNPs in the *VDR* gene (61). For example, stellate cells with the *VDR* A1012G GG genotype (rs4516035) had no reduction in TGF- β following VD3 treatment in contrast to subjects without this variant. These authors also showed that NAFLD subjects with the *VDR* A1012G GG genotype had lower *VDR* expression than the other genotypes for this SNP, suggesting this SNP may contribute to VDD-mediated pathogenesis in NAFLD (61).

Several other differentially expressed genes in our study also point to involvement of the MAPK and NF- κ B pathways in VDD NAFLD patients. The keratin 10 gene, *KT10*, which was decreased 5-fold, was the most significantly DE gene in VDD subjects. Previous studies have shown that VD3 upregulates *KT10* expression (62), and that MAPK is activated in

KT10 (–/–) knockout mice (63). Moreover, *KT10* expression in the basal epidermis in transgenic mice, leads to impaired NF-κB activity via inhibition of Akt, in these cells (64). Together these studies suggest that VDD mediated decreased expression of *KT10* leads to MAPK activation and potential enhanced Akt and NF-κB activity. In contrast, semaphorin 3B (gene *SEMA3B*; increased >3-fold in VDD subjects) was associated with decreased Akt phosphorylation via binding neuropilin-1, but also subsequent increased apoptosis (65), which we have recently shown to be associated with NASH (66). *SEMA3B* expression also leads to neuropilin-1 mediated MAPK activation, and subsequent release of the chemokine IL-8 *in vitro* (67). Although we did not observe increased serum IL-8 levels in our VDD subjects, in a related hepatic transcriptomic study, we found *IL8* to be one of the most significantly upregulated genes in patients with NASH compared to subjects with steatosis alone (unpublished observations).

We recognize limitations to the current study, including more sensitive measures of abdominal fat distribution and insulin sensitivity and the relatively small number of subjects with hepatic transcriptome data, which likely reduced the number of genes which were significantly differentially expressed between NAFLD subjects with VDD and those with sufficient VD levels. The lack of an association between circulating biomarkers (e.g., cytokines) and VDD status may be due to the timing of biospecimens collection in relation to VDD. In addition, the pleiotropic role of cytokines in diverse biological processes, most of which utilize the circulatory system as the mode of transportation, can make the identification of specific biological relationships (e.g., cytokines and VDD status) difficult to discern from a single peripheral measure. As usual in association studies of this type we cannot prove causality; whether these observations represent a novel mechanism linking VDD to inflammation, parenchymal damage and NASH severity will need to be confirmed by detailed mechanistic studies.

In summary, we have found that VDD in our NAFLD subjects was associated with a definitive diagnosis of NASH, increased lobular inflammation, more ballooning and the presence of fibrosis. Taken together the results of hepatic transcriptomic profiling in VDD subjects compared to subjects with VD3 20ng/ml suggests that the MAPK, NF-κB, TGF-β and Akt pathways may be involved in the increased histologic severity seen in our VDD NAFLD subjects. These data are in agreement with a recent study by our group in a rat model of NAFLD and VDD which also showed that VDD was associated with increased disease severity and up-regulation of hepatic inflammatory and TLR genes (9).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Source of funding:

This work was supported by a grant from the American College of Gastroenterology to KVK.

The Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) is supported by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (grants U01DK061718, U01DK061728, U01DK061731,

U01DK061732, U01DK061734, U01DK061737, U01DK061738, U01DK061730, U01DK061713), and the National Institute of Child Health and Human Development (NICHD).

Several clinical centers use support from the National Center for Advancing Translational Sciences (NCATS) in conduct of NASH CRN Studies (grants UL1TR000439, UL1TR000077, UL1TR000436, UL1TR000150, UL1TR000424, UL1TR000006, UL1TR000448, UL1TR000040, UL1TR000100, UL1TR000004, UL1TR000423, UL1TR000058, UL1TR000067, UL1TR000454). This work was supported in part by the Intramural Research Program of the National Cancer Institute.

Abbreviations

NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NAFLD NAS	activity score
IL	interleukin

References

1. Wang Y, Zhu J, DeLuca HF. Where is the vitamin D receptor? Arch Biochem Biophys. 2012; 523:123–33. [PubMed: 22503810]
2. Targher G, Scorletti E, Mantovani A, et al. Nonalcoholic fatty liver disease and reduced serum vitamin D(3) levels. Metab Syndr Relat Disord. 2013; 11:217–28. [PubMed: 23745619]
3. Ding C, Gao D, Wilding J, et al. Vitamin D signalling in adipose tissue. Br J Nutr. 2012; 108:1915–23. [PubMed: 23046765]
4. Holick MF. Vitamin D deficiency. N Engl J Med. 2007; 357:266–81. [PubMed: 17634462]
5. Smotkin-Tangorra M, Purushothaman R, Gupta A, et al. Prevalence of vitamin D insufficiency in obese children and adolescents. J Pediatr Endocrinol Metab. 2007; 20:817–23. [PubMed: 17849744]
6. Gordon CM, DePeter KC, Feldman HA, et al. Prevalence of vitamin D deficiency among healthy adolescents. Arch Pediatr Adolesc Med. 2004; 158:531–7. [PubMed: 15184215]
7. Rajakumar K, de las Heras J, Chen TC, et al. Vitamin D status, adiposity, and lipids in black American and Caucasian children. J Clin Endocrinol Metab. 2011; 96:1560–7. [PubMed: 21367931]
8. Shea MK, Houston DK, Tooze JA, et al. Correlates and prevalence of insufficient 25-hydroxyvitamin D status in black and white older adults: the health, aging and body composition study. J Am Geriatr Soc. 2011; 59:1165–74. [PubMed: 21668915]
9. Roth CL, Elfers CT, Figlewicz DP, et al. Vitamin D deficiency in obese rats exacerbates NAFLD and increases hepatic resistin and toll-like receptor activation. Hepatology. 2012; 55:1103–11. [PubMed: 21994008]
10. Roth CL, Elfers C, Kratz M, et al. Vitamin D deficiency in obese children and its relationship to insulin resistance and adipokines. J Obes. 2011; 2011:495101. [PubMed: 22254134]
11. Vaidya A, Williams JS, Forman JP. The Independent Association Between 25-Hydroxyvitamin D and Adiponectin and Its Relation With BMI in Two Large Cohorts: The NHS and the HPFS. Obesity (Silver Spring). 2012; 20:186–91. [PubMed: 21760630]
12. Wu CC, Chang JH, Chen CC, et al. Calcitriol treatment attenuates inflammation and oxidative stress in hemodialysis patients with secondary hyperparathyroidism. Tohoku J Exp Med. 2011; 223:153–9. [PubMed: 21350317]
13. Tanaka M, Tokunaga K, Komaba H, et al. Vitamin D receptor activator reduces oxidative stress in hemodialysis patients with secondary hyperparathyroidism. Ther Apher Dial. 2011; 15:161–8. [PubMed: 21426509]
14. Browning JD, Szczepaniak LS, Dobbins R, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology. 2004; 40:1387–95. [PubMed: 15565570]

15. Schwimmer J, Deutsch R, Kahen T, et al. Prevalence of fatty liver in children and adolescents. *Pediatrics*. 2006; 118:1388–93. [PubMed: 17015527]
16. Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med*. 2010; 363:1341–50. [PubMed: 20879883]
17. Musso G, Gambino R, Cassader M, Pagano G. A meta-analysis of randomized trials for the treatment of nonalcoholic fatty liver disease. *Hepatology*. 2010; 52:79–104. [PubMed: 20578268]
18. Ybarra J, Sanchez-Hernandez J, Perez A. Hypovitaminosis D and morbid obesity. *Nurs Clin North Am*. 2007; 42:19–27. [PubMed: 17270587]
19. Liangpunsakul S, Chalasani N. Serum vitamin D concentrations and unexplained elevation in ALT among US adults. *Dig Dis Sci*. 2011; 56:2124–9. [PubMed: 21503677]
20. Katz K, Brar PC, Parekh N, Liu YH, Weitzman M. Suspected nonalcoholic Fatty liver disease is not associated with vitamin d status in adolescents after adjustment for obesity. *J Obes*. 2010; 2010:496829. [PubMed: 21331294]
21. Eliades M, Spyrou E, Agrawal N, et al. Meta-analysis: vitamin D and non-alcoholic fatty liver disease. *Aliment Pharmacol Ther*. 2013; 38:246–54. [PubMed: 23786213]
22. Targher G, Bertolini L, Scala L, et al. Associations between serum 25-hydroxyvitamin D3 concentrations and liver histology in patients with non-alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis*. 2007; 17:517–24. [PubMed: 16928437]
23. Manco M, Ciampalini P, Nobili V. Low levels of 25-hydroxyvitamin D(3) in children with biopsy-proven nonalcoholic fatty liver disease. *Hepatology*. 2010; 51:2229. author reply 2230.
24. Nobili V, Giorgio V, Liccardo D, et al. Vitamin D levels and liver histological alterations in children with nonalcoholic fatty liver disease. *Eur J Endocrinol*. 2014; 170:547–53. [PubMed: 24412930]
25. Dasarathy J, Periyalwar P, Allampati S, et al. Hypovitaminosis D is associated with increased whole body fat mass and greater severity of non-alcoholic fatty liver disease. *Liver Int*. 2014; 34:e118–27. [PubMed: 24118743]
26. Bril F, Maximos M, Portillo-Sanchez P, et al. Relationship of vitamin D with insulin resistance and disease severity in nonalcoholic steatohepatitis. *J Hepatol*. 2015; 62:405–11. [PubMed: 25195551]
27. Hourigan SK, Abrams S, Yates K, et al. for the NASH CRN. The relationship between vitamin D status and non-alcoholic fatty liver disease in children. *J Pediatr Gastroenterol Nutr*. 2015; 60:396–404. [PubMed: 25710716]
28. Nakano T, Cheng YF, Lai CY, et al. Impact of artificial sunlight therapy on the progress of non-alcoholic fatty liver disease in rats. *J Hepatol*. 2011; 55:415–25. [PubMed: 21184788]
29. Sanyal AJ, Chalasani N, Kowdley KV, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med*. 2010; 362:1675–85. [PubMed: 20427778]
30. Neuschwander-Tetri BA, Clark JM, Bass NM, et al. Clinical, laboratory and histological associations in adults with nonalcoholic fatty liver disease. *Hepatology*. 2010; 52:913–24. [PubMed: 20648476]
31. Kleiner DE, Brunt EM, Van NM, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005; 41:1313–1321. [PubMed: 15915461]
32. Laha TJ, Strathmann FG, Wang Z, et al. Characterizing antibody cross-reactivity for immunoaffinity purification of analytes prior to multiplexed liquid chromatography-tandem mass spectrometry. *Clin Chem*. 2012; 58:1711–6. [PubMed: 22968104]
33. Absoud M, Cummins C, Lim MJ, et al. Prevalence and predictors of vitamin D insufficiency in children: a Great Britain population based study. *PLoS One*. 2011; 6:e22179. [PubMed: 21799790]
34. Kistler KD, Brunt EM, Clark JM, et al. for the NASH CRN Research Group. Physical activity recommendations, exercise intensity, and histological severity of nonalcoholic fatty liver disease. *Am J Gastroenterol*. 2011; 106:460–468. [PubMed: 21206486]
35. Goecks J, Nekrutenko A, Taylor J. The Galaxy Team. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol*. 2010 Aug 25.11(8):R86. [PubMed: 20738864]
36. Blankenberg D, Von Kuster G, Coraor N, et al. Galaxy: a web-based genome analysis tool for experimentalists. *Current Protocols in Molecular Biology*. 2010 Jan.Chapter 19(Unit 19.10.1–21)

37. Giardine B, Riemer C, Hardison RC, et al. Galaxy: a platform for interactive large-scale genome analysis. *Genome Res.* 2005; 15:1451–5. [PubMed: 16169926]
38. Flicek P, Amode MR, Barrell D, et al. Ensembl 2014. *Nuc Acids Res.* 2014; 42:749–55. (Database issue).
39. Trapnell C, Roberts A, Goff L, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc.* 2012; 7:562–78. [PubMed: 22383036]
40. Anders S, Pyl PT, Huber W. HTSeq – A Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England).* Epub ahead of print.
41. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* 2010; 26:139–140. [PubMed: 19910308]
42. Carbon S, Ireland A, Mungall CJ, et al. AmiGO: online access to ontology and annotation data. *Bioinformatics.* 2009; 25:288–9. [PubMed: 19033274]
43. Croft D, Mundo AF, Haw R, et al. The Reactome pathway knowledgebase. *Nuc Acids Res.* 2014; 42:D472–7. (Database issue).
44. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nature Protoc.* 2009; 4:44–57. [PubMed: 19131956]
45. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing; Vienna, Austria: 2015. <https://www.R-project.org>
46. Earthman CP, Beckman LM, Masodkar K, Sibley SD. The link between obesity and low circulating 25-hydroxyvitamin D concentrations: considerations and implications. *Int J Obesity.* 2012; 36:387–396.
47. Bikle D. Nonclassic actions of vitamin D. *J Clin Endocrinol Metab.* 2009; 94:26–34. [PubMed: 18854395]
48. Silswal N, Singh AK, Aruna B, et al. Human resistin stimulates the pro-inflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kappaB-dependent pathway. *Biochem Biophys Res Commun.* 2005; 334:1092–101. [PubMed: 16039994]
49. Jiang LL, Li L, Hong XF, et al. Patients with nonalcoholic fatty liver disease display increased serum resistin levels and decreased adiponectin levels. *Eur J Gastroenterol Hepatol.* 2009; 21:662–6. [PubMed: 19282764]
50. Lange CM, Bojunga J, Ramos-Lopez E, et al. Vitamin D deficiency and a CYP27B1-1260 promoter polymorphism are associated with chronic hepatitis C and poor response to interferon-alfa based therapy. *J Hepatol.* 2011; 54:887–93. [PubMed: 21145801]
51. Wong GL, Chan HL, Chan HY, et al. Adverse effects of vitamin D deficiency on outcomes of patients with chronic hepatitis B. *Clin Gastroenterol Hepatol.* 2015; 13:783–90. [PubMed: 25445773]
52. Bolland MJ, Grey A, Gamble GD, et al. The effect of vitamin D supplementation on skeletal, vascular, or cancer outcomes: a trial sequential meta-analysis. *Lancet Diabetes Endocrinol.* 2014; 2:307–20. [PubMed: 24703049]
53. Wactawski-Wende J, Kotchen JM, Anderson GL, et al. Calcium plus vitamin D supplementation and the risk of colorectal cancer. *N Engl J Med.* 2006; 354:684–96. [PubMed: 16481636]
54. Ding C, Wilding JP, Bing C. 1,25-dihydroxyvitamin D3 protects against macrophage-induced activation of NFκB and MAPK signaling and chemokine release in human adipocytes. *PLoS One.* 2013; 8:e61707. [PubMed: 23637889]
55. Mutt SJ, Karhu T, Lehtonen S, et al. Inhibition of cytokine secretion from adipocytes by 1,25-dihydroxyvitamin D via the NF-κB pathway. *FASEB J.* 2012; 26:4400–7. [PubMed: 22798425]
56. Meeker S, Seamons A, Paik J, et al. Increased dietary vitamin D suppresses MAPK signaling, colitis, and colon cancer. *Cancer Res.* 2014; 74:4398–408. [PubMed: 24938764]
57. Shakoory B, Fitzgerald SM, Lee SA, et al. The role of human mast cell-derived cytokines in eosinophil biology. *Interferon Cytokine Res.* 2004; 24:271–81.
58. Kraft S, Bieber T. Fc epsilon ri-mediated activation of transcription factors in antigen-presenting cells. *Int Arch Allergy Immunol.* 2001; 125:9–15. [PubMed: 11385283]

59. Durchdewald M, Angel P, Hess J. The transcription factor Fos: a Janus-type regulator in health and disease. *Histol Histopathol.* 2009; 24:1451–61. [PubMed: 19760594]
60. Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature.* 1998; 394:909–13. [PubMed: 9732876]
61. Beilfuss A, Sowa J-P, Sydor S, et al. Vitamin D counteracts fibrogenic TGF- β signaling in human hepatic stellate cells both receptor-dependently and independently. *Gut.* 2015; 64:791–9. [PubMed: 25134788]
62. Garach-Jehoshua O, Ravid A, Liberman UA, et al. Upregulation of the calcium-dependent protease, calpain, during keratinocyte differentiation. *Br J Dermatol.* 1998; 139:950–7. [PubMed: 9990355]
63. Reichelt J, Furstenberger G, Magin TM. Loss of keratin 10 leads to mitogen-activated protein kinase (MAPK) activation, increased keratinocyte turnover, and decreased tumor formation in mice. *Invest Dermatol.* 2004; 123:973–81.
64. Santos M, Perez P, Segrelles C, et al. Impaired NF-kappa B activation and increased production of tumor necrosis factor alpha in transgenic mice expressing keratin K10 in the basal layer of the epidermis. *J Biol Chem.* 2003; 278:13422–30. [PubMed: 12566451]
65. Castro-Rivera E, Ran S, Brekken RA, Minna JD. Semaphorin 3B inhibits the phosphatidylinositol 3-kinase/Akt pathway through neuropilin-1 in lung and breast cancer cells. *Cancer Res.* 2008; 68:8295–303. [PubMed: 18922901]
66. Maliken BD, Nelson JE, Klintworth HM, et al. Hepatic reticuloendothelial system cell iron deposition is associated with increased apoptosis in nonalcoholic fatty liver disease. *Hepatology.* 2013; 57:1806–13. [PubMed: 23325576]
67. Rolny C, Capparuccia L, Casazza A, et al. The tumor suppressor semaphorin 3B triggers a prometastatic program mediated by interleukin 8 and the tumor microenvironment. *J Exp Med.* 2008; 205:1155–71. [PubMed: 18458115]
68. Ghosh D. Human sulfatases: a structural perspective to catalysis. *Cell Mol Life Sci.* 2007; 64:2013–22. [PubMed: 17558559]
69. Ballatori N. Pleiotropic Functions of the Organic Solute Transporter Osta –Ost β . *Dig Dis.* 2011; 29:13–17. [PubMed: 21691099]
70. Xue Y, Shen L, Cui Y, et al. Tff3, as a novel peptide, regulates hepatic glucose metabolism. *PLoS One.* 2013; 8:e75240. [PubMed: 24086476]

Members of the Nonalcoholic Steatohepatitis Clinical Research Network

Baylor College of Medicine, Houston, TX: Stephanie H. Abrams, MD, MS; Ryan Himes, MD; Rajesh Krisnamurthy, MD; Leanel Maldonado, RN (2007-2012); Beverly Morris

Case Western Reserve University Clinical Centers:

- MetroHealth Medical Center, Cleveland, OH: Patricia Brandt; Srinivasan Dasarathy, MD; Jaividhya Dasarathy, MD; Carol Hawkins, RN; Arthur J. McCullough, MD
- Cleveland Clinic Foundation, Cleveland, OH: Srinivasan Dasarathy, MD; Arthur J. McCullough, MD; Mangesh Pagadala, MD; Rish Pai, MD; Ruth Sargent, LPN; Shetal Shah, MD; Claudia Zein, MD

Cincinnati Children's Hospital Medical Center, Cincinnati, OH: Kimberlee Bernstein, BS, CCRP; Kim Cecil, PhD; Stephanie DeVore, MSPH (2009-2011); Rohit Kohli, MD; Kathleen Lake, MSW (2009-2012); Daniel Podberesky, MD; Crystal Slaughter, BA, CCRP; Stavra Xanthakos, MD

Columbia University, New York, NY: Gerald Behr, MD; Joel E. Lavine, MD, PhD; Ali Mencin, MD; Nadia Ovchinsky, MD; Elena Reynoso, MD

Duke University Medical Center, Durham, NC: Manal F. Abdelmalek, MD; Mustafa Bashir, MD; Stephanie Buie; Anna Mae Diehl, MD; Cynthia Guy, MD; Christopher Kigongo; Yi-Ping Pan; Dawn Piercy, FNP (2004-2012); Melissa Wagner

Emory University, Atlanta, GA: Adina Alazraki, MD; Rebecca Cleeton, MPH; Saul Karpen, MD, PhD; Nicholas Raviele; Miriam Vos, MD, MSPH

Indiana University School of Medicine, Indianapolis, IN: Elizabeth Byam, RN; Naga Chalasani, MD; Oscar W. Cummings, MD; Cynthia Fleming, RN, MSN; Marwan Ghabril, MD; Ann Klipsch, RN; Smitha Marri, MD; Jean P. Molleston, MD; Linda Ragozzino, RN; Kumar Sandrasegaran, MD; Girish Subbarao, MD; Raj Vuppalanchi, MD

Johns Hopkins Hospital, Baltimore, MD: Kimberly Pfeifer, RN; Ann Scheimann, MD; Michael Torbenson, MD

Mount Sinai Kravis Children's Hospital, New York, NY: Ronen Arnon, MD; Mariel Boyd, CCRP

Northwestern University Feinberg School of Medicine/Ann & Robert H. Lurie Children's

Hospital of Chicago: Katie Amsden, Mark H. Fishbein, MD; Elizabeth Kirwan, RN; Saeed Mohammad, MD; Ann Quinn, RD (2010–2012); Cynthia Rigsby, MD; Peter F. Whittington, MD

Saint Louis University, St Louis, MO: Sarah Barlow, MD (2002–2007); Jose Derdoy, MD (2007–2012); Ajay Jain MD; Debra King, RN; Pat Osmack; Joan Siegner, RN; Susan Stewart, RN; Brent A. Neuschwander-Tetri, MD; Dana Romo

University of California San Diego, San Diego, CA: Brandon Ang; Sandra Arroyo; Cynthia Behling, MD, PhD; Archana Bhatt; Jennifer Collins; Iliana Doycheva, MD; Janis Durelle; Tarek Hassanein, MD (2004–2009); Joel E. Lavine, MD PhD (2002–2010); Rohit Loomba, MD, MHSc; Michael Middleton, MD, PhD; Kimberly Newton, MD; Phirum Nguyen; Mazen Noureddin, MD; Melissa Paiz; Heather Patton, MD; Jeffrey B. Schwimmer, MD; Claude Sirlin, MD; Patricia Ugalde-Nicalo

University of California San Francisco, San Francisco, CA: Bradley Aouizerat, PhD; Nathan M. Bass, MD, PhD (2002–2011); Danielle Brandman, MD; Linda D. Ferrell, MD; Shannon Fleck; Ryan Gill, MD, PhD; Bilal Hameed, MD; Alexander Ko; Camille Langlois; Emily Rothbaum Perito, MD; Aliya Qayyum, MD; Philip Rosenthal, MD; Norah Terrault, MD, MPH; Patrika Tsai, MD

University of California San Francisco- Fresno, Fresno, CA: PradeepAtla, MD; Cathy Hurtado; Rebekah Garcia; Sonia Garcia; Muhammad Sheikh, MD; Mandeep Singh, MD

University of Washington Medical Center and Seattle Children's Hospital, Seattle, WA: Kara Cooper; Simon Horslen, MB ChB; Evelyn Hsu, MD; Karen Murray, MD; Randolph Otto, MD; Deana Rich; Matthew Yeh, MD, PhD; Melissa Young

Virginia Commonwealth University, Richmond, VA: Sherry Boyett, RN, BSN; Laura Carucci, MD; Melissa J. Contos, MD; Michael Fuchs, MD; Amy Jones; Kenneth Kraft, PhD; Velimir AC Luketic, MD; Kimberly Noble; Puneet Puri, MD; Bimalijit Sandhu, MD (2007–2009); Arun J. Sanyal, MD; Carol Sargeant, RN, BSN, MPH (2004–2012); Jolene Schlosser; Mohhamad S. Siddiqui, MD; Ben Wolford; Melanie White, RN, BSN (2006–2009)

Virginia Mason Medical Center, Seattle, WA: Sarah Ackermann; Shannon Cooney; David Coy, MD, PhD; Katie Gelinas; Kris V. Kowdley, MD; Maximillian Lee, MD, MPH; Tracey Pierce; Jody Mooney, MS; James E. Nelson, PhD; Lacey Siekas; Cheryl Shaw, MPH; Asma Siddique, MD; Chia Wang, MD

Washington University, St. Louis, MO: Elizabeth M. Brunt, MD; Kathryn Fowler, MD

Resource Centers:

National Cancer Institute, Bethesda, MD: David E. Kleiner, MD, PhD

National Institute of Child Health and Human Development, Bethesda, MD: Gilman D. Grave, MD

National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD: Edward C. Doo, MD; Jay H. Hoofnagle, MD; Patricia R. Robuck, PhD, MPH (2002–2011); Averell Sherker, MD

Johns Hopkins University, Bloomberg School of Public Health (Data Coordinating Center), Baltimore, MD: Patricia Belt, BS; Jeanne M. Clark, MD, MPH; Erin Corless, MHS; Michele Donithan, MHS; Milana Isaacson, BS; Kevin P. May, MS; Laura Miriel, BS; Alice Sternberg, ScM; James Tonascia, PhD; Aynur Ünalp-Arida, MD, PhD; Mark Van Natta, MHS; Ivana Vaughn, MPH; Laura Wilson, ScM; Katherine Yates, ScM

STUDY HIGHLIGHTS

1. WHAT IS CURRENT KNOWLEDGE

- There is an inverse association between serum vitamin D levels with BMI and insulin resistance levels.
- VDD is associated with a pro-inflammatory immune response.
- VDD has emerged as a risk factor for diabetes mellitus, metabolic syndrome and NAFLD.
- The relationship between VDD and NASH pathogenesis is poorly documented.

2. WHAT IS NEW HERE

- VDD was independently associated with definitive NASH, increased NAS, lobular inflammation and ballooning and the presence of fibrosis after adjusting for age, sex, race, BMI, ALT, and diabetes status.
- Serum resistin levels are significantly increased in VDD NAFLD patients.
- Hepatic transcriptomic profiling suggests that the MAPK and NF- κ B pathways are activated in VDD NAFLD patients.

Figure 1. Multivariable logistic regression analysis for histologic features of NASH

Multiple logistic regression analysis was used to model the independent risk of the presence (yes versus no) of a definitive diagnosis of NASH (Panel A) and advanced histological disease features (Panels B–E) for VDD and including potential confounding variables age, male sex, race, diabetes, metabolic syndrome, BMI, ALT level and season of the year.

Table 1

Patient characteristics according to vitamin D level category

Characteristic *	Vitamin D deficient < 20 ng/ml	Vitamin D insufficient 20–30 ng/ml	Vitamin D sufficient >30 ng/ml	P value [#]	P trend [†]
Number	105 (55)	49 (26)	36 (19)		
Age (yrs)	46.5 ± 10.2	48.1 ± 11.5	50.5 ± 12.3	0.072	0.023
Male (No.)	54 (61)	20 (22)	15 (17)	0.378	0.215
BMI (kg/m ²)	35.4 ± 6.8	33.6 ± 6.5	33.5 ± 6.2	0.204	0.115
Obese (≥ 30 BMI)	80 (59)	32 (24)	23 (17)	0.167	0.083
Waist circum. (cm)	111 ± 13	107 ± 16	109 ± 14	0.237	0.337
Waist-to-hip ratio	0.95 ± 0.08	0.95 ± 0.08	0.94 ± 0.08	0.708	0.408
Diabetes mellitus	19 (54)	7 (20)	9 (26)	0.496	0.517
Metabolic Syndrome	71 (58)	26 (21)	26 (21)	0.122	0.113
Race (No.)				0.246	0.107
White	77 (52)	36 (25)	34 (23)		
Black	4 (80)	1 (20)	0 (0)		
Asian	7 (54)	5 (38)	1 (8)		
American Indian or Alaska Native	6 (86)	0 (0)	1 (14)		
Native Hawaiian or Pacific Islander	1 (100)	0 (0)	0 (0)		
Other	5 (50)	5 (50)	0 (0)		
Ethnicity (No.)				0.213	0.078
Non-Hispanic	87 (53)	43 (26)	34 (21)		
Hispanic	18 (69)	6 (23)	2 (8)		
Household income				0.370	0.791
<\$15,000	9 (50)	3 (17)	6 (33)		
\$15,000–\$29,999	13 (65)	6 (30)	1 (5)		
\$30,000–\$49,999	24 (60)	11 (28)	5 (12)		
\$50,000	58 (53)	29 (26)	23 (21)		
Dietary vitamin D (mg/day)	131 ± 93	156 ± 116	187 ± 180	0.163	0.059
Supplemental vitamin D (mg/day)	109 ± 168	85 ± 152	206 ± 201	0.067	0.130
Dietary calcium (mg/day)	728 ± 444	722 ± 385	719 ± 439	0.991	0.978
Supplemental calcium (mg/day)	136 ± 321	109 ± 241	431 ± 519	0.020	0.013

Characteristic*	Vitamin D deficient < 20 ng/ml	Vitamin D insufficient 20-30 ng/ml	Vitamin D sufficient >30 ng/ml	P value [#]	P trend [†]
Metabolic Equiv. (wk)	128 ± 48	127 ± 65	128 ± 57	0.730	0.873
Avg. Sunlight (mo) [‡]	152 ± 70	197 ± 73	196 ± 58	0.0001	<0.001

* Values are means ± sd or n(%)

[#] Differences *between* vitamin D level categories were assessed using the Kruskal Wallis test for continuous variables and Fisher's Exact test for categorical data.

[†] Trends *across* categories were analyzed using non-parametric trend test (Stata nptrend test) for continuous variables or Cochran-Mantel-Haenszel Stratified Test of Association for categorical data

Table 2

Laboratory value differences among different vitamin D level categories

Variable *	Vitamin D deficient (<20 ng/ml)	Vitamin D insufficient (20–30 ng/ml)	Vitamin D sufficient (>30 ng/ml)	P value #	P trend †
Glucose (mg/dL)	105 ± 29	106 ± 33	109 ± 32	0.786	0.643
Insulin (μU/mL)	25.1 ± 21.6	21.5 ± 13.9	21.6 ± 12.1	0.558	0.390
HOMA-IR	6.7 ± 5.9	5.7 ± 4.3	6.0 ± 4.1	0.682	0.585
HbA1c (%)	6.0 ± 1.1	6.0 ± 1.1	5.9 ± 0.9	0.828	0.628
ALT (U/L)	72 ± 43	74 ± 45	96 ± 68	0.276	0.161
AST (U/L)	48 ± 26	50 ± 28	63 ± 44	0.309	0.227
AST/ALT	0.75 ± 0.31	0.73 ± 0.24	0.72 ± 0.22	0.945	0.800
Direct bilirubin (mg/dL)	0.82 ± 0.45	0.77 ± 0.43	0.75 ± 0.30	0.751	0.690
Total bilirubin (mg/dL)	0.15 ± 0.09	0.14 ± 0.06	0.16 ± 0.07	0.130	0.104
GGT (U/L)	62 ± 65	55 ± 39	83 ± 109	0.708	0.546
Uric acid (mg/dL)	6.6 ± 1.8	6.5 ± 1.3	6.5 ± 1.5	0.954	0.917
Platelets (K/cmm)	239 ± 66	238 ± 60	228 ± 63	0.508	0.260
Leukocytes (K/cmm)	7.2 ± 2.2	6.3 ± 1.8	6.8 ± 2.4	0.017	0.049
Hemoglobin (g/dL)	14.5 ± 1.5	14.3 ± 1.4	14.3 ± 1.1	0.296	0.142
Calcium (mg/dL)	9.4 ± 0.4	9.4 ± 0.5	9.6 ± 0.4	0.349	0.231
Serum ferritin (ng/mL)	244 ± 228	303 ± 349	395 ± 501	0.327	0.139
Total cholesterol (mg/dL)	197 ± 46	195 ± 45	199 ± 45	0.930	0.969
Triglycerides (mg/dL)	184 ± 121	189 ± 199	180 ± 120	0.878	0.780
LDL (mg/dL)	122 ± 39	118 ± 40	123 ± 42	0.842	0.993
HDL (mg/dL)	42 ± 11	43 ± 10	46 ± 10	0.163	0.057
IL-6 (pg/ml)	8.7 ± 10.5	24 ± 62	9.5 ± 17.2	0.671	0.467
IL-1β (pg/ml)	0.53 ± 0.46	0.34 ± 0.28	0.59 ± 0.60	0.174	0.686
TNF-α (pg/ml)	9.6 ± 17.7	7.2 ± 3.7	7.3 ± 3.7	0.970	0.970
IL-8 (pg/ml)	3.9 ± 3.3	3.3 ± 3.1	5.3 ± 6.1	0.156	0.605
Resistin (ng/ml)	17.0 ± 7.7	15.8 ± 9.4	12.5 ± 6.0	0.044	0.013
Adiponectin (mg/ml)	10.6 ± 4.9	11.8 ± 7.6	12.4 ± 8.0	0.880	0.619
Vitamin D 25(OH)D2	3.5 ± 7.9	1.6 ± 2.4	1.7 ± 2.9	0.540	0.286
Vitamin D 25(OH)D3	13.8 ± 4.0	24.1 ± 2.6	37.0 ± 6.4	0.0001	<0.001

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

* Values are means $\pm$ sd

Differences between vitamin D level categories were assessed using the Kruskal Wallis test.

† Trends across categories were analyzed using the non-parametric trend test (Stata nptrend test).

Table 3

Factors affecting serum vitamin D 25(OH)D3 levels defined by regression analysis

Variable	Univariate regression			Multivariable regression [†]		
	Coefficient	S.E.	P value	Coefficient	S.E.	P value
Age	0.15	0.06	0.17			0.018
Male sex	-1.39	1.44	-0.07			0.335
Race	-1.06	0.55	-0.14			0.054
Hispanic ethnicity	-4.55	2.06	-0.16	-5.36	2.55	0.038
BMI (kg/m2)	-0.21	0.11	-0.14	-0.28	0.12	0.018
Avg. Sunlight	0.04	0.01	0.31	0.04	0.01	0.002
Income level	0.35	0.72	0.04			0.626
Metabolic Equiv. (wk)	-0.01	0.02	-0.04			0.648
Dietary vitamin D (mg/day)	0.01	0.01	0.17			0.047
Suppl. vitamin D (mg/day)	0.01	0.004	0.13			0.129

[†] stepwise forward linear regression using p<0.1 cutoff for inclusion into the model

Table 4
Top 10 differentially expressed genes in hepatic tissue between VDD and non-VDD subjects

Gene Name	Gene Symbol	logFold change	Raw P value	Adj. P value	Potential relationship to NAHL pathogenesis
Keratin 10	<i>KRT10</i>	-2.33	4.71E-08	9.72E-04	KRT10 loss causes MAPK activation (63) Enhanced Akt and NFκB activity (64)
Sema Domain, Immunoglobulin Domain (Ig), Short Basic Domain, Secreted	<i>SEMA3B</i>	1.72	2.97E-07	0.003	Induces apoptosis (65) Activates MAPK and induces IL8 (67)
Small Nucleolar RNA, C/D Box 3C	<i>SNORD3C</i>	-2.99	7.06E-06	0.035	Unknown
Arylsulfatase D	<i>ARSD</i>	-0.46	8.31E-06	0.035	Localized to the endoplasmic reticulum, function unknown (68)
Immunoglobulin Kappa Variable 4-1	<i>IGKV4-1</i>	1.10	8.69E-06	0.035	FcεRI mediated NF-κB, MAPK and TLR activation (57, 58)
Small Nucleolar RNA, C/D Box 3D	<i>SNORD3D</i>	-2.29	2.49E-05	0.077	Unknown
Solute Carrier Family 51, Beta Subunit	<i>SLC51B</i>	1.55	2.88E-05	0.077	Basolateral bile acid transporter; increased enterohepatic bile reabsorption activates VDR and MAPK signaling (69).
Immunoglobulin Lambda Variable 8-61	<i>IGLV8-61</i>	1.69	2.97E-05	0.077	FcεRI mediated NF-κB, MAPK and TLR activation (57, 58)
V-Fos/FBJ Murine Osteosarcoma Viral Oncogene Homolog	<i>FOS</i>	1.82	3.51E-05	0.080	Forms AP-1; activated by FcεRI, MAPK; induces cytokine and chemokine release; activates TGFβ (59)
Trefoil Factor 3	<i>TFF3</i>	-2.13	4.35E-05	0.089	Decreased hepatic TFF3 is associated with greater insulin resistance (70)