Vitamin D levels and liver histological alterations in children with nonalcoholic fatty liver disease

Valerio Nobili[1], Valentina Giorgio[1], Daniela Liccardo[1], Giorgio Bedogni[1,2], Giuseppe Morino[3], Anna Alisi[1] and **Stefano Cianfarani[4,5]**

[1]Hepato-Metabolic Disease Unit, Bambino Gesù Children's Hospital – IRCCS, Rome, Italy, [2]Clinical Epidemiology Unit, Liver Research Center, Basovizza, Trieste, Italy, [3]Dietetics Unit, Bambino Gesù Children's Hospital – IRCCS, Rome, Italy, [4]D.P.U.O. 'Bambino Gesù' Children's Hospital – 'Tor Vergata' University, Piazza S. Onofrio 4, 00165 Rome, Italy and [5]Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

Correspondence should be addressed to S Cianfarani
Email
stefano.cianfarani@opbg.net

Abstract

Objective: To investigate the association between plasma vitamin D (VD) levels and histological liver damage in children with nonalcoholic fatty liver disease (NAFLD).

Subjects and methods: In this cross-sectional study, carried out in a tertiary care center for obesity, 73 consecutive overweight and obese children with persistently elevated serum aminotransferase levels and diffusely hyperechogenic liver on ultrasonography were selected for liver biopsy. Nonalcoholic steatohepatitis (NASH) and fibrosis were histologically diagnosed using NAFLD Clinical Research Network (CRN) criteria. The plasma levels of 25-OH-VD were measured by HPLC. Bone mineral density (BMD) of lumbar spine was evaluated by dual-energy X-ray absorptiometry. Multiple linear regression analysis was used to evaluate the association between 25-OH-VD levels and the predictors of interest after correction for age, gender, waist circumference, BMI, and other potential confounders.

Results: The children (64% males) were aged 8–18 years, and their median BMI was 2.45 SDS. Both parathyroid hormone levels and BMD were within the normal range. All cases of fibrosis were detected in children with NASH. On multivariable linear regression with correction for age, gender, and BMI, 25-OH-VD levels were found to be 9 (95% CI 12–6) ng/ml lower in children with NASH than in those without NASH ($P<0.001$) and 9 (12–6) ng/ml lower in children with stage 1 fibrosis than in those with stage 0 fibrosis and 9 (13–6) ng/ml lower in children with stage 2 than in those with stage 0 fibrosis ($P<0.001$ for both).

Conclusion: VD levels are inversely associated with NASH and fibrosis in children with NAFLD.

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Introduction

Vitamin D (VD) is a key nutrient for both healthy and chronically ill children (1). Sources of VD are diet and dietary supplements as well as skin 7-dehydrocholesterol after exposure to UVB radiation. Despite this large availability, it is estimated that one billion people worldwide are VD deficient (2).

VD2, ergocalciferol, derives from plant sources and dietary supplements. VD3, cholecalciferol, is produced in human skin or derives from animal sources or dietary supplements. VD3 undergoes a three-step activation before interacting with its specific receptor (VDR). After the conversion of skin 7-dehydrocholesterol into pre-VD, 25-hydroxylation occurs in the liver and further 1-hydroxylation occurs in the kidneys. VD then interacts with VDR and regulates the expression of more than 200 genes, mostly involved in apoptosis, cell growth, and cell differentiation.

Nonalcoholic fatty liver disease (NAFLD) is the leading chronic liver disease worldwide, with an increasing prevalence that mirrors that of obesity (3). The prevalence

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of NAFLD in obese children is estimated to be between 36 and 44%, regardless of the method used to diagnose the disease (4, 5). It is worth noting that VD deficiency is more common in obese subjects than in normal-weight subjects (6). Furthermore, a recent meta-analysis has shown that NAFLD patients have decreased serum VD levels (7).

In adults affected by NAFLD, VD levels have been reported to be inversely associated with liver steatosis, necroinflammation, and fibrosis (8). In rats exposed to Westernized diets, VD deficiency exacerbates NAFLD through the activation of the Toll-like receptor, possibly by means of endotoxin exposure, causing insulin resistance (IR), overexpression of hepatic resistin, and upregulation of hepatic inflammatory genes (9, 10).

To date, no robust data on the association between VD levels and NAFLD in childhood are available because of the difficulty in obtaining liver tissue specimens to be related to circulating VD levels. We have previously reported preliminary data suggesting an inverse association between VD levels and liver fibrosis and necroinflammation in obese children (11). The aim of the present study was to confirm and extend that preliminary observation by carrying out a large-scale study in children with biopsy-proven NAFLD. We investigated the association of VD levels with the histopathological features of NAFLD, taking into account potential confounders such as age, gender, waist circumference, and BMI.

Subjects and methods

Subjects

A total of 73 overweight or obese Caucasian children with biopsy-proven NAFLD were consecutively enrolled in this cross-sectional study between January and March 2012. The setting was a tertiary referral center for the study and treatment of obesity and the metabolic syndrome (MS). Children with persistently elevated serum aminotransferase levels and/or diffusely hyperechogenic liver on ultrasonography were selected for liver biopsy. Exclusion criteria were hypothyroidism, Wilson's disease, HBV or HCV infection, cystic fibrosis, celiac disease, α-1-antitrypsin deficiency, autoimmune hepatitis, use of known steatogenic drugs, and parenteral nutrition. Patients taking vitamin and/or mineral supplements and/or medications known to influence VD status were excluded from the study.

VD levels were also assessed in two control groups: Control group A, 64 (40 males and 24 females) age-matched (median age: 12.8 years, 10–15 years) normal-weight children (mean BMI: 20±2.1) recruited from the Ear, Nose and Throat Pediatric Outpatient Clinic, and Control group B, 21 (13 males and eight females), age-matched (median age: 11.5 years, 6–15 years) obese children (mean BMI: 29.3±2.9) without NAFLD.

The study was approved by the Ethics Committee of the Bambino Gesù Children's Hospital, and informed consent was obtained from the parents or legal guardians of the patients or the patients themselves when aged 18 years.

Anthropometric and laboratory assessment

Weight, height, and waist circumference were measured following standard guidelines (12). BMI was calculated and converted to SDS using Italian reference data (13).

Fasting blood samples collected at the time of liver biopsy were obtained to measure serum levels of glucose, insulin, HbA1c, cholesterol, LDL, HDL, triglycerides, creatinine, calcium, phosphate, VD, parathyroid hormone (PTH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase (GGT). Simultaneous spot urinary samples were collected to measure urinary calcium levels. IR was estimated from the homeostasis model assessment (HOMA) (14). Glucose levels were measured by standard methods and insulin levels by means of RIA (MYRIA Technogenetics, Milan, Italy). Glomerular filtration rate (GFR) was estimated from Schwartz's formula (15). Blood fatty acids were analyzed in a drop of whole blood absorbed on a strip and transmethylated for gas chromatography. The levels of 25-OH-VD were measured by HPLC (Bio-Rad). This method is used to measure 25-OH VD3 and 25-OH VD2 levels. VD deficiency was defined as a value of 25-OH-VD <20 ng/ml (16). Intact PTH levels were measured using chemiluminescence immunoassay (Siemens, Munich, Germany). Normal values of PTH in our laboratory ranged between 9 and 55 pg/ml. The MS was diagnosed according to the International Diabetes Federation (17).

Dual-energy X-ray absorptiometry

Dual-energy X-ray absorptiometry measurements of lumbar spine were performed using a QDR-4500 scanner (Hologic, Inc., Waltham, MA, USA) to obtain bone mineral density (BMD, g/cm^2) at the L1–L4 level. BMD was calculated using the reference values provided by the scanner software (18).

Liver ultrasonography

Liver ultrasonography was carried out at the most 1 month before liver biopsy using an Acuson S2000 system

(Siemens) with linear and convex transducers (frequency bandwidth 4–14 MHz). Absent steatosis (grade 0) was defined as normal liver echo-texture; mild steatosis (grade 1) as slight and diffuse increase in fine parenchymal echoes with normal visualization of diaphragm and portal vein borders; moderate steatosis (grade 2) as moderate and diffuse increase in fine echoes with slightly impaired visualization of diaphragm and portal vein borders; and severe steatosis (grade 3) as fine echoes with poor or no visualization of diaphragm, portal vein borders, and posterior portion of the right lobe (19).

Liver histopathology

Liver tissue specimens were fixed in buffered formalin, embedded in paraffin, sliced into 3 mm sections, and stained with hematoxylin and eosin, periodic Schiff acid with and without diastase, and Van Gieson's trichrome stains. The histological features of NAFLD were classified using the NAFLD Clinical Research Network (CRN) system (20). Steatosis was graded as follows: 0, involving $<5\%$ of hepatocytes; 1, involving up to 33%; 2, involving 33–66%; and 3, involving $>66\%$. Lobular inflammation was graded as follows: 0, no foci; 1, <2 foci per 200× field; 2, 2–4 foci per 200× field; and 3, >4 foci per 200× field. Portal chronic inflammation was also evaluated as follows: 0, no and 1, yes. Hepatocyte ballooning was graded as follows: 0, none; 1, few balloon cells; and 2, many/prominent balloon cells. Fibrosis was staged as follows: F0, no fibrosis; F1, perisinusoidal or periportal; F2, perisinusoidal and portal/periportal; F3, bridging; and F4, cirrhosis. The nonalcoholic steatohepatitis score (NAS) was obtained by summing the steatosis, lobular inflammation, and ballooning scores. Nonalcoholic steatohepatitis (NASH) was diagnosed by a liver pathologist following the NASH CRN recommendations (20).

Statistical analysis

Most of the continuous variables were not normally distributed, and all are reported as 50th, 25th, and 75th percentiles. Categorical variables are reported as counts and percentages. Between-group comparisons of medians were made using quantile regression. Multiple linear regression was used to evaluate the association between VD levels (continuous, ng/ml) and the four predictors of interest (PTH, NAS, NASH, and fibrosis) after correction for age (continuous, years), BMI (continuous, SDS), and gender (discrete: 0, female and 1, male). In separate regression models, we also addressed the independent effects of GFR, HOMA, waist circumference, and the MS. Among the predictors, PTH was modeled as continuous (ng/dl), NAS as continuous (arbitrary units), NASH as discrete (0, no and 1, yes), and fibrosis as discrete (0, F0; 1, F1; and 2, F2). Multivariable fractional polynomials were used to test whether the relationships of VD levels with continuous predictors were linear (21). We found all relationships to be linear, and we modeled them as such. Statistical analysis was carried out using Stata, version 12.1 (Stata Corp., College Station, TX, USA). Statistical significance was set at a P value <0.05.

Results

A total of 73 children (64% males) aged 8–18 years were consecutively enrolled into the study. The children were mostly males ($n=47$, 64%), and their measurements are given in Table 1 with and without stratification for VD status. Of the 73 children with NAFLD, 34 (47%) had low VD levels, whereas 39 (53%) had normal VD levels.

Not surprisingly, children with low VD levels had higher PTH levels ($P<0.001$) than those with normal VD levels, but PTH values were within the normal range in all the cases. Although children with low VD levels had lower ALT levels ($P<0.001$), this is not clinically relevant, as we have shown the lack of association between ALT levels and liver histopathology (22). VD levels in both control groups were within the normal range (Group A: mean, 29 ± 3.3 ng/ml and Group B: mean, 29.1 ± 8.3).

Among the study participants, 53 (73%) children had a large waist circumference, two (3%) high blood pressure, three (4%) high glucose levels, 22 (30%) high triglyceride levels, and 26 (36%) low HDL levels and 10 (14%) had three or more of the above, i.e. the MS (17). The histopathological features of the children are given in Table 2.

NASH was present in 67% of the children and fibrosis of any degree (F1 or F2) in 67% of the cases. All cases of fibrosis occurred in NASH patients.

The associations between VD levels and PTH, NAS, NASH, and fibrosis after correction for gender, age, waist circumference, and BMI are depicted in Fig. 1. The corresponding regression models are given in Supplementary Material, see section on supplementary data given at the end of this article.

An increase of 1 pg/ml in VD levels was associated with a mean decrease of 0.2 ng/ml in PTH levels ($P<0.01$). This effect was stable after correction for age, gender, and BMI alone and in association with GFR, HOMA, waist circumference, and the MS (see Supplementary Table 1,

Table 1 Measurements of the study children ($n=73$).

	Normal VD ($n=39$)			Low VD ($n=34$)			All ($n=73$)		
	P_{50}	P_{25}	P_{75}	P_{50}	P_{25}	P_{75}	P_{50}	P_{25}	P_{75}
Age (years)	14	11	15	12	11	16	13	11	15
BMI (kg/m^2)	31.8	28.5	34.4	30.3	27.9	34.4	31.3	28.5	34.4
BMI (SDS)	2.47	2.18	2.84	2.24	2.03	2.87	2.45	2.09	2.84
Waist circumference (cm)	90	88	100	90	87	103	90	88	100
BMC L1–L4 (g)	24	23.5	25	24	23	25	24	23	25
BMD L1–L4 (g/cm^2)	0.67	0.63	0.71	0.65	0.57	0.81	0.67	0.62	0.77
Glucose (mg/dl)	87	79	90	85	77	88	86	78	90
Insulin (μU/ml)	21	13	27	17	15	22	19	15	23
HOMA	4	3	5	4	3	5	4	3	5
HbA1c (mmol/mol)	30	27	33	30	28	33	30	28	33
Cholesterol (mg/dl)	178	130	196	166	149	188	172	137	190
LDL (mg/dl)	101	79	124	84	75	123	99	77	123
HDL (mg/dl)	42	34	46	43	37	50	42	37	49
Triglycerides (mg/dl)	102	60	150	86	59	178	95	60	164
Creatinine (g/dl)	0.6	0.4	0.8	0.6	0.6	0.7	0.6	0.5	0.7
eGFR (ml/min)	114	87	152	98	81	134	109	85	141
Serum calcium (mg/dl)	10.0	9.7	10.2	9.8	9.5	10.0	10.0	9.5	10.1
Serum phosphate (mg/dl)	6.1	5.8	6.4	6.1	5.7	6.3	6.1	5.7	6.4
Urinary calcium (mg/dl)	76	52	102	93	74	110	89	64	110
25-OH-vitamin D (ng/ml)	23	22	29	14*	12	15	20	14	23
PTH (pg/ml)	16	14	22	28*	16	32	18	15	31
AST (U/l)	27	24	43	24	21	30	25	21	41
ALT (U/l)	43	27	57	27*	20	43	32	21	50
GGT (U/l)	18	16	33	17.5	15	36	18	15	34

*$P<0.001$ vs normal vitamin D (median regression). BMC, bone mineral content.

see section on supplementary data given at the end of this article).

An increase of 1 unit in the NAS was associated with a mean decrease of 4.2 pg/ml in VD levels ($P<0.001$). This effect was stable after correction for the other predictors (see Supplementary Table 2, see section on supplementary data given at the end of this article). Among the other predictors, only the MS was independently associated with the NAS.

Coherently with the previous finding, NASH was associated with lower VD levels, i.e. −9.0 pg/ml ($P<0.001$) when compared with that in children without NASH. The HOMA was independently associated with VD levels ($P<0.05$), and the same was found for the MS ($P<0.01$) (see Supplementary Table 3, see section on supplementary data given at the end of this article).

Finally, F1 or F2 fibrosis was associated with lower VD levels when compared with F0 fibrosis, with similar values of −8.8 and −9.3 pg/ml ($P<0.001$). The size of this effect was similar to that observed with NASH because only subjects with NASH had fibrosis. Thus, it had to be expected that in this case HOMA ($P<0.05$) and the MS ($P<0.01$) were also inversely associated with fibrosis after correction for gender, age, and BMI (see Supplementary Table 4, see section on supplementary data given at the end of this article).

Discussion

The present study confirms and extends our preliminary observation of an inverse association between serum VD levels and histological liver damage in children with

Table 2 Histopathological findings at liver biopsy.

	n	Percentage (%)
Nonalcoholic steatohepatitis score (NAS)		
3	16	21.9
4	26	35.6
5	30	41.1
6	1	1.4
Total	73	100.0
Nonalcoholic steatohepatitis (NASH)		
No	24	32.9
Yes	49	67.1
Total	73	100.0
Fibrosis		
F0	24	32.9
F1	30	41.1
F2	19	26.0
Total	73	100.0

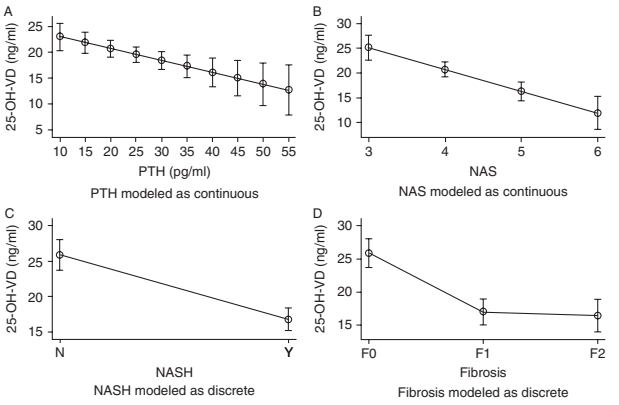

Figure 1

Relationships between vitamin D (25-OH-VD), parathyroid hormone (PTH, A), nonalcoholic steatohepatitis score (NAS, B), nonalcoholic steatohepatitis (NASH, C), and liver fibrosis (D) after correction for age, gender, and BMI in children with nonalcoholic fatty liver disease (see Supplementary Material for the corresponding regression models). Effect sizes (means and 95% CI) from multiple linear regression with gender, age, and BMI as covariates.

NAFLD (11). Both NAFLD and VD deficiency are common in obese subjects. While obesity is a risk factor for both conditions (1, 3), a growing body of evidence suggests a possible causal association between VD deficiency and NAFLD (10).

The biological effects of VD are mediated by VDR, a ligand-dependent transcription factor belonging to the family of nuclear hormone receptors. After binding with $1,25(OH)_2VD$, VDR dimerizes with the retinoid X receptor and binds to specific genomic sequences known as VD-response elements. VDR action has traditionally been associated with calcium homeostasis and bone mineralization. In this respect, it is of interest that none of the children included in the present study had osteopenia or osteoporosis contrarily to adults with NAFLD (23), although this may partly be explained by their younger age.

VDR is also expressed in many tissues that are not involved in calcium homeostasis. The enzyme 1α-hydroxylase, for example, is responsible for the production of $1,25(OH)_2VD$ in many organs beside the kidneys. This extrarenal production of $1,25(OH)_2VD$, together with a wide VDR availability, allows a local paracrine/autocrine regulation. In fact, $1,25(OH)_2VD$ controls the expression of many genes, playing a key role in cell proliferation, differentiation, inflammation, and immunity (24, 25).

In the liver, VDR is expressed in cholangiocytes, and this expression has been shown to be inversely associated with steatosis severity, lobular inflammation, and NAFLD score in adult patients with NASH (25). Moreover, both animal and human data suggest that $1,25(OH)_2VD$ may have anti-fibrogenic effects (25, 26). Selected *VDR* polymorphisms have been reported to be associated with primary biliary cirrhosis (27). VD levels have been shown to be related to more severe fibrosis and reduced response to interferons in viral liver diseases (28, 29). Finally, in a recent cross-sectional study of about 6500 Korean men participating in a health-screening program, patients with NAFLD had lower VD levels than those without NAFLD and such a difference persisted after correction for BMI and the MS (30).

A possible role for VD in liver fibrosis is supported by the fact that VDR is expressed in hepatic stellate cells (HSCs) (31). Such cells play a critical role in the pathogenesis of liver fibrosis, as they are responsible for the deposition of extracellular matrix (ECM) proteins. After activation, HSCs transform from quiescent vitamin A-storing cells to myofibroblast-like cells and accumulate ECM proteins, mostly type 1 collagen. Interestingly, there is *in vitro* evidence that VD administration may inhibit HSC activation by different pro-fibrogenic pathways (26, 32). The recent findings that phototherapy improves liver histology (33) and that VD supplementation prevents liver fibrosis (26) in animal models of NAFLD support a potential role of VD in the prevention and treatment of NASH.

Unexpectedly, no association between BMI and VD levels was found in our cohort of obese children. As VD is fat soluble and can be easily stored in adipose tissue, a link between obesity and low circulating VD levels has been reported (34). A possible explanation is that our study population consisted of only obese individuals with the same degree of excess weight.

This is the first study to systematically address the association between VD levels and histological liver damage in children with NAFLD, but a series of limitations should be considered. First, this is a cross-sectional study and as such cannot prove causation. However, the results are consistent with the available experimental and clinical data suggesting that VD deficiency is involved in the pathogenesis of NAFLD. Second, as this study was carried out in a tertiary care center, its findings may not be extended to the general population. Third, the lack of information about the physical activity of the study cohort does not allow to us to exclude this potential confounder. Lastly, our study population consisted of only Caucasian children, while NASH is more frequent and possibly more severe in other ethnic groups (3). Therefore,

further studies are certainly needed to evaluate VD–NAFLD association among non-Caucasian children.

Conclusion

We detected a significant inverse relationship between VD levels and histological liver damage in children with NAFLD, independently of age, gender, BMI, and other potential confounders. Whether VD plays a key role in the development of NAFLD should be determined by means of longitudinal cohort studies and randomized controlled trials with VD supplementation.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-13-0609.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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