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VITAMIN D DEFICIENCY, A NONINVASIVE MARKER OF STEATOHEPATITIS IN PATIENTS WITH OBESITY AND BIOPSY PROVEN NONALCOHOLIC FATTY LIVER DISEASE

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Abstract

Context. Nonalcoholic fatty liver disease (NAFLD) includes simple steatosis, steatohepatitis (NASH) which can evolve with progressive fibrosis, cirrhosis and hepatocellular carcinoma. As liver biopsy cannot be used as a screening method, noninvasive markers are needed.

Objective. The aim of this study was to test if there is a significant association between vitamin D deficit and the severity of NAFLD.

Design. The patients were divided into two groups (vitamin D insufficiency/deficiency) and statistical analyses were performed on the correlation of clinical and biochemical characteristics with histopathological hepatic changes.

Subjects and methods. We prospectively studied 64 obese patients referred for bariatric surgery between 2014 and 2016 to our Surgical Unit. Anthropometric, clinical measurements, general and specific biological balance were noted. NAFLD diagnosis and activity score (NAS) were evaluated on liver biopsies.

Results. Increased serum fibrinogen was correlated with NASH (p=0.005) and higher NAS grade. T2DM was positively correlated with liver fibrosis (p=0.002). 84.37% of the patients had vitamin D deficit and 15.62% were vitamin D insufficient. Lobular inflammation correlated with vitamin D deficit (p=0.040). Fibrosis (p=0.050) and steatohepatitis (p=0.032) were independent predictors of low vitamin D concentration.

Conclusions. Vitamin D status in conjunction with other parameters - such as T2DM - or serum biomarkers – namely fibrinogen level and PCR level - may point out the aggressive forms of NAFLD and the need for liver biopsy for appropriate management.

Key words: Vitamin D deficiency, steatohepatitis, NAFLD, obesity.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) comprises a whole spectrum of diseases, including simple steatosis, steatohepatitis (NASH) which can evolve with progressive fibrosis, cirrhosis and hepatocellular carcinoma. It is highly associated with obesity, insulin resistance and metabolic syndrome and it is estimated to affect about 20-40% of the general population in developed countries (1).

Hepatic steatosis is a non-progressive disease that does not alter life expectancy (2). Steatohepatitis instead, is the progressive form of NAFLD, characterized by lobular inflammation, hepatocytes degeneration and ballooning, under the condition of macrovesicular hepatic steatosis (3). The dangerousness of this specific form of NAFLD is given by its increased chance of progressing to liver fibrosis, cirrhosis and hepatocellular carcinoma. NAFLD occurs in 15% of non obese persons, in 65% of grade I and II obese people and in 85% of morbidly obese population (1). 25% of these cases will develop NASH (4) and 15% have an increased risk of developing liver cirrhosis (5); however, the incidence of this condition is under-reported and there are wide variations in the overall incidence (6).

Although liver biopsy is the gold standard in diagnosis of NAFLD it cannot be used as a screening method. Therefore, numerous noninvasive markers were studied over time in order to guide clinicians in assessing patients with obesity and NAFLD in terms of liver damage severity. Such an easy to dose and non-expensive marker is serum 25 (OH) vitamin D, the most stable of its circulating forms that also reflects the status of vitamin D in humans (7). There is already evidence showing the link between low serum vitamin D, obesity and the presence of metabolic syndrome or any of its various components, which are further closely related to NAFLD (8). The aim of this study was to test the hypothesis that there is a significant association between vitamin D deficit and the severity of NAFLD.

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MATERIALS AND METHODS

We conducted a prospective study of 64 obese patients referred for bariatric surgery to the IIIrd Surgery Unit of "St. Spiridon" Hospital Iasi between November 2014 and November 2016. We included obese patients randomized, in a consecutive manner, who were eligible for bariatric surgery. Patients with history of alcohol consumption (over 20g / day for women and more than 30 g / day in men), chronic hepatitis B or C or other chronic liver diseases or consumption of drugs known as hepatotoxic were excluded. We also excluded patients with endocrine, renal and other chronic conditions or medical therapies that could affect cardiovascular or metabolic function. The patients enrolled signed the informed consent approved for this study by the Ethics Committee of "St. Spiridon" Hospital and "Gr. T. Popa" University of Medicine and Pharmacy, Iasi. All subjects had a complete work-up including medical history, clinical examination, anthropometric measurements, both general and specific biological balance. Fasting blood samples were collected in the morning of surgical procedure to assess serum Chol, HDL-Chol, Triglycerides, Insulin, Gly, 25(OH) vitamin D. Insulin resistance was assessed by homeostatic model assessment-insulin resistance index (HOMA-IR = fasting glucose (mmol/L) X fasting insulin (mU/L)/22.5). CRP, Fibrinogen level, NLR and PLR scores (obtained by dividing the neutrophil counts and the platelet counts by the lymphocyte count respectively) were evaluated, with prognostic value for liver inflammation. 25 (OH) vitamin D serum level was measured using chemiluminescence assay. We used a single a value of serum 25 (OH) vitamin D, considering it to be the exact value that reflects vitamin D status when evaluating by liver biposy the hepatic changes related to obesity. Of associated comorbidities, type II diabetes mellitus (T2DM) and arterial hypertension (HTA) were noted as components of the metabolic syndrome (MS).

All patients underwent a liver biopsy during bariatric surgery, with samples measuring at least 0.4/0.4 cm. The liver biopsies were formalin-fixed and paraffin-embedded. Serial sections were stained with hematoxylin and eosin (H&E). All specimens were analyzed by the same experienced pathologist who was blinded to clinical and biological data except for the fact that the hepatic biopsies were taken from bariatric patients. The samples were evaluated using NAFLD criteria by Kleiner *et al.* The NAFLD activity score (NAS) was as well evaluated using Brunt *et al.* scoring system (9,10).

Statistical analyses were performed using SAS 9.1 software. Continuous data were expressed as means (± standard deviation [SD]) and noncontinuous data in percentages. Comparison between groups used analysis of variance (ANOVA) and Kruskal-Wallis test. Multiple logistic analyses were performed for NAS score dependent variable and vitamin D level dependent variable.

RESULTS

Our study group consisted of 49 women and 15 men. BMI ranged between 35 and 58 with an average of 45.06. 17% of the patients had II grade obesity and 83% of them were morbidly obese. Respecting treatment indications set by guidelines (11), patients with grade II obesity in our study had at least one of the severe and hardly controllable comorbidities, such as: type 2 DM (3 patients), hyperlipidemia (1 patient), arterial hypertension (2 patients) or concomitant combinations of these pathologies (5 patients), which is why they were refereed to bariatric surgical procedure. Different forms of NAFLD were diagnosed in all cases from our target group formed by obese patients with indication of bariatric surgery. Simple steatosis was found in 37.5% of the cases and nonalcoholic steatohepatitis (NASH) in 51.56% of them. 60.6% of the patients with NASH also had different degrees of fibrosis. As 10.9% of the subjects had characteristic hepatic cell injuries which consisted in association of lobular inflammation and hepatocyte ballooning in the abscence of macrovesicular steatosis, we considered those cases as NASH with "vanished steatosis".

Patients with NASH had significantly higher average of fibrinogen levels (p=0.005); PCR serum levels were significantly higher (p=0.005) in subjects with NASH + "vanished steatosis". The female sex was correlated with NASH, either in the presence or absence of steatosis (Table 1).

Considering liver histopathological changes separately, statistical analysis indicated increased fibrinogen and PCR serum levels in patients presenting lobular inflammation. We found a statistically significant correlation (p=0.018 and p=0.019 respectively) between increased values of these two inflammatory markers and liver fibrosis (Table 2).

The subgroup of NASH patients consisted in 33 cases, 60.6% of them presenting also different degrees of fibrosis; analyzing this subgroup, the results showed that there is a significant positive correlation between higher levels of serum TG and higher TG/

HDL-Chol index and the progressive fibrotic form of steatohepatitis. T2DM was also positive correlated with the presence of liver fibrosis (Table 3). In a linear regression model, age and sex showed to be good predictors in the determinism of steatohepatitis with fibrosis (Table 4).

The NAFLD score was evaluated for all patients, 50% of them had NAS grade 0 (score < 3), 43.75 % NAS grade 1 (score 3-6) and only 6.25% had NAS grade 2 (score 7-9). None of the subjects presented grade 3 NAS (score 10-12). The statistical analysis showed a significant correlation between female sex and increased serum fibrinogen level and a higher NAS grade (Table 5). Multivariate analysis confirms gender and fibrinogen levels as markers which can significantly influence the NAS score (Table 6).

All patients included in our study had low levels of 25(OH) vitamin D. 84.37% had vitamin D deficit, with an average of 12.58 ng/mL and values ranging between 3.45 ng/mL and 19.78 ng/mL. 15.62% of the patients were vitamin D insufficient, with an average of 25.34 ng/mL. Table 7 summarizes the clinical and biological characteristics of patients, dividing them into two groups according to their plasma 25-OH D

Table 1. Clinical and biochemical characteristics of study population

Parameter	NAFLD			
	Simple steatosis (n=24)	NASH (n=33)	NASH + "vanished steatosis"(n=7)	p-value for F_{ANOVA} test
Age (year)	38.38 ± 13.40	43.18 ± 10.83	42.71 ± 10.90	0.310
Sex* (M/F)	2/22	13/20	0/7	**0.003**
T2DM* (%)	8.3	24.2	28.6	0.115
HTA* (%)	28.1	35.7	50.0	0.343
BMI (kg/m^2)	44.35 ± 6.82	45.82 ± 6.50	43.91 ± 7.56	0.643
HOMA-IR	4.42 ± 2.39	5.71 ± 3.58	5.28 ± 2.46	0.300
IR-present* (%)	50.0	36.4	28.6	0.225
Total cholesterol (mg/dL)	195.52 ± 45.95	200.83 ± 35.37	217.17 ± 28.57	0.503
HDL-cholesterol (mg/dL)	43.42 ± 8.95	44.09 ± 11.53	45.14 ± 7.76	0.921
Triglycerides (mg/dL)	144.75 ± 55.48	167.67± 101.35	123.86 ± 35.16	0.342
TG/HDL chol score	3.53 ± 1.64	4.42 ± 4.75	2.81 ± 0.99	0.454
25(OH) vit. D (ng/mL)	16.19 ± 6.25	13.78 ± 5.92	12.80 ± 7.63	0.264
Fibrinogen (mg/dL)	379.83 ± 46.89	421.00 ± 44.84	403.43 ± 52.00	**0.005**
PCR (mg/dL)	0.47 ± 0.45	1.07 ± 0.94	1.48 ± 1.08	**0.005**
PLR	119.82 ± 24.63	112.49 ± 37.83	118.26 ± 13.17	0.674
NLR	2.09 ± 0.67	2.02 ± 0.81	2.51 ± 0.66	0.287

Results are shown as mean ± SD and *Kruskal-Wallis test.

Table 2. Clinical and biological characteristics related to various histopathological changes

Parameter	Histopathological changes					
	Mild/medium steatosis (n=40)	Severe steatosis (n=17)	Lobular inflammation (n=44)	Hepatocyte ballooning (n=49)	Fibrosis (n=25)	p-value for F_{ANOVA} test
Age (year)	40.65±12.36	42.35±11.75	42.89±11.04	40.98±11.21	44.76±10.99	0.678
Sex* (M/F)	9/31	6/11	14/30	14/35	9/16	0.073
T2DM* (%)	22.5	5.9	25.0	20.4	36.0	0.127
HTA* (%)	35.0	29.4	40.9	34.7	40.0	0.465
BMI (kg/m^2)	45.12±6.29	45.41±7.53	45.35±6.40	45.22±5.97	44.92±6.84	0.879
HOMA-IR	5.07±3.48	5.41±2.40	5.38±3.43	5.36±3.24	5.91±3.80	0.709
IR-present* (%)	50.0	23.5	40.9	38.8	32.0	0.400
Cholesterol (mg/dL)	194.80± 43.54	205.79± 32.75	205.65± 36.00	199.45± 40.21	205.60± 36.67	0.407
HDL chol. (mg/dL)	43.28± 9.99	45.06± 11.66	44.09± 10.69	43.41± 10.52	44.40± 12.38	0.560
Triglycerides (mg/dL)	147.63± 57.57	182.47± 128.20	157.64± 92.11	164.04± 88.97	171.56± 114.26	0.160
TG/HDL chol score	3.65±1.84	4.99±3.77	4.10±4.21	4.23±3.98	4.60±5.40	0.222
25(OH) vit. D (ng/mL)	15.31±5.48	13.60±7.47	13.86±5.87	14.29±6.31	13.58±5.73	0.339
Fibrinogen (mg/dL)	409.45± 47.70	390.06± 49.36	416.95± 43.60	409.53± 48.13	421.32± 47.92	**0.018**
PCR (mg/dL)	0.89±0.94	0.66±0.40	1.06±0.95	0.98±0.89	1.21±1.02	**0.019**
PLR	118.86± 33.12	107.85± 31.88	113.20± 33.73	117.88± 34.58	111.93± 28.63	0.250
NLR	1.89±0.69	2.11±0.77	2.02±0.77	2.15±0.82	2.10±0.56	0.291

Results are shown as mean ± SD and *Kruskal-Wallis test.

Table 3. Differences between Steatohepatitis and Steatohepatitis + Fibrosis

Parameter	Steatohepatitis (n=13)	Steatohepatitis+ Fibrosis (n=20)	p-value for F_{ANOVA} test
Age (year)	39.46 ± 10.16	45.60 ± 10.80	0.113
Sex* (M/F)	4/9	9/11	0.410
T2DM* (%)	0.0	40.0	**0.002**
HTA* (%)	46.2	45.0	0.948
BMI (kg/m^2)	46.62 ± 6.77	45.30 ± 6.44	0.575
HOMA-IR	5.09 ± 2.82	6.12 ± 4.02	0.430
IR-present* (%)	46.2	30.0	0.348
Total cholesterol (mg/dL)	193.63 ± 21.29	204.67 ± 41.16	0.489
HDL-cholesterol (mg/dL)	43.15 ± 7.19	44.70 ± 13.18	0.713
Triglycerides (mg/dL)	141.00 ± 45.09	185.00 ± 27.58	**0.050**
TG/HDL chol score	3.44 ± 1.48	5.07 ± 1.33	**0.045**
25(OH) vitamin D (ng/mL)	13.93 ± 7.41	13.69 ± 4.93	0.909
Fibrinogen (mg/dL)	417.08 ± 42.86	423.55 ± 47.00	0.692
PCR (mg/dL)	1.03 ± 0.95	1.10 ± 0.96	0.828
PLR	114.74 ± 47.43	111.03 ± 31.36	0.788
NLR	2.02 ± 1.13	2.02 ± 0.54	0.996

Results are shown as mean ± SD and *Kruskal-Wallis test.

Table 4. Model Summary Predictors for Steatohepatitis + Fibrosis

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	0.324(a)	0.105	0.086	0.443	0.105	50.613	1	48	0.022
2	0.422(b)	0.178	0.143	0.429	0.073	40.197	1	47	0.046
3	0.438(c)	0.192	0.120	0.434	0.014	0.379	2	45	0.687
4	0.438(d)	0.192	0.100	0.439	0.000	0.025	1	44	0.875
5	0.463(e)	0.215	0.084	0.443	0.023	0.603	2	42	0.552
6	0.609(f)	0.370	0.065	0.448	0.156	0.907	9	33	0.531

a. Predictors: (Constant), age; **b.** Predictors: (Constant), age, Sex; **c.** Predictors: (Constant), age, Sex, HTA, T2DM; **d.** Predictors: (Constant), age, Sex, HTA, T2DM, BMI; **e.** Predictors: (Constant), age, Sex, HTA, T2DM, BMI, IR, HOMA_IR; **f.** Predictors: (Constant), age, Sex, HTA, T2DM, BMI, IR, HOMA_IR, PLR, TG/HDLscore, 25(OH) vitamin D, TG, Chol, HDL, PCR, PLR, NLR.

Table 5. Clinical and biochemical characteristics related to NAS grade

Parameter	NAS score			
	Grade 0 (n=32)	Grade 1 (n=28)	Grade 2 (n=4)	p-value for F_{ANOVA} test
Age (year)	41.38 ± 13.24	40.32 ± 10.57	48.00 ± 9.52	0.490
Sex* (M/F)	2/30	10/18	3/1	**0.001**
Diabetes* (%)	12.5	25.0	25.0	0.241
HTA* (%)	28.1	35.7	50.0	0.343
BMI (kg/m^2)	44.81 ± 7.19	45.46 ± 6.59	44.23 ± 2.77	0.905
HOMA-IR	4.72 ± 2.47	5.71 ± 3.78	5.14 ± 1.93	0.473
IR-present* (%)	56.3	60.7	75.0	0.501
Total cholesterol (mg/dL)	203.27 ± 43.39	196.24 ± 24.34	198.00 ± 75.45	0.841
HDL-cholesterol (mg/dL)	44.72 ± 8.96	42.86 ± 9.77	45.50 ± 21.21	0.746
Triglycerides (mg/dL)	138.94 ± 50.99	172.07± 109.36	152.50 ± 22.65	0.298
TG/HDL chol score	3.28 ± 1.52	4.58 ± 5.04	4.29 ± 2.99	0.372
25(OH) vit. D (ng/mL)	15.21 ± 6.49	14.22 ± 6.45	12.05 ± 1.37	0.595
Fibrinogen (mg/dL)	384.78 ± 46.32	419.64 ± 44.71	442.50 ± 33.44	**0.004**
PCR (mg/dL)	0.84 ± 1.02	0.90 ± 0.72	1.26 ± 0.61	0.667
PLR	117.79 ± 22.96	113.71 ± 39.30	115.64 ± 33.06	0.883
NLR	2.09 ± 0.64	2.13 ± 0.90	1.98 ± 0.18	0.924

Results are shown as mean ± SD and *Kruskal-Wallis test.

Table 6. Multiple logistic analysis. NAS score dependent variable

Term	B Coefficient	S.E.	95% CI	t	Sig.
Constant	-0.361	1.631	-1.580÷5.245	0.221	0.826
Age	0.007	0.009	-0.027÷0.012	0.800	0.430
Sex	-0.636	0.229	-0.352÷0.550	2.775	**0.009**
Diabetes	0.243	0.279	-0.953÷0.176	0.870	0.391
HTA	0.180	0.200	-0.454÷0.365	0.903	0.373
BMI	-0.022	0.013	-0.028÷0.025	1.709	0.097
HOMA-IR	-0.088	0.057	-0.080÷0.148	1.543	0.132
IR-present	0.532	0.278	-1.025÷0.068	1.914	0.064
Chol	-0.001	0.003	-0.006÷0.004	0.504	0.618
HDL	0.021	0.019	-0.055÷0.026	1.104	0.278
Triglycerides	-0.003	0.005	-0.007÷0.014	0.519	0.607
TG/HDL	0.205	0.192	-0.549÷0.262	1.068	0.293
25(OH)Vit.D	-0.013	0.015	-0.017÷0.044	0.880	0.382
Fibrinogen	0.006	0.002	-0.004÷0.004	3.314	**0.002**
PCR	0.140	0.094	-0.114÷0.276	1.485	0.147
PLR	-0.005	0.004	-0.003÷0.014	1.337	0.190
NLR	0.072	0.173	-0.516÷0.227	0.414	0.681

Table 7. Clinical and biochemical characteristics related to vitamin D level

Parameter	Vitamin D level		p-value for F_{ANOVA} test
	Deficiency (n=54)	Insufficiency (n=10)	
Age (year)	41.44 ± 12.15	40.70 ± 11.04	0.858
Sex* (M/F)	13/41	2/8	0.777
Diabetes* (%)	22.2	0.0	**0.033**
HTA* (%)	31.5	40.0	0.603
BMI (kg/m^2)	45.34 ± 6.93	43.59 ± 5.12	0.452
HOMA-IR	5.38 ± 3.22	4.11 ± 2.05	0.238
IR-present* (%)	61.1	50.0	0.514
Total cholesterol (mg/dL)	197.21 ± 37.56	218.13 ± 46.65	0.171
HDL-cholesterol (mg/dL)	43.30 ± 10.21	47.50 ± 9.47	0.231
Triglycerides (mg/dL)	154.85 ± 87.17	151.20 ± 71.42	0.898
TG/HDL chol score	4.00 ± 3.82	3.47 ± 2.02	0.672
Fibrinogen (mg/dL)	407.78 ± 50.05	381.30 ± 32.92	0.114
25(OH) vitamin D (ng/mL)	12.59 ± 4.39	25.34 ± 2.98	**0.001**
PCR (mg/dL)	2.00 ± 0.0	1,39 ± 0.49	**0.001**
PLR	115.46 ± 32.07	118.07 ± 27.90	0.811
NLR	2.11 ± 0.68	2.07 ± 1.07	0.905

Results are shown as mean ± SD and *Kruskal-Wallis test.

Table 8. Relation between severity liver disease and plasma vitamin D level

Parameter	Vitamin D level		p-value for F_{ANOVA} test
	Deficiency (n=54)	Insufficient (n=10)	
Mild/medium steatosis* (%)	63.0	60.0	0.859
Severe steatosis* (%)	25.9	30.0	0.790
Lobular inflammation* (%)	74.1	40.0	**0.040**
Hepatocyte ballooning* (%)	75.9	80.0	0.777
Fibrosis* (%)	42.6	20.0	0.162
Simple steatosis* (%)	33.3	60.0	0.116
NASH* (%)	55.6	30.0	0.134
NASH + "vanished steatosis" * (%)	11.1	10.0	0.917

Results are shown as mean ± SD and *Kruskal-Wallis test.

Table 9. Multiple logistic analysis of anthropometric data, clinical and biological markers and histopathological liver changes. Dependent Variable: Vitamin D Level

Term	B Coefficient	S.E.	95% CI	t	Sig.
Constant	3.945	1.597	0.686÷7.204	2.518	**0.020**
Age	-0.004	0.008	-0.020÷0.012	0.504	0.619
Sex	-0.251	0.174	-0.613÷0.112	1.437	0.165
Diabetes	0.059	0.255	-0.471÷0.590	0.232	0.818
HTA	0.131	0.158	-0.198÷0.460	0.828	0.417
BMI	-0.011	0.011	-0.033÷0.011	1.029	0.315
Steatosis	-0.075	0.166	-0.420÷0.269	0.453	0.655
Inflammations	0.274	0.353	-0.460÷1.008	0.776	0.446
Hepatocyte	0.044	0.317	-0.615÷0.703	0.139	0.891
Fibrosis	-1.014	0.487	-2.026÷0.002	2.083	**0.050**
Steatohepatitis	-1.089	0.473	-2.073÷0.105	2.302	**0.032**
SH+Fibrosis	-0.151	0.675	-1.554÷1.252	0.224	0.825
HOMA-IR	-0.027	0.043	-0.116÷0.063	0.625	0.538
IR-present	0.097	0.234	-0.390÷0.583	0.414	0.683
Chol	0.001	0.003	-0.004÷0.006	0.445	0.661
HDL	0.027	0.018	-0.011÷0.065	1.497	0.149
Triglycerides	-0.008	0.006	-0.020÷0.004	1.345	0.193
TG/HDL	0.335	0.192	-0.063÷0.734	1.750	0.095
25(OH)Vit.D	0.002	0.014	-0.027÷0.030	0.121	0.905
Fibrinogen	0.002	0.002	-0.002÷0.005	0.944	0.356
PCR	-0.099	0.238	-0.593÷0.395	0.417	0.681
PLR	-0.001	0.004	-0.010÷0.008	0.171	0.866
NLR	0.031	0.160	-0.301÷0.363	0.194	0.848

levels (vitamin D insufficiency: 20–30 ng/mL and vitamin D deficiency: <20 ng/mL). Aside from the significantly different plasma vitamin D levels among the 2 groups, and patients with vitamin D deficiency having a significantly higher prevalence of T2DM (p=0.033), patients were well-matched for age and clinical or biological metabolical parameters, including BMI, HOMA-IR, HDL-Chol, Chol, TG. The statistical analysis also revealed a significant correlation between vitamin D deficit and higher levels of PCR (p=0.001).

As summarized in Table 8, the only difference in histological severity of liver disease among the two groups was related to lobular inflammation that significantly correlated with vitamin D deficit (p=0.040). Despite having more lobular inflammation, but with no correlation with any of the other histological parameters, severe steatosis (p=0.790), hepatocyte ballooning (p=0.777), fibrosis (p=0.162), there was no significant correlation between plasma 25-OH D deficit and definite NASH. The multivariate analysis showed that fibrosis and steatohepatitis were independent predictors of low vitamin D concentration (Table 9).

DISCUSSION

Vitamin D has long been regarded only as regulatory factor for phospho-calcium metabolism and bone homeostasis. However, many studies have shown more varied properties, namely immunomodulatory role (12), involvement in cellular differentiation and proliferation (16), hormone secretion (13) and antiinflammatory and antifibrotic effects (14,15). All these roles as well as the complex implications in other common pathologies to our days, such as metabolic syndrome and insulin resistance, support the importance of maintaining optimal vitamin D levels (17). Values below 20 ng/mL define vitamin D deficiency and its level between 20 and 30 ng/mL is recognized as insufficiency (18). Vitamin D deficiency is pandemic, affecting more than one billion people worldwide, the mechanisms underlying this phenomenon being still unknown although intensely studied (19).

The results of our study did not show an inverse correlation between BMI and vitamin D level, as most previous publications found (20, 21). However, as 100% of the study subjects had abnormal low levels of vitamin D, with more than 80% deficiency, we can rally to the literature data showing intense association between obesity and low levels of vitamin D (22). The 25 (OH) vitamin D deficit encountered in patients with morbid obesity and NAFLD may be explained by the increased amount of adipose tissue in which the vitamin D is distributed in obese patients; this will decrease the circulating serum of vitamin D level, given that vitamin D, synthesized in the skin or from food sources will either take the 25-hydroxylation hepatic pathway or be

stored in adipocytes (23). Another explanation could be related to sedentary lifestyle, poor micro-nutrient nutrition and lack of exposure to sunlight of obese people (24, 25). Patients in our study were included throughout all the seasons, which gave homogeneity to our studied group. Even so, in our group of study we did not notice consistent differences between vitamin D status depending on the season in which the patient was enrolled.

NAFLD is considered the hepatic manifestation of the metabolic syndrome (26). All patients in our cohort presented NAFLD under its various forms, 51.5% of cases having NASH; an interesting occurrence was noted in 7 patients, in whom histopathological examination revealed liver cell ballooning and lobular inflammation without macrovesicular steatosis. As previously claimed by other authors, this suggests that liver cell damage may occur in the absence of steatosis or that steatosis has vanished (27).

The deficit of 25 (OH) vitamin D is highly associated with NAFLD, most authors showing an inverse correlation between serum vitamin D and the presence of this disease; some of these studies used non-invasive methods (ultrasound) for detecting the hepatic damage (28, 29). As histopathological examination of liver biopsy sample is the gold standard for diagnosis of NAFLD, the results of these studies can be affected by using non-invasive methods with lower specificity and sensitivity. The aim of the study was not to find the mechanisms by which vitamin D deficiency can be linked to the progressive forms of NAFLD, but to assess whether this deficit is statistically significant in relation with the progressive forms of NAFLD involving inflammation and fibrosis (steatohepatitis) and if it can be used as a non-invasive marker to place the obese patients in a risk category of progressive NAFLD.

Studies using liver biopsy show strong results both in the accuracy of diagnosis and by reference to NASH, the aggressive form of NAFLD. Part of the authors sustain the hypothesis that vitamin D deficit is associated with a greater severity of NAFLD (24, 30, 31). There are also recent studies that argue that there is no association between vitamin D deficiency and the presence or the severity of NAFLD, many of them using liver biopsy for diagnosis (32, 33).

Our study also shows accuracy of the results given that NAFLD diagnosis has been proven by histopathological examination of liver biopsy samples; moreover, the evaluation of each patient included the assessment of NAFLD activity score. In our cohort, the deficit of vitamin D significantly correlates with hepatic lobular inflammation (74.1% *vs*. 40%; p = 0.040), the pathological hallmark of steatohepatitis (Table 8). When proceeding with multiple logistic analysis we found that fibrosis and steatohepatitis were independent predictors of low vitamin D concentration (CI: -2.073÷0.105; p=0.032, CI: -2.026÷0.002, p= 0.050) (Table 9).

Regarding inflammatory serum markers, there is a significant positive correlation between vitamin D deficit and increased levels of PCR (p=0.001) (Table 7). More, serum fibrinogen is significantly increased in patients with NASH (Fibrinogen: 421.00 ± 44.84, p=0.005) and serum PCR significantly increased in patients with NASH and "vanished steatosis" (1.48 ± 1.08, p=0.005) (Table 1). Higher levels of fibrinogen significantly also correlated with a greater NAFLD activity score (Table 5 and 6). These results suggest that the deficit of vitamin D may be related to the inflammatory status given by steatohepatitis, the progressive form of NAFLD.

Although extensively studied the causal relation between low levels of vitamin D and NAFLD or the mechanisms that underlie the connection between these two pahological entities are not established. Two theories are discussed regarding the pathogenic mechanisms: the deficit may further damage the liver or liver pathological changes accentuate the vitamin D deficit. For the first assumption there are many studies showing a significant association between low serum levels of 25 (OH) vitamin D and insulin resistance or DM II development; data in literature describe even a predictive value of vitamin D on the glycemic status and future possible development of insulin resistance of the patients (34, 35). This may be an explanation relevant to the conditions under which IR and DM II are important factors in NAFLD onset and progression to aggressive forms that involve hepatic inflammation and fibrosis (36, 37). In our study, the statistical analysis showed a significant correlation (p=0.033) between the vitamin D deficit and the presence of diabetes (Table 7). Our results also showed that the number of diabetic cases is significantly higher (p=0.002) in the subgroup of patients having NASH and fibrosis (Table 3). Even so, after collating these data, considering vitamin D deficit as an important piece in the pathogenic circle linking diabetes, NAFLD and liver fibrosis may be an exaggerated conclusion.

Another pathological hypothesis - that hepatic injury accentuates vitamin D deficiency - has been refuted by studies showing that oral administration of

vitamin D in patients with NAFLD led to normalization of its serum level, showing that hepatic 25-hydroxylation normally occurs even in pathological conditions induced by NAFLD (38). Besides this, at least for our study group, steatohepatitis - as progressive form of NAFLD - does not imply liver failure, so we cannot claim that in our patients diminishing liver function is the cause of the impaired status of vitamin D.

One of the great challenges in obese patient management is assessing the degree of liver damage; so far, neither non-invasive scores based on serological tests nor imaging methods managed to distinguish between simple and aggressive forms of the disease. This is crucial, given that only steatohepatitis has the potential to progress to fibrosis and liver cirrhosis and hepatic fibrosis is the strongest predictor of mortality related to cardiovascular disease and liver complications in patients with histopathological proven NAFLD (39, 40). Our study revealed a statistically significant correlation between vitamin D deficiency and the presence of steatohepatitis in patients with biopsy proven NAFLD. We cannot either ignore the positive correlation between vitamin D deficit and hepatic lobular inflammation, increased inflammatory serum markers and the presence of diabetes in our cohort of obese patients, changes which are themselves correlated with biopsy proven steatohepatitis or liver fibrosis. The results of our study allow us to conclude that vitamin D status in conjunction with other clinical parameters - such as T2DM - or serum biomarkers –namely fibrinogen level and PCR level - may participate in framing obese patients in a risk category for the presence of steatohepatitis and liver fibrosis. Subsequently, these patients may undergo liver biopsy for positive diagnosis and appropriate management.

Conflict of interest

The authors declare that they have no conflict of interest.

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