Iranian Journal of Pharmaceutical Research (2019), 18 (1): 496-505
Received: April 2017
Accepted: May 2018

Original Article

Reduction of Some Atherogenic Indices in Patients with Non-Alcoholic Fatty Liver by Vitamin D and Calcium Co-Supplementation: A Double Blind Randomized Controlled Clinical Trial

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Abstract

The role of non-alcoholic fatty liver disease (NAFLD) as a potential independent cardiovascular disease (CVD) risk factor has recently gained considerable attention because CVD is the common cause of death in NAFLD patients. We aimed to estimate the effects of vitamin D supplementation alone or in combination with calcium on atherogenic indices, liver function tests, and grade of disease in patients with NAFLD. One-hundred twenty NAFLD patients were randomized in a double-blind, placebo-controlled clinical trial as follows: D (1000 IU vitamin D), CaD (500 mg as calcium carbonate plus 1000 IU vitamin D) or P (placebo), once daily with meals over 12 weeks. Adjusted for all the baseline measures, reduction in serum ALT, AST, LDL-C/HDL-C, TC/HDL-C, and non-HDL-C were significantly higher in the CaD compared with the P group ($p < 0.001$, $p = 0.03$, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively). Also, mean difference of serum ALT, LDL-C/HDL-C, TC/HDL-C, and TG/HDL-C were significantly higher in the CaD than D group ($p < 0.001$, $p = 0.006$, $p < 0.001$ and $p = 0.03$, respectively). Serum non-HDL-C was marginally decreased in the CaD compared with the D group ($p = 0.06$). With considering the BMI changes as covariate, reduction in the grade of fatty liver was significantly higher in the CaD and D groups than the P ($p < 0.001$). The present study suggests that supplemental calcium combined with vitamin D, but not vitamin D alone, may reduce serum atherogenic indices, liver function tests, and grade of disease in patients with NAFLD.

Keywords: Calcium; Vitamin D; Atherogenic indices; Non-alcoholic fatty liver; Supplementation.

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Introduction

Non- alcoholic fatty liver disease (NAFLD) is considered as liver manifestations of metabolic syndrome (MetS), which is an atherogenic condition (1). Most patients are overweight and/or obese, as well as have dyslipidemia, insulin resistance (IR), hypertension, and high waist circumference (WC) (2). Prevalence of atherosclerosis in NAFLD patients is high and this condition is independent on obesity and other established risk factors (3). The role of NAFLD as a potential independent cardiovascular disease (CVD) risk factor has recently gained considerable attention because CVD is the common cause of death in NAFLD patients (4). Dyslipidemia, as a common risk factor of NAFLD and CVD, up-regulates the transcription factor sterol regulatory element binding protein-1c (SREBP-1c). Insulin and SREBP-1c synergistically stimulate genes involved in de-novo synthesis of lipids. Furthermore, SREBP-1c inhibits oxidation of free fatty acids and leads to increase in hepatic lipid content (5, 6). To compensate for the increased hepatic triglycerides (TG), the liver forms an atherogenic lipid profile, consisting of high TG levels, low high-density lipoprotein (HDL) cholesterol, increased small and dense low-density lipoprotein (LDL) particles, increased very low-density lipoprotein (VLDL) cholesterol levels, and elevated apolipoprotein B100 concentration; all of which are strongly associated with adverse cardiovascular outcomes (7-9). Non-high-density lipoprotein cholesterol (non-HDL-C) which reflects the cholesterol in all lipoprotein particles has become increasingly recognized as an important measure of atherogenesis (10). Among the CVD risk factors, vitamin D deficiency is the new one (11). Vitamin D deficiency is a pandemic problem and studies suggested that this might contribute to the worldwide increased prevalence of CVD (12, 13). Several mechanisms have been proposed to account for this inverse relationship including anti-inflammatory effects, immune response modulation, fibrotic pathways, and cytokine's secretion (14, 15). One study showed that calcium carbonate supplementation lead to a positive change in lipid profile and atherogenic indices in centrally obese patients (16). Roles of calcium and vitamin D are inter-twinning. To our the best knowledge, there is no study to assess the effects of calcium plus vitamin D on atherogenic indices in NAFLD patients compared with vitamin D, alone. The purpose of this study was to compare the effects of vitamin D supplementation with and without calcium for 12-wk on atherogenic indices in NAFLD patients controlled by dietary intake and physical activity levels.

Experimental

A double-blind, placebo-controlled clinical trial was designed that aimed to the effects of vitamin D supplementation with and without calcium on weight loss, body fat, liver function enzymes, and atherogenic indices in NAFLD patients which were controlled by dietary intake and physical activity levels. Totally, 120 NAFLD patients were selected from who were attending the Rasool-Akram Hospital, Tehran, Iran. Patients aged 18-65 y, BMI of 25-35 kg/m^2, serum 25 (OH) D level< 15 ng/mL and willingness to control sun exposure time, dietary and physical activity levels were included. The patients taking any supplement, drug for blood glucose and/or lipid control during at least the last 3 month, having pregnancy or given birth in the past year or planning a pregnancy in the next 6 month, having lactation, weight loss of ≥10% of body weight within the six month before enrollment in the study, participation in a competitive sport, abnormal thyroid hormone concentration, intake of medications that could affect body weight and/or energy expenditure, allergy, smoking, diagnosis of chronic diseases including inflammatory diseases, heart, liver and renal failure, cancer, acute myocardial infarction, diabetes, stroke, or serious injuries and any other conditions that were not suitable for the trial as evaluated by the physician were excluded. The study was approved by the Ethical Committee of Iran University of Medical Sciences, Tehran, Iran. All subjects provided their signed consent before the study enrollment. At the screening visits, new diagnosed NAFLD patients proven by ultra-sonography underwent an examination by an Internist. After measuring baseline variables, eligible participants were randomly assigned by using a computer-generated random-numbers

method by the project coordinator. The patients were randomly assigned to one of the three groups: D group (n = 37) received one tablet containing 1000 IU of vitamin D (25µg/d as Calciferol; Jalinus Arya Co, Iran), CaD group (n = 37) received one tablet containing 500 mg/d as calcium carbonate plus 1000 IU vitamin D as calciferol (Jalinus Arya Co, Iran), placebo (P) group (n = 36) received placebo (25 µg/d as lactose; Jalinus Arya Co. Iran) after lunch with a glass of water for 12 weeks. Shape, color, and packaging of the placebo were exactly similar to the supplements. The dietary program was designed to introduce a 500-kcal energy deficit based on estimated energy requirements at the start of the study by an expert dietitian. Daily 30 min moderate walking advised to all the participants. Dietary intake and physical activity level were assessed by a 3-d dietary food recall questionnaire and International Physical Activity questionnaire (IPAQ) that were completed three times during the study period (baseline and at the end of the 6th weeks and 12 th weeks of intervention). These dietary intake data were analyzed by the N4 software (Nutritionist 4, First Data Bank, San Bruno, CA, USA). Compliance with the supplement consumption instructions was monitored once a week by telephone interviews. Researchers advised to the participants to control the direct sun-light exposure to less than 1 hour/day. Duration of sun-light exposure was monitored once a week in each telephone interview.

Measurements

Anthropometric and biochemical measurements of all subjects were taken at the baseline and after 12 wk. Blood samples of the subjects were taken from the antecubital vein after 10-12 h fasting. After centrifugation for 20 min (3000 g), the serum samples were frozen simultaneously and stored at -80 °C until analyzed. In order to eliminate the probable effects of sex hormones on blood lipids, blood sampling was not performed between days 1 and 5 of the menstrual cycle in women. At the baseline and after 12 weeks of treatment, the liver fat accumulation and serum lipid profile were measured for the groups. Lipid profile and liver enzymes were measured by an enzymatic method (Pars Azmoon Co. kit, Tehran, Iran) using Liasys autoanalyzer. 25-OH vitamin D was measured by ELISA kit (SE120139, Sigma Aldrich).

Body weight was taken to the nearest 0.1 kg by using a calibrated Seca scale to the nearest 100 gram with light clothing and no shoes. Body height was measured to the nearest 0.5 cm by using a wall-mounted stadiometer (Seca) in a barefoot and freestanding position. WC was measured with a rigid measuring tape and recorded to the nearest 0.5 cm. WC was measured at the halfway between the lower rib and the iliac crest (17). Body fat was measured by BIA (Bioelectrical Impedance Analyzer) setting at the beginning and end of the study. BIA (model BIA-109 RJL/ Akern Systems, Detroit, Michigan, USA) was connected to surface electrodes on the right hand and foot. BMI (Body Mass Index) was calculated according to the formula: BMI = weight/ height^2 (kg/m^2). The demographic data were collected during the initial anthropometric assessment.

Grades of fatty liver classification

Liver ultrasound device Siemens brand Sonoline G50 series and 3.5 to 5 MHz probe made of Germany was used for liver sonography. Liver steatosis was classified through sonographic echogenisity of liver as: 1) normal: echogenicity as the same as renal cortex 2) grade I: mild steatosis; increased hepatic echogenicity with visible periportal and diaphragmatic echogenicity 3) grade II: moderate steatosis; increased hepatic echogenicity with imperceptible periportal echogenicity, without obscuration of diaphragm 3) grade III: sever steatosis; increased hepatic echogenicity with imperceptible periportal echogenicity and obscuration of diaphragm (19).

Sample size and statistical Analysis

In design of the study, we considered a power of 80% with a two-sided test with α =0.05 (type I error) and standard deviation (SD) difference of 3.5 for WC and 2 for BMI. On the basis of SDs, reported in similar studies (19), the number of subjects needed to be treated in order to detect this difference was 36/group. Given an anticipated dropout rate of 10 percent, we set the enrollment target at 40 subjects.

All data were expressed by means ± SE.

The level of significance was set at P <0.05. Statistical analyses were performed with IBM SPSS Statistics software (version 18; IBM Corp). Normal distribution of the variables was checked by Kolmogorov Smirnov Test. One-way ANOVA followed by post-hoc test was used to test whether the differences between the mean values of the items studied in the groups were significant. The comparison of mean values of variables before and after the intervention in each group was examined by paired t-tests. ANCOVA test was used to compare mean difference of outcomes, adjusted for the baseline measures as covariate. Chi-square test was used to compare the qualitative variables before and at the end of the study.

Results

Mean age of participants was not significantly different among groups (P group = 4410.8 ± yr; D group=39.811 ± yr; CaD group=38.3 ± 10.1 yr, $p > 0.05$). There wasn't significant difference in the sex of participants. 64.7% in the P, 59.5% in the D and 62.3% of participants in the CaD group were male. Time of sun-light exposure (hr/day) was not significantly different among the groups ($p > 0.05$). There was no significant difference in energy and nutrient intake at the beginning of the study among groups and also no significant change was seen at the end of the study (Table 1). The level of physical activity was not different among groups at the baseline, as well as at the end of the study. At baseline, there were no statistically significant differences in physical characteristics and biochemical measurements among the intervention groups (Table 2). BMI reduction in each group was shown after 12 wk of the study (P group= -1.4 ± 0.29 kg/m^2, D group= -1.1 ± 0.13 kg/m^2, CaD group= -1.3 ± 1.17 kg/m^2, $p < 0.001$), but differences among the groups was not significant after 12 wk of the study, adjusted to the baseline measures (p =0.65). Reductions in body fat mass was significant in each group after 12 wk of the study (P group= -2.36 ± 0.49%, D group= -2.25 ± 0.36%, CaD group= -3.67 ± 0.46%, p <0.001). Adjusting to the baseline measures, there was significant difference in WHR among the groups after 12 wk of the study ($p < 0.001$). Adjusting to the baseline measures, serum ALT and AST levels were significantly different among the groups after 12 wk of the study ($p < 0.001$ and p =0.04, respectively). Reduction in serum ALT level was significantly higher in the CaD (-14.4 ± 1.7 µmol/L) compared with the P (0.5 ± 2.06 µmol/L, $p < 0.001$), and the D (-3.5 ± 1.8 µmol/L, p <0.001) group at the end of the study. Reduction in serum AST level was significantly higher in the CaD than the P group (-5.5 ± 0.6 µmol/L vs. -2.5 ± 1.02 µmol/L, p = 0.03).

In the CaD supplemented group, serum LDL-C/HDL-C, TC/HDL-C, TG/HDL-C and non-HDL-C levels were significantly decreased after 12-wk of supplementation ($p < 0.001$, $p <$ 0.001, p <0.001 and p = 0.001, respectively). In the D supplemented group, serum TG/HDL-C was significantly decreased at the end of the study (p <0.001). Atherogenic indices didn't change in the P group ($p > 0.05$). Adjusted for the baseline measures, mean difference of LDL-C/HDL-C, TG/HDL-C and TC/HDL-C were significantly different among the groups (p <0.001, p =0.001 and p <0.001, respectively). Post-hoc analysis showed that mean difference of LDL-C/HDL-C was significantly higher in the CaD than P group (p <0.001), as well as D group (p = 0.006). Serum TC/HDL-C and TG/HDL-C level were significantly decreased in the CaD than P ($p < 0.001$ and $p < 0.001$), as well as D group ($p < 0.001$ and p =0.03). Mean difference of non-HDL-C was significantly different among the groups, adjusted for the baseline measures (p = 0.001). Serum non-HDL-C was significantly decreased in the CaD than P group ($p < 0.001$). Serum non-HDL-C was marginally decreased in the CaD compared with the D supplemented group (p =0.06) (Table 2).

By splitting to the sex of participants, significant differences were shown in ALT, TG/HDL-C, TC/HDL-C, LDL-C/HDL-C and non-HDL-C alterations from baseline up to the end of the study in females among the groups (p = 0.001, p = 0.007, $p < 0.001$, p = 0.003 and p = 0.005, respectively). Mean difference of serum ALT level was significantly higher in the CaD than P group (p = 0.003), as well as in the CaD than D supplemented group (p = 0.004). Mean difference of TG/HDL-C was significantly higher in the CaD (p = 0.01) and

Table1. Daily dietary intake of energy and some nutrients of the studied groups.

Dietary intake	P (n = 36)	D (n = 37)	CaD (n = 37)	*p* value*
Total Energy:(Kcal)				
Before intervention	2157.5 ± 437.1	2160.2 ± 385.2	2162.3 ± 426.4	0.4
After intervention	1657.8 ± 391.3	1667.5 ± 345.9	1658.5 ± 364.7	0.5
Total protein(g/day)				
Before intervention	75.8 ± 19.9	79.8 ± 20	76.6 ± 19.5	0.1
After intervention	60.1 ± 17.1	62.4 ± 22.7	60.5 ± 19.3	0.1
Total carbohydrate(g/day)				
Before intervention	298.42 ± 72.6	296.72 ± 52.33	295.9 ± 68.5	0.31
After intervention	223.9 ± 68.52	216.3 ± 63.21	219.8 ± 62.3	0.8
Total fat(g/day)				
Before intervention	73.3 ± 20	72.8 ± 24.3	73.9 ± 22.6	0.8
After intervention	57.9 ± 19.5	60.3 ± 24.7	59.7 ± 23.3	0.51
SFA[1](g/day)				
Before intervention	13.6 ± 6.5	13 ± 6.3	12.8 ± 5.9	0.42
After intervention	3.4 ± 6.1	3.5 ± 6.6	3.9 ± 6.5	0.52
PUFA[2](g/day)				
Before intervention	43.4 ± 14.7	37.7 ± 17.9	39.2 ± 16.4	0.9
After intervention	33.4 ± 19.2	32.1 ± 21.4	33.6 ± 20.8	0.4
MUFA[3] (g/day)				
Before intervention	6.1 ± 4.5	12.5±3.1	11.9 ± 4.2	0.54
After intervention	21.1 ± 2.3	24.7±2.7	22.2 ± 3.1	0.6
Fiber (g/day)				
Before intervention	16.7 ± 7.6	14.8 ± 5.8	15.4 ± 6.7	0.3
After intervention	13.9 ± 9.8	13.8 ± 5.5	13.5 ± 7.6	0.7
Vitamin C (mg/day)				
Before intervention	127.8 ± 85.8	107.3 ± 75.9	1165 ± 81.8	0.35
After intervention	130.9 ± 83.3	110.3 ± 74.8	124.6 ± 79.9	0.34
Vitamin E (mg/day)				
Before intervention	10.7 ± 8.1	13.1 ± 7.5	12.4 ± 7.9	0.25
After intervention	10.6 ± 7.9	12.7 ± 7.1	11.5 ± 6.9	0.3
calcium (mg/day)				
Before intervention	777 ± 386	773 ± 586	758 ± 432	0.8
After intervention	725 ± 454	729 ± 533	792 ± 214	0.63
Vitamin D (µg/day)				
Before intervention	0.39 ± 0.37	0.53 ± 0.6	0.51 ± 0.4	0.32
After intervention	0.37 ± 0.35	0.4 ± 0.47	0.39 ± 0.2	0.75

Data are expressed as means ±SD. *Differences between groups were evaluated by independent t- test

[1]SFA: Saturated Fatty Acid, [2]PUFA: Polyunsaturated Fatty Acid, MUFA: Monounsaturated Fatty Acid

P: placebo group; D: vitamin D supplemented group as calciferol; CaD: calcium plus vitamin D supplemented group.

Table 2. Anthropometric and biochemical measures at baseline and week 12 and changes from baseline up to the end of the study[1].

Variables	P (n = 36)	D (n = 37)	CaD (n = 37)	*p* value
BMI, kg/m²				
- Baseline	31.3 ± 0.58	30.3 ± 0.64	30.5 ± 0.93	0.69[†]
- Week 12	29.9 ± 0.46	29.2 ± 0.67	29.2 ± 0.87	0.65
- Treatment effect	-1.4 ± 0.29	-1.1 ± 0.13	-1.3 ± 1.2	0.78[‡]
WHR				
- Baseline	0.98 ± 0.01	0.97 ± 0.01	0.99 ± 0.01	0.8
- Week 12	0.97 ± 0.01	0.96 ± 0.01	1.02 ± 0.006	<0.001
- Treatment effect	-0.01 ± 0.01	-0.017 ± 0.01	0.03 ± 0.01	0.001
BF, %				
- Baseline	34.9 ± 1.3	35.3 ± 1.2	38.4 ± 0.98	0.07
- Week 12	32.6 ± 1.3	33 ± 1.2	34.7 ± 0.98	0.1
- Treatment effect	-2.36 ± 0.49	-2.25 ± 0.36	-3.67 ± 0.46	0.05
ALT, µmol/L				
- Baseline	46.5 ± 3.01	45.9 ± 2.4	50.2 ± 2.6	0.44
- Week 12	47.3 ± 2.6	42.4 ± 2.6	35.8 ± 1.4	<0.001
- Treatment effect [a, b]	0.5 ± 2.1	-3.5 ± 1.8	-14.4 ± 1.7	<0.001
AST, µmol/L				
- Baseline	31.1 ± 1.6	30.4 ± 1.3	32.5 ± 1.2	0.48
- Week 12	28.6 ± 1.8	26.3 ± 1.5	26.9 ± 1.2	0.04
- Treatment effect [a]	-2.5 ± 1.02	-4.2 ± 0.71	-5.52 ± 0.61	0.028
LDL-C/HDL-C				
-Baseline	3.1 ± 0.16	3.1 ± 0.15	3.56 ± 0.15	0.08
-Week 12	3.2 ± 0.14	3 ± 0.13	3 ± 0.11	0.5
-Treatment effect [a, b]	0.07 ± 0.07	-0.14 ± 0.09	-0.56 ± 0.11	<0.001
TG/HDL-C				
- Baseline	5.7 ± 0.54	5.7 ± 0.45	6.2 ± 0.34	0.7
- Week 12	5.5 ± 0.44	4.6 ± 0.33	4.5 ± 0.25	0.5
- Treatment effect [a, c]	-0.22 ± 0.25	-1.1 ± 0.29	-1.7 ± 0.21	0.001
TC/HDL-C				
- Baseline	5.2 ± 0.24	5.3 ± 0.24	5.8 ± 0.22	0.16
- Week 12	5.3 ± 0.22	5.03 ± 0.17	4.7 ± 0.12	0.07
- Treatment effect [a, b]	0.15 ± 0.12	-0.26 ± 0.14	-1.04 ± 0.14	<0.001
Non-HDL-C				
- Baseline	148.5 ± 5.8	151.1 ± 5.5	163.3 ± 6	0.16
- Week 12	154.9 ± 5.1	150.7 ± 5.5	154.2 ± 5.2	0.83
- Treatment effect [a]	6.4 ± 3.3	-0.4 ± 2.6	-9.1 ± 2.5	0.001

Table 2. Continued.

Variables	P (n = 36)	D (n = 37)	CaD (n = 37)	*p* value
25 (OH) D[a], ng/ml				
- Baseline	10 ± 0.63	9.9 ± 0.64	9.9 ± 0.64	0.9
- Week 12	11 ± 0.78	21.4 ± 0.73	27.1 ± 1.1	<0.001
- Treatment effect [a, b, c]	0.98 ± 0.35	11.5 ± 0.96	17.3 ± 0.99	<0.001

[1]Values are means ± SE. $p<0.05$ was considered as significant. [a] To convert 25(OH) D values to ng/mL, divide by 2.5.

WHR, waist to hip ratio; BF, body fat; TC, total cholesterol; TG, triglyceride; ALT, alanine amino-transferase; AST, aspartate amino-transferase.

P: placebo group; D: vitamin D supplemented group as calciferol; CaD: calcium plus vitamin D supplemented group.

[†]p values are related to the differences among the groups; evaluated by one-way ANOVA.

[‡]P values are for the supplemented groups relative to the placebo group by using an ANCOVA with baseline values as covariate.

[a] Significant difference between the CaD and placebo group; [b] Significant difference between the CaD and D group; [c] Significant difference between the D and placebo group ($p<0.05$).

D ($p = 0.03$) than P group. TC/HDL-C and non-HDL-C were significantly decreased in the CaD ($p < 0.001$ and $p = 0.008$) and D ($p = 0.004$ and $p = 0.03$) than P group. Alterations in serum TC/HDL-C was significantly higher in the CaD than D supplemented group ($p =0.003$), after 12 weeks of treatment. Mean difference of LDL-C/HDL-C was significantly higher in the CaD ($p = 0.007$) and D ($p = 0.01$) than P group. Serum LDL-C/HDL-C was significantly decreased in the CaD compared with D supplemented group ($p < 0.001$) after 12 weeks of treatment.

In males, significant differences were shown in mean difference of serum ALT, TG/HDL-C, TC/HDL-C, LDL/HDL-C and non-HDL-C at the end of the treatment ($p =0.001$, $p = 0.04$, $p <0.001$, $p < 0.001$ and $p = 0.02$, respectively). ALT alteration was significantly higher in the CaD than P group ($p = 0.001$), as well as the D group ($p = 0.01$). After 12 weeks of supplementation, TG/HDL-C, TC/HDL-C and LDL-C/HDL-C were significantly decreased in the CaD compared with the P group ($p = 0.03$, $p = 0.01$ and $p = 0.03$, respectively). Mean difference of TG/HDL-C, TC/HDL-C and LDL-C/HDL-C were significantly higher in the CaD than P group ($p = 0.04$, $p =0.002$ and $p =0.007$, respectively). Furthermore, mean difference of TC/HDL-C, LDL-C/HDL-C and non-HDL-C were significantly higher in the CaD than D supplemented group ($p =0.002$, $p =0.001$ and $p = 0.02$, respectively).

With considering the BMI changes as covariate, reduction in the grade of fatty liver was significant in the CaD and D supplemented groups relative to the P ($p < 0.001$). In the P group, no change was seen in the grade of fatty liver in 89% of participants (N = 32) at the end of the study. In the D supplemented group, 89% of participants (N = 33) had 1 degree reduction in the grade of fatty liver. In the CaD supplemented group, 62% (N = 23) and 10.8% (N = 4) of participants had 1 degree and 2 degree reduction in fatty liver grade, respectively.

Discussion

To the best of our knowledge, this is the first randomized, placebo-controlled, double-blind clinical trial to evaluate the beneficial effects of oral calcium plus vitamin D supplementation, compared to vitamin D alone, on atherogenic indices in NAFLD patients. Despite the well-documented role of calcium in cell metabolism, its role in the development of cardiovascular disease is still inconclusive (20-23). Several studies suggest that calcium supplementation might be associated with an increased risk of coronary heart disease (20, 23), whereas others underline a significant effect on lowering high blood pressure and hyperlipidemia (22-26). Our results suggest that CaD supplementation has

more beneficial effects on atherogenic factors including LDL-C/HDL-C, TC/HDL-C, TG/HDL-C and non-HDL-C in NAFLD patients. Reduction in serum ALT level was significantly higher in the CaD supplemented group than others. Non-HDL-C was significantly decreased in the CaD than P group. This effect was marginal in the CaD compared with the D group.

Previous studies have shown that ratios of cholesterol ester-rich lipoprotein levels (TC/HDL-C and LDL-C/HDL-C), as well as TG/HDL-C are strong predictors of insulin resistance and LDL particle diameter (small-dense LDL-C), and ultimately CVD (27-29). Our results are in agreement with the previous studies which showed an inverse association between serum levels of TG and HDL-C in adults with pre-diabetes (30), as well as lipid profile, insulin sensitivity and liver enzyme tests with vitamin D in NAFLD patients (31). Moreover, some studies showed a negative correlation between serum 25 (OH) D levels and TG, as well as low HDL-C levels (32-34). In contrast, one cross-sectional study on healthy participants with a mean BMI of 23.8 kg/m^2 showed no significant correlation between serum lipid profile and vitamin D levels (35). It may be suggested that serum levels of vitamin D are related with lipid profile and atherogenic indices in patients with inflammatory conditions. Several mechanisms are suggested for these effects. Low serum levels of 25 (OH) D leads to hyperparathyroidism which decrease peripheral removal of TG and activate microsomal TG-transfer protein and induce hypertriglyceridemia (35, 36). Also, it is suggested that vitamin D may regulate macrophage function on reverse cholesterol transport and improve free fatty acids-induced insulin resistance and inflammation (37, 38).

Effects of calcium supplementation were measured on lipid profile and atherogenic index of plasma (AIP) in centrally obese men (16). Oral 1500 mg/day calcium supplementation for 8 weeks resulted in 22% reduction in TG and 19.2% in HDL-C levels. Significant reduction in serum TC and LDL-C levels were only found in dyslipidemic and centrally obese subjects. AIP decreased significantly by 51% with calcium carbonate treatment.

Non-HDL-C is an excellent diagnostic marker for lipid disorders and CVD risk (39, 40). It presents full impact of all plasma lipid components involved in atherogenesis including chylomicrones, VLDL and their remnants, IDL, LDL and Lp (a), except HDL (41). The concentration of non-HDL-C is calculated using total cholesterol minus HDL-C. A meta-analysis of lipid-lowering therapies showed a 1:1 correlation between the lowering of non-HDL-C and CVD risk (42). One study found that 1 mg/dL increase in non-HDL-C, increases the risk of CVD death by 5% (39). In a cross-sectional study, each 10 nmol/L increase in 25(OH) D was associated with a decrease in non-HDL-C concentration (-0.89 mg/dl) (43). Our results are in agreement with the mentioned study. Alteration in serum non-HDL-C was significantly higher in the D supplemented than the P group. On the other hand, CaD supplementation had more effects on serum non-HDL-C reduction than D, alone. But this effect was marginally significant due to small sample size. There are conflicting results with a non-selected cohort study in south Italy in which a significant association was shown between TC, LDL-C, HDL-C, non-HDL-C, TG and serum calcium in men and postmenopause women (44). It seems that the relationship between calcium and/or vitamin D supplementation and CVD is influenced by the type of intervention, duration, supplement dosage, and participants (45). To our knowledge this is the first study about the effects of vitamin D alone and/or in combination with calcium on atherogenic indices in NAFLD patients and showed that CaD supplementation had positively effect on lipid profile and atherogenic indices compared with the D and P groups. Also, a larger sample size and longer duration are needed before reaching conclusive results. There are some limitations to our results; most importantly that NAFLD was diagnosed by biochemical and ultra sonographic findings in our patients, which is not possible to distinguish between simple fatty liver and NASH. Liver biopsy is the gold standard to NAFLD diagnosis, but it is an invasive and expensive method. We should note that the levels of ALT and AST were quasi in normal range. Therefore, the grade of fatty accumulation was not great in the majority of the patients. Another study with the higher levels of these enzymes is needed to assess the

effects of Calciferol with and/or without calcium on NAFLD progression.

Ethical considerations

Ethical issue principles including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

Acknowledgment

Authors are very thankful from all the participants and physicians in this study. Authors declare that there is no conflict of interest. Registered under ClinicalTrials.gov Identifier no. IRCT201408312709N29.

References

(1) Liu H and Lu HY. Nonalcoholic fatty liver disease and cardiovascular disease. *World J. Gastroenterol.* (2014) 20: 8407–15.

(2) Hashizume H, Sato K, Yamazaki Y, Horiguchi N, Kakizaki S and Mori M. A prospective study of long-term outcomes in female patients with nonalcoholic steatohepatitis using age- and body mass index-matched cohorts. *Acta Med. Okayama* (2013) 67: 45–53.

(3) Blackett PR and Sanghera DK. Genetic determinants of cardiometabolic risk: a proposed model for phenotype association and interaction. *J. Clin. Lipidol*. (2013) 7: 65–81.

(4) Huang Y, Bi Y, Xu M, Ma Z, Xu Y, Wang T, Li M, Liu Y, Lu J, Chen Y, Huang F, Xu B, Zhang J, Wang W, Li X and Ning G. Nonalcoholic fatty liver disease is associated with atherosclerosis in middle-aged and elderly Chinese. *Arterioscler. Thromb. Vasc. Biol.* (2012) 32: 2321-6.

(5) Ahmed MH, Abu EO and Byrne CD. Non-Alcoholic Fatty Liver Disease (NAFLD): new challenge for general practitioners and important burden for health authorities? *Prim. Care Diabetes* (2010) 4: 129-37.

(6) Dogru T, Genc H, Tapan S, Ercin CN, Ors F, Aslan F, Kara M, Sertoglu E, Bagci S, Kurt I and Sonmez A. Elevated asymmetric dimethylarginine in plasma: an early marker for endothelial dysfunction in non-alcoholic fatty liver disease? *Diabetes Res. Clin. Pract.* (2012) 96: 47-52.

(7) Alkhouri N, Kistangari G, Campbell C, Lopez R, Zein NN and Feldstein AE. Mean platelet volume as a marker of increased cardiovascular risk in patients with nonalcoholic steatohepatitis. *Hepatology* (2012) 55: 331.

(8) Motta AB. Report of the international symposium: polycystic ovary syndrome: first Latin-American consensus. *Int. J. Clin. Pract*. (2010) 64: 544–57.

(9) Gawrieh S, Baye TM, Carless M, Wallace J, Komorowski R, Kleiner DE, Andris D, Makladi B, Cole R, Charlton M, Curran J, Dyer TD, Charlesworth J, Wilke R, Blangero J, Kissebah AH and Olivier M. Hepatic gene networks in morbidly obese patients with nonalcoholic fatty liver disease. *Obes. Surg*. (2010) 20: 1698–709.

(10) Orakzai SH, Nasir K, Blaha M, Blumenthal RS and Raggi P. Non-HDL cholesterol is strongly associated with coronary artery calcification in asymptomatic individuals. *Atherosclerosis* (2009) 202: 289–95.

(11) Kassi E, Adamopoulos C, Basdra EK and Papavassiliou AG. Role of Vitamin D in Atherosclerosis. *Circulation* (2013) 128: 2517-31.

(12) Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH and Weaver CM. Endocrine Society. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J. Clin. Endocrinol. Metab*. (2011) 96: 1911–30.

(13) Holick MF. Vitamin D deficiency. *N. Engl. J. Med*. (2007) 357: 266–81.

(14) Danik JS and Manson JE. Vitamin D and cardiovascular disease. *Curr. Treat. Options. Cardiovasc. Med*. (2012) 14: 414–24.

(15) Wallis DE, Penckofer S and Sizemore GW. The "sunshine deficit" and cardiovascular disease. *Circulation* (2008) 118: 1476–85.

(16) Thanda Sein M, Swe Latt T and Ohnmar. Effect of Oral Calcium Supplementation on Lipid Profile and Atherogenic Index of Plasma. *JAFES* (2014) 29: 135-40.

(17) Mason C and Katzmarzyk PT. Effect of the site of measurement of waist circumference on the prevalence of the metabolic syndrome. *Am. J. Cardiol*. (2009) 103: 1716-20.

(18) Goodman E, Daniels SR, Morrison JA, Huang B, Dolan LM. Contrasting prevalence of and demographic disparities in the World Health Organization and National Cholesterol Education Program Adult Treatment Panel III definitions of metabolic syndrome among adolescents. *J. Pediatr*. (2004) 145: 445-51.

(19) Rumack CM, Wilson SR and Charboneau JW. (eds.) *Diagnostic Ultrasound*. 3th ed. Elsevier Mosby, (2005) 95–6.

(20) Rautiainen S, Wang L, Manson JE and Sesso HD. The role of calcium in the prevention of cardiovascular disease: a review of observational studies and randomized clinical trials. Curr. Atheroscler. Rep. (2013) 15: 362-64.

(21) Bolland MJ, Barber PA, Doughty RN, Mason B, Horne A, Ames R, Gamble GD, Grey A and Reid IR. Vascular events in healthy older women receiving calcium supplementation: randomised controlled trial. BMJ. (2008) 336: 262–6.

(22) Shidfar F, Moghayedi M, Jafarian Kerman SR, Hosseini S and Shidfar S. Effects of a calcium supplement

on serum lipoproteins, apolipoprotein B, and blood pressure in overweight men. *Int. J. Endocrinol. Metab*. (2010) 8: 194-200.

(23) Chai W, Cooney RV, Franke AA and Bostick RM. Effects of calcium and vitamin D supplementation on blood pressure and serum lipids and carotenoids: a randomized, double-blind, placebo-controlled, clinical trial. *Ann. Epidemiol*. (2013) 23: 564-570.

(24) Margolis KL, Ray RM, Van Horn L, Manson JE, Allison MA, Black HR, Beresford Sh AA, Connelly SA, Curb JD, Grimm RH, Kotchen ThA, Kuller LH, Wassertheil-Smoller S, Thomson CA and Torner JC. Effect of Calcium and Vitamin D Supplementation on Blood Pressure. *Hypertension* (2008) 52: 847-55.

(25) Lorvand Amiri H, Agah Sh, Tolouei Azar J, Hosseini Sh, Shidfar F and Mousavi SN. Effect of daily calcitriol supplementation with and without calcium on disease regression in non-alcoholic fatty liver patients following an energy-restricted diet: Randomized, controlled, double-blind trial. *Clinical Nutrition*. (2016). https://doi.org/10.1016/j.clnu.2016.09.020.

(26) Kawano Y, Yoshimi H, Matsuoka H, Takishita S and Omae T. Calcium supplementation in patients with essential hypertension: assessment by office, home and ambulatory blood pressure. *J. Hypertens*. (1998) 16: 1693-9.

(27) Nobili V, Alkhouri N, Bartuli A, Manco M, Lopez R, Alisi A and Feldstein AE. Severity of liver injury and atherogenic lipid profile in children with nonalcoholic fatty liver disease. *Pediatr. Res*. (2010) 67: 665-70.

(28) McLaughlin T, Reaven G, Abbasi F, Lamendola C, Saad M, Waters D, Simon J and Krauss RM. Is there a simple way to identify insulin-resistant individuals at increased risk of cardiovascular disease? Am. J. Cardiol. (2005) 96: 399–404.

(29) Quijada Z, Paoli M, Zerpa Y, Camacho N, Cichetti R, Villarroel V, Arata-Bellabarba G and Lanes R. The triglyceride/HDL-cholesterol ratio as a marker of cardiovascular risk in obese children; association with traditional and emergent risk factors. Pediatr. Diabetes. (2008) 9: 464–71.

(30) Na Kwon H and Lim H. Relationship between Serum Vitamin D Status and Metabolic Risk Factors among Korean Adults with Prediabetes. *PLoS One* (2016) 11: e0165324.

(31) Lorvand Amiri H, Agah Sh, Mousavi SN, Hosseini AF and Shidfar F. Regression of Non-Alcoholic Fatty Liver by Vitamin D Supplement: A Double-Blind Randomized Controlled Clinical Trial. *Arch. Iran Med*. (2016) 19: 631-638.

(32) Mansuri S, Badawi A, Kayaniyil S, Cole DE, Harris SB, Mamakeesick M, Maguire J, Zinman B, Connelly PW and Hanley AJ. Associations of circulating 25(OH)D with cardiometabolic disorders underlying type 2 diabetes mellitus in an aboriginal Canadian community. *Diabetes. Res. Clin. Pract*. (2015) 109: 440-9.

(33) Lu L, Yu Z, Pan A, Hu FB, Franco OH, Li H, Li X, Yang X, Chen Y and Lin X. Plasma 25-hydroxyvitamin D concentration and metabolic syndrome among middle-aged and elderly Chinese individuals. *Diabetes Care* (2009) 32: 1278-83.

(34) Chon SJ, Yun BH, Jung YS, Cho SH, Choi YS, Kim SY, Lee BS and Seo SK. Association between vitamin D status and risk of metabolic syndrome among Korean postmenopausal women. *PLoS One* (2014) 21: e89721.

(35) Huang CY, Chang HH, Lu CW, Tseng FY, Lee LT and Huang KC. Vitamin D status and risk of metabolic syndrome among non-diabetic young adults. *Clin. Nutr*. (2015) 34: 484-9.

(36) Mead JR, Irvine SA and Ramji DP. Lipoprotein lipase: structure, function, regulation, and role in disease. *J. Mol. Med (Berl)*. (2002) 80: 753-69.

(37) Matsuura F, Wang N, Chen W, Jiang XC and Tall AR. HDL from CETP-deficient subjects show enhanced ability to promote cholesterol efflux from macrophages in an apoE- and ABCG1-dependent pathway. *J. Clin. Invest*. (2006) 116: 1435–42.

(38) Zhou QG, Hou FF, Guo ZJ, Liang M, Wang GB and Zhang X. 1, 25-Dihydroxyvitamin D improved the free fatty-acid-induced insulin resistance in cultured C2C12 cells. *Diabetes. Metab. Res*. (2008) 24: 459–64.

(39) Liu J, Sempos C and Donahue R.P. Joint distribution of non-HDL and LDL cholesterol and coronary heart disease risk prediction among individuals with and without diabetes. *Diabetes Care*. (2005) 28: 1916-1921.

(40) Katarzyna B. Non-HDL Cholesterol and Evaluation of Cardiovascular Disease Risk. *EJIFCC*. (2010) 21: 64–7.

(41) Bittner V. Non-HDL cholesterol measurement, interpretation and significance. *Adv. Stud. Med*. (2007) 7: 1-8.

(42) Robinson JG, Wang S, Smith BJ and Jacobson TA. Meta-analysis of the relationship between non-high-density lipoprotein cholesterol reduction and coronary heart disease risk. *J. Am. Coll. Cardiol*. (2009) 53: 316-22.

(43) Birken CS, Lebovic G, Anderson LN, McCrindle BW, Mamdani M, Kandasamy S, Khovratovich M, Parkin PC and Maguire JL. Association between Vitamin D and Circulating Lipids in Early Childhood. *PLoS One* (2015) 10: e0131938.

(44) Gallo L, Faniello MC, Canino G, Tripolino C, Gnasso A and Cuda G. Serum Calcium Increase Correlates with Worsening of Lipid Profile: An Observational Study on a Large Cohort From South Italy. *Medicine (Baltimore)*. (2016) 95: e2774.

(45) Mao PJ, Zhang C, Tang L, Xian YQ, Li YS, Wang WD, Zhu XH, Qiu HL, He J and Zhou YH. Effect of calcium or vitamin D supplementation on vascular outcomes: a meta-analysis of randomized controlled trials. *Int. J. Cardiol*. (2013) 169: 106-11.

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