The relationship of vitamin D with non-traditional risk factors for cardiovascular disease in subjects with metabolic syndrome

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Abstract

Introduction: Several studies implicate an inverse relationship between 25-hydroxy vitamin D (25(OH)Vit D) serum levels and metabolic syndrome (MetS). We sought to investigate a possible relationship between 25(OH)Vit D and emerging risk factors associated with MetS, such as small dense low-density lipoprotein cholesterol (sdLDL-C) concentration, lipoprotein-associated phospholipase A2 (Lp-PLA2) activity and high-sensitivity C-reactive protein (hsCRP) levels.

Material and methods: We studied 110 consecutive otherwise healthy individuals. Of these, 52 were diagnosed with MetS and 58 who did not meet the MetS criteria served as controls. Low-density lipoprotein (LDL) subclass analysis was performed by polyacrylamide gel electrophoresis. Lp-PLA2 activity was determined in total plasma by the trichloroacetic acid precipitation procedure. Serum 25(OH)Vit D was determined quantitatively by an enzyme immunoassay method.

Results: Metabolic syndrome subjects had significantly lower 25(OH)Vit D levels (11.8 [0.6-48.3] ng/ml; 29.5 [1.5-120.75] nmol/l) compared with controls (17.2 [4.8-62.4] ng/ml; 43 [12-156] nmol/l, \( p = 0.027 \)). Univariate regression analysis showed that 25(OH)Vit D concentration was inversely related to triglycerides \(( r = -0.416, p = 0.003 )\) and sdLDL-C \(( r = -0.305, p = 0.004 )\). There was no association of 25(OH)Vit D with waist circumference, blood pressure, high-density lipoprotein cholesterol (HDL-C), fasting glucose, Lp-PLA2 and hsCRP. In multivariate regression analysis the relationship between 25(OH)Vit D and sdLDL-C became insignificant when triglycerides were included in the model.

Conclusions: Subjects with MetS exhibit lower 25(OH)Vit D serum levels compared with non-MetS individuals. Low 25(OH)Vit D is associated with higher sdLDL-C levels possibly through elevated triglycerides. No association between 25(OH)Vit D and Lp-PLA2 or hsCRP was found.

Key words: vitamin D, metabolic syndrome, small dense low-density lipoprotein cholesterol, lipoprotein-associated phospholipase A2 activity, high-sensitivity C-reactive protein.

Introduction

In recent years emphasis has been placed on the role of vitamin D (Vit D) in areas beyond bone metabolism and calcium homeostasis [1]. In this
context, Vit D deficiency, which is very common worldwide, has been associated with risk factors for cardiovascular disease (CVD), the metabolic syndrome (MetS) and even with cancer, autoimmune diseases, infections and overall mortality [1, 2].

Metabolic syndrome is a constellation of CVD risk factors, i.e. abdominal obesity, atherogenic dyslipidemia (high triglycerides and reduced high-density lipoprotein cholesterol [HDL-C]), disturbed carbohydrate metabolism, elevated blood pressure (BP), along with a prothrombotic and proinflammatory profile [3]. Moreover, MetS has been associated with emerging risk factors, such as increased levels of atherogenic small dense low-density lipoprotein cholesterol (sdLDL-C) at relatively ‘normal’ levels of LDL-C [4] and elevated lipoprotein-associated phospholipase A2 (Lp-PLA2) activity [5]. Both increased sdLDL-C and Lp-PLA2 activity have been associated with increased CVD risk [5, 6]. Also, high-sensitivity C-reactive protein (hsCRP) has been proposed as a novel parameter to assess CVD risk [7].

Interestingly, a number of recent, population-based, cross-sectional studies suggest distinct metabolic roles for 25(OH)Vit D and parathyroid hormone (PTH) in MetS [2, 8-10]. The third National Health and Nutrition Examination Survey (NHANES III) and NHANES 2003-2004 studies have shown a significant inverse association between serum 25(OH)Vit D concentrations and MetS as a whole, as well as with each one of its diagnostic criteria [2, 9]. The same relation was also demonstrated in a European study [11]. Overall, there is a clear association between 25(OH)Vit D and HDL-C and triglycerides in the vast majority of studies.

The purpose of this study was to evaluate whether 25(OH)Vit D levels could be associated with emerging CVD risk factors of MetS, such as sdLDL-C levels, Lp-PLA2 activity and hsCRP concentration.

Material and methods

Patients

One hundred ten otherwise healthy consecutive individuals, who visited the Outpatient Lipid Clinic of the University Hospital of Ioannina, Ioannina, Greece, for a regular check-up, were included in the present study. Of these, 52 fulfilled 3 or more of the American Heart Association (AHA) criteria [12] for the diagnosis of MetS (waist circumference > 102 cm in men, > 88 cm in women, fasting serum triglycerides > 150 mg/dl, HDL-C < 40 mg/dl in men, < 50 mg/dl in women, blood pressure > 130/85 mm Hg, fasting serum glucose > 100 mg/dl), while 58 age- and sex-matched individuals with less than 3 criteria for the diagnosis of MetS served as controls. Blood pressure was measured in triplicate in the right arm after patients had rested for 10 min in a sitting position. Measurements were performed by trained clinicians using an electronic sphygmomanometer (WatchBP Office, Microlife WatchBP AG, Widnau, Switzerland). Individuals found to be diabetic (1 random measurement of plasma glucose > 200 mg/dl; 11 mmol/l plus symptoms of hyperglycemia, 2 measurements of fasting glucose levels > 126 mg/dl; 7 mmol/l, or plasma glucose > 200 mg/dl; 11 mmol/l 2 h after a 75 g oral glucose tolerance test [OGTT]), or with history of CVD were excluded. Other exclusion criteria were the presence of thyroid dysfunction (adverse levels of thyroid stimulating hormone), liver or kidney disease (defined as a positive medical history or a threefold increase in serum aminotransferases and serum creatinine levels of > 1.6 mg/dl; 141.4 μmol/l, respectively) and the administration of drugs that may interfere with glucose or lipid (e.g. statins, fibrates and niacin) as well as calcium metabolism (e.g. multivitamin preparations or drugs for osteoporosis). Subjects with homeostasis model assessment (HOMA) index values above the 75th percentile (i.e. 2.0) were considered to have insulin resistance [13]. However, HOMA index was treated as a continuous variable in this study. Cut-off values for serum 25(OH)Vit D levels in adults include: > 30 ng/ml (> 75 nmol/l) for vitamin D sufficiency, 20-28 ng/ml (50-70 nmol/l) for insufficiency and < 20 ng/ml (< 50 nmol/l) for deficiency.

All specimens were collected during the same season of the year (spring) so as to exclude any sunlight effect on Vit D levels. All participants were of Greek origin, had similar dietary habits with usual calcium content and comparable amounts of sun exposure, since none was institutionalized or home-bound or had a special dress code.

All participants gave written informed consent before enrollment and the study was approved by the Ethics Committee of the University Hospital of Ioannina. All experiments were performed with the understanding and consent of each subject. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Analytical methods

All lipid and lipoprotein determinations were carried out after an overnight fast. Serum levels of total cholesterol, HDL-C and triglycerides were determined enzymatically on the Olympus AU600 Clinical Chemistry analyzer (Olympus Diagnostica, Hamburg, Germany). Serum LDL-C was calculated using the Friedewald formula (provided that triglycerides were < 350 mg/dl; 3.95 mmol/l). Serum apolipoprotein AI and B (apo AI and apo B) levels were measured with a Behring Holding BN 100 Nephelometer (Liederbach, Germany). Insulin levels were determined by a microparticle enzyme immunoassay on an AXSYM analyzer (Abbott Diag-
nóstika, Wiesbaden-Delkenheim, Germany) with a coefficient of variation of 4.2% to 9.0%. HOMA index was calculated as follows: fasting insulin (mIU/l) × fasting glucose (mg/dl)/405.

Serum concentrations of hsCRP were measured by the high sensitivity CRP method (Dade Behring, Marburg, Germany) based on particle enhanced immunonephelometry; the reference range is 0.175 mg/l to 55 mg/l.

LDL subclass analysis was performed by electrophoresis, using high-resolution, 3% polyacrylamide tube gel and the Lipoprint LDL System (QuantiMetrix, Redondo Beach, Calif) according to the manufacturer’s instructions. Low-density lipoprotein subclass was calculated with the electrophoretic mobility (RF) between the very low density lipoprotein (VLDL) fraction (RF, 0.0) and the HDL fraction (RF, 1.0). LDL is distributed from RF 0.32 to 0.64 as 7 bands, whose RFs are 0.32, 0.38, 0.45, 0.51, 0.56, 0.6 and 0.64 (LDL-1 to LDL-7, respectively).

LDL-1 and LDL-2 are defined as large, buoyant LDL, and LDL-3 to LDL-7 are defined as sdLDL. Mean particle size was provided by the Lipoprint LDL System.

Lp-PLA2 activity in total plasma was determined by the trichloroacetic acid precipitation procedure using [3H]-platelet-activating factor (PAF) (100 μM final concentration) as a substrate. The reaction was performed for 10 min at 37°C and Lp-PLA2 activity is reported as nmol PAF degraded per min per ml of plasma or mg of LDL subfraction protein.

Serum 25(OH)Vit D was determined quantitatively by an enzyme immunoassay method using reagents from DRG Instruments GmbH kit (Germany). The sensitivity of the method was 1.28 ng/ml (3.2 nmol/l) and the intra- and inter assay variation is 7% for each of the level of 72 nmol/l and 84 nmol/l (28.8 ng/ml and 33.6 ng/ml), respectively. Parathyroid hormone levels were determined by IMMULITE 2500 Intact PTH.

**Results**

The clinical and laboratory characteristics of study participants are shown in Table I. There were no differences in age and sex distribution between study groups. As anticipated, subjects with MetS exhibited significantly elevated weight, body mass index (BMI), waist circumference, systolic and diastolic BP, triglycerides, apo B, fasting plasma glucose, insulin and HOMA index, but lower HDL-C and apo AI compared with control subjects. Total cholesterol, LDL-C and PTH levels did not differ significantly between groups. Subjects with MetS presented with significantly higher hsCRP, sdLDL-C and Lp-PLA2 and smaller LDL size compared with participants without MetS. Importantly, the MetS group exhibited significantly lower 25(OH)Vit D serum levels compared with controls (11.8 [0.6-48.3] ng/ml; 29.5 [1.5-120.75] nmol/l vs. 17.2 [4.8-62.4] ng/ml; 43 [12-156] nmol/l, p = 0.027) (Table I).

In MetS subjects univariate analysis showed that 25(OH)Vit D was significantly and inversely associated with triglycerides (r = −0.416, p = 0.003), but not with the other diagnostic criteria of MetS (i.e. waist circumference, BP, HDL-C and fasting glucose) (Table II). In addition, 25(OH)Vit D was inversely related to sdLDL-C levels (r = 0.03) and PTH (r = −0.376, p = 0.04), but not significantly associated with LDL size, Lp-PLA2 and hsCRP (Table II).

We performed stepwise multivariate linear regression analysis with sdLDL-C as the dependent variable and sex, age, smoking, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting glucose, HOMA index, triglycerides, HDL-C, LDL-C, apo B, hsCRP and 25(OH)Vit D as independent variables. In this analysis, sdLDL-C levels were significantly influenced only by triglycerides but not by 25(OH)Vit D concentration (Table III).

**Discussion**

To our knowledge this is the first time that the association of 25(OH)Vit D with emerging CVD risk factors of MetS and a set of independent variables (or predictors) that were significantly correlated with the dependent variable in the univariate analysis after checking for normality and linearity. A p value < 0.05 was considered to be significant. All analyses were carried out with the SPSS 18 software package.
25(OH)Vit D concentration. No association between 25(OH)Vit D and Lp-PLA2 or hs-CRP was noted.

The large NHANES III and NHANES 2003-2004 have shown a significant inverse association between serum 25(OH)Vit D concentration and MetS as a whole, as well as with each one of its components [2, 9]. Our results are in accordance with these studies showing that subjects with MetS have significantly lower 25(OH)Vit D levels compared with non-MetS. A possible explanation for this observation could be the sequestration of the fat soluble Vit D in the adipose tissue [14], which is in abundance in MetS subjects. This makes stores less available to become biologically activated [15]. However, we cannot exclude the possibility that obesity and associated co-morbid conditions could reduce levels of outdoor physical activity and sun exposure, and subsequently lead to Vit D deficiency. Yet, it is still unknown whether Vit D deficiency

Table I. Clinical and laboratory characteristics of study participants

| Parameter | Metabolic syndrome (n = 52) | Non-metabolic syndrome (n = 58) | Value of p |
|-----------|-----------------------------|--------------------------------|------------|
| Age [years] | 52 ±10 | 50 ±12 | NS |
| Sex (male/female) | 24/28 | 26/32 | NS |
| Smoking (yes/no) | 15/37 | 17/41 | NS |
| Weight [kg] | 83 ±13 | 78 ±15 | 0.01 |
| BMI [kg/m²] | 30.2 ±3.0 | 28.2 ±4.6 | 0.01 |
| Waist circumference [cm] | 102 ±8 | 95 ±14 | 0.007 |
| SBP [mm Hg] | 135 ±15 | 124 ±18 | 0.002 |
| DBP [mm Hg] | 88 ±9 | 80 ±10 | < 0.001 |
| T-Chol [mg/dl] | 236 ±37 | 226 ±46 | NS |
| HDL-C [mg/dl] | 49 ±11 | 57 ±12 | 0.001 |
| LDL-C [mg/dl] | 152 ±29 | 148 ±39 | NS |
| Triglycerides [mg/dl] | 152 (78-350) | 97 (37-294) | < 0.001 |
| Non HDL-C [mg/dl] | 186 ±35 | 169 ±45 | 0.031 |
| Apo AI [mg/dl] | 141 ±26 | 154 ±25 | 0.03 |
| Apo B [mg/dl] | 119 ±17 | 105 ±25 | 0.04 |
| sdLDL-C [mg/dl] | 9.0 (0.01-66.0) | 3.5 (0.01-28.0) | 0.006 |
| LDL size [Å] | 265.0 ±7.0 | 270.0 ±2.5 | 0.001 |
| Lp-PLA2 activity [nmol/ml/min] | 60.0 ±16.0 | 510 ±14.6 | 0.019 |
| Fasting glucose [mg/dl] | 104 ±16 | 92 ±11 | < 0.001 |
| Insulin [μU/ml] | 11 (2-57) | 8 (2-33) | 0.045 |
| HOMA index | 2.4 (0.5-20.0) | 1.8 (0.4-7.0) | 0.045 |
| hsCRP [mg/l] | 2.7 (0.2-6.8) | 2.1 (0.2-6.2) | 0.045 |
| 25(OH)Vit D [ng/ml] | 118 (0.6-48.3) | 172 (4.8-62.4) | 0.027 |
| PTH [pg/ml] | 42 (19-125) | 53 (11-96) | NS |
| Total Ca [mg/dl] | 9.7 ±0.4 | 9.5 ±0.3 | NS |
| e-GFR [ml/min/1.73 m²] | Cockcroft-Gault | 111 ±30 | 106 ±21 | NS |
| MDRD | 80 ±15 | 83 ±10 | NS |

BMI – body mass index, SBP – systolic blood pressure, DBP – diastolic blood pressure, T-Chol – total cholesterol, HDL-C – high-density lipoprotein cholesterol, LDL-C – low-density lipoprotein cholesterol, Apo – apolipoprotein, sdLDL-C – small dense LDL-C, Lp-PLA2 – lipoprotein-associated phospholipase A2, HOMA index – homeostasis model assessment insulin resistance index, 25(OH)Vit D – 25-hydroxy vitamin D, PTH – parathyroid hormone, hsCRP – high-sensitivity C-reactive protein, Ca – calcium, e-GFR – estimated glomerular filtration rate, MDRD – Modification of Diet in Renal Disease

To convert values for triglycerides to mmol/l multiply by 0.01129. To convert values for cholesterol to mmol/l multiply by 0.02586. To convert values for glucose to mmol/l multiply by 0.05551. To convert values for 25(OH)Vit D to mmol/l multiply by 2.5. To convert values for Ca²⁺ to mmol/l multiply by 0.25.
has an important pathogenetic role in CVD or this association is non-causal and confounded by other factors. On the other hand, Vit D deficiency could have an indirect effect on the development of obesity, which is a basic characteristic of MetS. Of note, PTH (which is elevated in Vit D deficiency) increases the cytosolic calcium level in isolated adipocytes [17], thus impeding the catecholamine-induced lipolysis [18] and promoting the expression of fatty acid synthetase [19].

There is also evidence that apart from low Vit D, elevated PTH levels could be associated with glucose intolerance and insulin resistance [20-22]. Reis et al. showed that MetS was positively related with PTH concentration among older men but not women [8, 9], while Lee et al. did not find any relationship between MetS and PTH levels in men and no evidence of an age interaction [11]. In our study, PTH levels were numerically but not significantly lower in MetS compared with non-MetS subjects.

Dyslipidemia is a hallmark of MetS and may substantially contribute to the increased CVD risk observed in this population. Previous studies have examined the relationship between 25(OH)Vit D and HDL-C and triglyceride concentration [9, 23-26] and reported overall a positive correlation between 25(OH)Vit D and HDL-C and an inverse correlation between 25(OH)Vit D and triglyceride levels. Our study confirmed the inverse relationship between 25(OH)Vit D and triglycerides, but we could find no significant association between 25(OH)Vit D and HDL-C. Similarly, a secondary analysis of the NHANES III (n = 15,088) showed that the adjusted prevalence of high serum triglyceride levels was higher in the first compared with the fourth quartile of serum 25(OH)Vit D levels (odds ratio 1.47, p < 0.001) [26]. After adjustment for age, gender and race 32.9% of participants in the first quartile of 25(OH)Vit D concentration (< 21 ng/ml; < 52 nmol/l) had triglycerides ≥ 150 mg/dl (≥ 1.69 mmol/l) compared with 23.8% of those in the fourth quartile (≥ 37 ng/ml; ≥ 92 nmol/l) (p < 0.001) [26]. These findings are in accordance with a previous smaller sub-study of NHANES III (n = 8421) [2], but not with a more recent study which also utilized data from NHANES between 2003 and 2004 (n = 1654) and found a positive relationship between Vit D and HDL-C (p = 0.004), but no association with triglycerides [9]. In a large European cohort (n = 6810 British white subjects) 25(OH)Vit D was inversely associated with triglyceride levels (p = 0.004) after adjustment for insulin growth factor (IGF)-1, as well as obesity and social and lifestyle variations [23]. In contrast, a positive association between 25(OH)Vit D and high triglycerides (p = 0.001) was reported for the female participants in another study (n = 1070; 660 women) [8].

Vitamin D may affect serum lipid levels both directly and indirectly. In vitro studies have shown that 1,25(OH)₂Vit D (the active metabolite of Vit D) may have a direct dose-dependent effect on adipogenesis, with low doses of 1,25(OH)₂Vit D having

| Parameter | r | p |
|-----------|---|---|
| Waist circumference | 0.119 | 0.422 |
| Blood pressure | | |
| SBP | 0.234 | 0.109 |
| DBP | 0.009 | 0.949 |
| Log [triglycerides] | -0.416 | 0.003 |
| HDL-C | 0.127 | 0.390 |
| Fasting glucose | 0.048 | 0.747 |
| HOMA index | 0.083 | 0.632 |
| Log [sdLDL-C] | -0.305 | 0.03 |
| LDL size | 0.275 | 0.165 |
| Lp-PLA2 activity | 0.064 | 0.746 |
| hsCRP | -0.096 | 0.562 |
| PTH | -0.376 | 0.04 |

| Parameter | β | p | 95% CI |
|-----------|---|---|-------|
| Sex | 0.068 | 0.779 | -0.355, 0.462 |
| Age | -0.091 | 0.770 | -0.031, 0.024 |
| Smoking | -0.046 | 0.875 | -0.577, 0.498 |
| SBP | -0.128 | 0.710 | -0.017, 0.012 |
| DBP | 0.008 | 0.975 | -0.018, 0.018 |
| Fasting glucose | -0.121 | 0.692 | -0.022, 0.015 |
| HOMA index | 0.172 | 0.561 | -0.049, 0.086 |
| Log [triglycerides] | 0.689 | 0.019 | 0.146, 1.311 |
| HDL-C | -0.109 | 0.719 | -0.025, 0.018 |
| LDL-C | 0.227 | 0.619 | -0.011, 0.018 |
| Apo B | 0.119 | 0.781 | -0.018, 0.024 |
| Log [hsCRP] | 0.214 | 0.330 | -0.267, 0.726 |
| Log [25(OH)Vit D] | -0.001 | 0.996 | -0.363, 0.361 |
a stimulating effect and high doses an inhibitory effect [27, 28]. Furthermore, the indirect actions of Vit D could be mediated through its effect on serum PTH and/or on the calcium balance. High levels of Vit D lead to increased calcium absorption, less calcium in the intestine and accordingly decreased formation of calcium-fatty acid soaps excreted in the feces and subsequently increased fat absorption, leading to increased serum triglyceride levels [29]. However, the effect of the intestinal calcium on fat absorption is too small to significantly affect serum triglycerides in humans [30]. Moreover, an effect of Vit D on serum lipids could be mediated through suppression of PTH secretion, since the PTH has been reported to reduce lipolysis at least in vitro [19]. In addition, Vit D may influence serum lipids by affecting serum calcium levels, given that an elevated calcium level may reduce hepatic triglyceride formation and/or secretion [31, 32]. In our study total calcium levels did not differ between the two groups. Furthermore, Vit D may have an effect both on insulin secretion and insulin sensitivity and thereby indirectly influence lipid metabolism [33].

Importantly, we report a significant inverse relationship between 25(OH)Vit D serum levels and sdLDL-C. On the other hand, 25(OH)Vit D was not significantly associated with LDL size despite a suggestive \( r = 0.275 \). Multivariate regression analysis showed that sdLDL-C levels were influenced only by serum triglycerides and not by 25(OH)Vit D levels. To explain the different results of the univariate and multivariate analyses, we should consider the results of previous studies which showed that the most important single determinant of LDL particle distribution is the pool of triglyceride-rich lipoproteins [4]. In general, the higher the triglyceride levels, the smaller the LDL size [34]. The formation of sdLDL particles is mostly observed in the presence of a hypertriglyceridemic state, with an increased exchange of triglycerides from triglyceride-rich lipoproteins to LDL and HDL particles in exchange of cholesteryl esters (CE) through the action of cholesteryl ester transfer protein (CETP). This process leads to the generation of very low-density lipoprotein (VLDL) particles enriched in CE and to triglyceride-rich LDL particles. These triglyceride-rich lipoproteins are good substrates for hepatic lipase (HL), which has a higher binding affinity for lipoproteins smaller than VLDL, regulating total plasma LDL concentrations as well as the production of sdLDL-C from larger, more buoyant precursors [34]. Based on the inverse relationship between Vit D and serum triglyceride levels found in our and other studies, we conclude that low Vit D is indirectly related to higher sdLDL-C levels, possibly by contributing to an elevation of serum triglycerides.

We also searched for a possible relationship between 25(OH)Vit D and Lp-PLA2, as well as hsCRP, which are considered as powerful predictors of CVD [35]. We could find no association between 25(OH)Vit D and Lp-PLA2 activity or hsCRP, two sensitive surrogates of low grade inflammation of the arteries related to atherosclerosis. This finding may imply that whatever the relation between 25(OH)Vit D and MetS, it may not be an atherogenic one.

The main limitation of this study is that a causal relationship between 25(OH)Vit D and emerging risk factors in subjects with MetS could not be assessed because of its cross-sectional design. Moreover, the relatively small number of participants does not allow us to generalize these results.

In conclusion, MetS subjects have lower 25(OH)Vit D levels compared with non-MetS. Lower 25(OH)Vit D levels are associated with higher sdLDL-C concentration in subjects with MetS, possibly through elevated triglycerides. Future prospective studies are needed to address a possible effect of Vit D supplementation on the metabolic characteristics of patients with MetS.

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