Clinical Nutrition ESPEN 29 (2019) 198–202

Contents lists available at ScienceDirect

Clinical Nutrition ESPEN

journal homepage: http://www.clinicalnutritionespen.com

Original article

Effect of combined vitamin D administration plus dietary intervention on oxidative stress markers in patients with metabolic syndrome: A pilot randomized study

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ARTICLE INFO

Article history:
Received 14 September 2018
Accepted 6 October 2018

Keywords:
Vitamin D
Metabolic syndrome
Oxidative stress

SUMMARY

Background: Metabolic syndrome (MetS) patients can have low 25-hydroxyvitamin D 25(OH)VitD levels, which may be associated with increased oxidative stress. There is little data on the effect of 25(OH)VitD administration plus dietary intervention on oxidative stress markers in these patients.
Aim: To study the effect of 25(OH)VitD administration plus dietary intervention on oxidative stress markers in MetS patients.
Methods: This is a pre-specified analysis of a previously published study (NCT01237769 ClinicalTrials.gov). MetS participants (n = 50, 52 ± 10 years) were given dietary instructions and were randomized to 25(OH)VitD 2.000 IU/day p.o. (Suppl group) or no supplementation (No-Suppl group). Serum 25(OH)VitD, oxidized LDL (ox-LDL), paraoxonase activity (PON-1), arylesterase activity (ARYL) and urine 8-isoprostane (8-iso-PGF_{2a}) levels were measured at baseline and after 3 months.
Results: MetS patients had low baseline 25(OH)VitD levels, which increased by 90% in the Suppl group [from 16.1 (3.3–35.1) to 30.6 (8.4–67.6) ng/mL, p = 0.001] and by 33.3% in the No-Suppl group [from 9.9 (4.0–39.6) to 13.2 (3.5–36.8) ng/mL, p = NS] after intervention. Ox-LDL, PON-1 and ARYL did not change significantly at follow-up in both groups, except for urine 8-iso-PGF_{2a} levels that decreased by 22.7% in the Suppl group [from 48.8 (26.8–137.1) to 37.7 (12.3–99.0) ng/mmol creatinine, p = 0.015] and by 14.4% in No-Suppl group [from 45.8 (16.6–99.3) to 39.2 (13.3–120.1) ng/mmol creatinine, p = NS]. The reduction in 8-iso-PGF_{2a} levels did not differ significantly between the 2 groups.
Conclusion: The administration of 25(OH)VitD plus dietary intervention in patients with MetS was not associated with meaningful reductions in oxidative stress markers compared with dietary intervention alone.

1. Introduction

Over the past 20 years significant progress has been made to identify the multi-modal actions of vitamin D (VitD) [1]. In this context, VitD deficiency [25(OH)VitD levels <20 ng/mL] [2] has actually been related to various chronic musculoskeletal, cardiovascular, infectious, autoimmune and malignant diseases [1] as well as metabolic syndrome (MetS) [3]. MetS is also associated with increased oxidative stress, which in turn is related with endothelial dysfunction, atherosclerosis and cardiovascular diseases (CVD) [4].

In VitD deficient subjects the oxidant/anti-oxidant status seems to lean in favor of oxidative stress [5], while correcting hypovitaminosis D may inhibit oxidative stress and inflammation [6].

Some studies indicated that supplementing obese VitD deficient subjects with VitD may attenuate oxidative stress, particularly when combined with exercise [7,8]. Other studies in patients with chronic diseases [9–13] and even asymptomatic VitD deficient individuals [14] showed that VitD supplementation improved several

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https://doi.org/10.1016/j.clnesp.2018.10.004
2405-4577/

markers of oxidative stress. To date, however, there is lack of data regarding the effect of VitD supplementation on oxidative stress markers in patients with MetS.

In this study we implemented a dietary intervention programme in all MetS participants and aimed to examine whether increasing VitD by oral supplementation (Suppl group) could alter oxidative stress markers compared with no supplementation (No-Suppl group).

2. Material and methods

2.1. Study population

This is a pre-specified analysis of a previously published study (NCT01237769 ClinicalTrials.gov) [15]. This study had a PROBE (prospective, randomised, open-label, blinded end-point) design as previously described [15]. Briefly, consecutive subjects with MetS attending the Outpatient Lipid and Obesity Clinic of the University Hospital of Ioannina, Ioannina, Greece, were included in the study. The study protocol was approved by the ethics committee of our institution and was conducted following the guidelines outlined in the Declaration of Helsinki. In this analysis we included some extra participants (5 in Suppl Group and 4 in No-Suppl Group) since we could not retrieve serum samples from all patients participating in the original study to measure oxidative stress parameters.

Patients with diabetes, chronic kidney or liver disease, triglycerides >500 mg/dL (5.65 mmol/L) as well as those on calcium and/or VitD supplements and lipid-lowering medications were excluded. Eligible patients were randomly allocated (through a computer-generated sequence of random numbers) by sex and age as baseline factors to either only dietary instructions (No-Suppl group) or to receive 2000 IU VitD/day po (Vitamin D3, Lamberts) plus dietary instructions (Suppl group) for 3 months (see CONSORT 2010 flow diagram). Investigators enrolled study participants and assigned them to the interventions according to a computer-generated sequence of random numbers. All patients were instructed to follow a 12-week dietary intervention programme according to NCEP ATP III guidelines [16]. At baseline visit, a dietician provided a low-fat diet (<30% of total calories) taking into account each subject's personal activities and energy requirements so as to achieve a 500 kcal reduction in daily energy intake with no differences in diet composition in both study groups. The compliance to dietary instructions was assessed by completing food diaries and through discussion during follow up visits, while compliance with study medication was checked by tablet count at week 12; patients were considered compliant if they received 80–100% of the prescribed tablets.

In order to minimise the effect of sunlight on 25(OH)VitD levels, all specimens were collected during March to September, a season during which the duration of sunlight is approximately similar in Greece.

2.2. Laboratory measurements

All laboratory assays were performed after an overnight fast and were blindly assessed regarding treatment allocation. Serum 25(OH)VitD levels were measured quantitatively by an enzyme immunoassay method using the reagents from DRG Instruments GmbH kit (DRG, Marburg, Germany). This method has an analytical sensitivity is 1.28 ng/mL and the intra- and inter assay variation is 13% at the level of 18 and 16 ng/mL, respectively. Plasma levels of oxidized low-density lipoprotein (ox-LDL) were measured by a competitive enzyme-linked immunosorbent assay using a specific murine monoclonar antibody (4E6) according to the instructions provided by the manufacturer (Mercodia, Uppsala, Sweden) as previously described. Intra- and inter-assay coefficients of variation were 6.0% and 7.0%, respectively. The paraoxonase-1 (PON-1) activities in serum were measured using paraoxon (paraoxonase activity) or phenylacetate (arylesterase activity), as a substrate. Both PON-1 activities were determined in the presence of 2 mM Ca^{2+} in 100 mM Tris-HCl buffer (pH 8.0) for paraoxon and in 20 mM Tris-HCl buffer (pH 8.0) for phenyl acetate. Urine levels of 8-isoprostane (8-epiPGF2a) were determined by a competitive enzyme immunoassay (commercial 8-isoprostane EIA kit, Cayman Chemicals, Ann Arbor, MI), following manufacturer instructions. The 8-epiPGF2a levels in urine were expressed as ng/mmol creatinine.

2.3. Statistical analysis

This was a pilot study and therefore formal power calculation was not performed. The Kolmogorov–Smirnoff test was used for evaluation of the distribution of each variable (Gaussian or not). Data are presented as mean ± standard deviation for variables with a Gaussian distribution and as median (range, min–max) for those with a non-Gaussian distribution along with their 95% confidence intervals (95% CI). The paired samples t-test or the Wilcoxon Signed Ranks test was used to assess the effect of treatment in each group. To make comparisons between treatment groups we performed analysis of covariance (ANCOVA) or the Kruskal–Wallis test for non-parametric variables adjusted for baseline values. The significance was set at $p < 0.01$ due to multiple comparisons (Bonferoni correction). The statistical analyses were performemd using the SPSS 18.0 statistical package for Windows (SPSS Inc., 1989–2004, Chicago, IL).

3. Results

The clinical and laboratory characteristics of study participants ($n = 50$) are shown in Table 1. No significant differences in baseline characteristics were noted between the 2 groups except for arylesterase activity which was significantly lower in the Suppl vs No-Suppl group. There were also no differences in dietary intake between groups at baseline and after the intervention (data not shown). All patients in the Suppl group were compliant with study medication. Most participants (74%, 72% in Suppl and 76% in No-Suppl group) were VitD deficient at baseline (25(OH)VitD <20 ng/mL).

Three months after intervention, a similar small weight reduction (1–2 kg) was achieved in both groups, indicating poor compliance to dietary instructions (Table 2). 25(OH)VitD levels increased significantly by 90% [from 16.1 (3.3–35.1) to 30.6 (8.4–67.6) ng/mL, $p = 0.001$] in the Suppl group and by 33.3% [from 9.9 (4.0–39.6) to 13.2 (3.5–36.8) ng/mL, p = NS] in the No-Suppl group ($p = 0.002$ for the comparison between groups).

Plasma ox-LDL levels and serum paraoxonase and arylesterase activities of PON-1 did not significantly change in both groups. Same results were noted after correction for lipid and apolipoprotein levels (Table 2). Urine 8-iso-PGF_{2a} levels significantly decreased by 22.7% in the Suppl group [from 48.8 (26.8–137.1) to 37.7 (12.3–99.0) ng/mmol creatinine, $p = 0.015$], whereas a non-significant reduction by 14.4% was observed in the No-Suppl group [from 45.8 (16.6–99.3) to 39.2 (13.3–120.1) ng/mmol creatinine, p = NS]. However, the reduction in 8-iso-PGF_{2a} levels did not differ significantly between the 2 groups (Table 2).

4. Discussion

In this pre-specified analysis we found that VitD supplementation plus dietary intervention did not meaningfully alter several

Table 1
Baseline characteristics of study participants.

	No-Suppl group		Suppl group		P
	Mean ± SD or median (range)	95% CI	Mean ± SD or median (range)	95% CI	
N (males/females)	25 (10/15)		25 (15/10)		0.12
Age (years)	52 ± 15	40.0–59.2	53 ± 7.0	48.1–58.2	0.59
Smoking, %	24		16		0.44
Body weight (kg)	90 ± 14	81.1–99.4	89 ± 13	80.4–102.6	0.95
BMI (kg/m^2)	34.0 ± 7.6	28.7–37.5	32.0 ± 5.0	28.3–35.8	0.19
WC (cm)	112.0 ± 8.5	104.4–115.9	106.0 ± 11.0	99.8–119.0	0.34
SBP (mm Hg)	137 ± 13	127.7–146.3	135 ± 14	123.7–145.0	0.49
DBP (mm Hg)	87 ± 9	81.6–92.8	86 ± 8	81.1–90.2	0.78
25(OH)VitD (ng/mL)	9.9 (4.0–39.6)	5.5–15.7	16.1 (3.3–35.1)	10.3–24.8	0.12
Ox-LDL (U/L)	67.2 ± 16.9	59.2–79.3	70.3 ± 15.2	64.8–87.6	0.50
Paraoxonase (U/L)	82.2 (16.1–207.4)	50.5–108.4	80.6 (19.5–287.4)	65.9–153.7	0.33
Arylesterase (U/mL)	97.7 ± 22.7	78.9–105.2	79.3 ± 26.0	59.2–87.3	0.013
Urine 8-epi PGF_{2a} (ng/mmol creatinine)	45.8 (16.6–99.3)	34.6–61.1	48.8 (26.8–137.1)	43.4–95.1	0.05
Ox-LDL/LDL (U/mg)	0.060 ± 0.008	0.41–0.58	0.050 ± 0.010	0.46–0.65	0.15
Ox-LDL/ApoB (U/mg)	0.070 ± 0.008	0.57–0.74	0.080 ± 0.040	0.70–1.08	0.027
Paraoxonase/HDL (U/mg)	0.17 ± 0.12	1.01–2.32	0.19 ± 0.15	1.41–3.2	0.31
Paraoxonase/ApoA1 (U/mg)	0.06 ± 0.04	0.34–0.82	0.08 ± 0.07	0.45–1.26	0.20

Suppl: supplementation, SD: standard deviation, CI: confidence interval, BMI: body mass index, WC: waist circumference, SBP: systolic blood pressure, DBP: diastolic blood pressure, 25(OH)Vit D: 25-hydroxy vitamin D, Urine 8-epi PGF2α: urine 8-isoprostane, ox-LDL: oxidized LDL, ApoA1/B: Apolipoprotein A1/B.

Table 2
Changes 3 months after treatment compared with baseline and between groups.

	Baseline		3 Months		P	Change, %	p vs No Suppl
	Mean ± SD or median (range)	95% CI	Mean ± SD or median (range)	95% CI			
Weight (kg)							
No Suppl	89.0 ± 13.4	81.1–99.4	87.0 ± 12.4	74.6–98.7	0.005	−2.2	
Suppl	89.3 ± 16.6	80.4–102.6	88.0 ± 17.1	74.5–94.7	0.020	−1.4	0.450
BMI (kg/m^2)							
No Suppl	33.4 ± 6.0	28.7–37.5	32.4 ± 5.3	26.9–35.5	0.008	−2.9	
Suppl	31.2 ± 5.3	28.3–35.8	32.4 ± 5.0	26.7–32.8	0.030	−3.8	0.191
WC (cm)							
No Suppl	110.0 ± 9.0	104.4–115.9	107.6 ± 9.6	95.6–114.3	0.002	−2.2	
Suppl	107.7 ± 12.7	99.8–119.0	106.3 ± 13.8	95.3–109.6	0.070	−1.3	0.150
25(OH)VitD (ng/mL)							
No Suppl	9.9 (4.0–39.6)	5.5–15.7	13.2 (3.5–36.8)	8.0–23.5	0.175	+33.3	
Suppl	16.1 (3.3–35.1)	10.3–24.8	30.6 (8.4–67.6)	25.7–41.5	0.001	+90.0	0.002
Urine 8-epi PGF2α (ng/mmol creatinine)							
No Suppl	45.8 (16.6–99.3)	34.6–61.1	39.2 (13.3–120.1)	14.5–73.3	0.407	−14.4	
Suppl	48.8 (26.8–137.1)	43.4–95.1	37.7 (12.3–99)	35.6–61.1	0.015	−22.7	0.293
Ox-LDL (U/L)							
No Suppl	67.2 ± 16.9	59.2–79.3	67.3 ± 19.3	56.9–92.5	0.938	+0.1	
Suppl	70.3 ± 15.2	64.8–87.6	75.9 ± 21.2	67.9–89.9	0.062	+7.9	0.144
Paraoxonase (U/L)							
No Suppl	82.2 (16.1–207.4)	50.5–108.4	95.7 (25.0–202.5)	45.9–138.4	0.068	+16.4	
Suppl	80.6 (19.5–287.4)	65.9–153.7	97.2 (21.0–236.0)	58.0–147.0	0.778	+20.6	0.865
Arylesterase (U/mL)							
No Suppl	97.7 ± 22.7	78.9–105.2	91.7 ± 22.8	67.1–111.7	0.196	−6.3	
Suppl	79.3 ± 26	59.2–87.3	81.2 ± 22.7	64.3–84.3	0.602	+2.4	0.122
OxLDL/LDL (U/mg)							
No Suppl	0.06 ± 0.008	0.41–0.58	0.06 ± 0.02	0.42–0.78	0.685	0.0	
Suppl	0.05 ± 0.01	0.46–0.65	0.05 ± 0.01	0.46–0.60	0.538	0.0	0.272
OxLDL/ApoB (U/mg)							
No Suppl	0.07 ± 0.008	0.57–0.74	0.08 ± 0.02	0.66–0.91	0.135	+14.3	
Suppl	0.08 ± 0.04	0.70–1.08	0.07 ± 0.01	0.69–0.82	0.346	−12.5	0.319
Paraoxonase/HDL (U/mg)							
No Suppl	0.17 ± 0.12	1.01–2.32	0.19 ± 0.12	0.85–2.8	0.078	+11.7	
Suppl	0.19 ± 0.15	1.41–3.20	0.19 ± 0.15	1.22–3.19	0.321	0.0	0.321
Paraoxonase/ApoA1 (U/mg)							
No Suppl	0.06 ± 0.04	0.34–0.82	0.07 ± 0.04	0.28–1.01	0.109	+16.6	
Suppl	0.08 ± 0.07	0.45–1.26	0.07 ± 0.05	0.43–1.1	0.211	−12.5	0.967

Suppl: supplementation, SD: standard deviation, CI: confidence interval, Suppl: supplementation, BMI: body mass index, WC: waist circumference, 25(OH)Vit D: 25-hydroxy vitamin D, Urine 8-epi PGF2α: urine 8-isoprostane, ox-LDL: oxidized LDL.

oxidative stress markers compared with dietary intervention alone in patients with MetS.

Subjects with MetS have decreased 25(OH)VitD levels probably due to their sedentary lifestyle, reduced 25(OH)VitD skin synthesis and VitD sequestration in their excessive fat tissue [17]. Indeed, in our study 74% of MetS participants were VitD deficient.

MetS has been associated with increased oxidative stress, which plays a crucial role in the formation, progression and rupture of

atherosclerotic plaques [4]. Of note, oxidation of LDL by free radicals and formation of ox-LDL particles happens in the very early but critical steps of atherosclerosis [18]. Paraoxonase activity and arylesterase activity (i.e. a PON-1 activity more closely related to PON-1 mass) are used as indicators inversely associated with oxidative stress, since they inhibit LDL oxidation and protect against cardiovascular diseases [19]. Another useful and validated oxidative stress marker is isoprostanes, especially the biologically active 8-iso-PGF_{2a}, which are produced by the random oxidation of tissue phospholipids by oxygen radicals [20]. In this study we evaluated these oxidative stress markers at baseline and 3 months after intervention in MetS patients. Notably, to date there is no unique method which can accurately measure oxidative stress.

VitD deficiency has been associated with increased oxidative stress in obese individuals, patients with chronic diseases and the elderly [21]. Some data suggest that VitD deficiency may promote vascular oxidative stress and induce hypertension as well as changes in cardiac gene expression [22]. VitD deficiency may also play a possible role in the link between oxidative stress and diabetes development and progression [23].

VitD supplementation has generally been associated with improvement of oxidative stress and inflammation [6], though not consistently. Some studies in obese VitD deficient subjects showed that VitD supplementation in combination with exercise could possibly ameliorate oxidative stress as assessed by urinary 8-isoprostane, hydrogen peroxide, tumor necrosis factor-alpha and other factors [7,8]. Other studies in patients with chronic diseases found that VitD administration was associated with improvement of several oxidative stress markers. In particular, patients with diabetes showed reductions in plasma nitric oxide (NO), glutathione (GSH) and malondialdehyde (MDA) levels and increase in total antioxidant status (TAS) [9,10]. Furthermore, women with polycystic ovary syndrome exhibited decreases in MDA and GSH levels and an increase in TAS [11,12], while patients with non-alcoholic fatty liver disease experienced a decrease in MDA levels and an increase in TAS [13]. Moreover, VitD deficient individuals exhibited reduction in MDA levels and increase in TAS, while in other studies a decrease in total oxidant status (TOS) and fibrinogen, but not ox-LDL levels were found [5,14]. On the contrary, in type 2 diabetic patients VitD administration was not associated with improvement of oxidative stress markers (superoxide dismutase or plasma 8-isoprostanes) [24].

Conflicting data among studies may be related to differences in a) studied populations, such as comorbidities and number of participants, b) baseline VitD status as well as dose, route and duration of treatment, c) study design and d) measured oxidative stress markers.

There is little data on the association between VitD and oxidative stress markers assessed in this study (ox-LDL, PON-1 activities, and urinary 8-isoprostanes). In particular, a recent study found that serum ox-LDL levels were significantly higher in type 2 diabetic patients with hypovitaminosis D compared with those with normal VitD status [25]. On the other hand, a study in VitD deficient but otherwise healthy persons and matched controls showed that ox-LDL and LDL levels did not differ between groups and there was no association between VitD and ox-LDL levels [5]. In the same study, treatment with 50,000 IU VitD/week per os for 8 weeks was not associated with changes in ox-LDL levels [5]. Similarly in our study, VitD supplementation was not associated with any significant change in ox-LDL levels in MetS subjects.

Regarding PON-1 activity, a previous study showed that supplementing asymptomatic VitD deficient people with 300,000 IU VitD intra-muscular (IM) monthly for 3 months was not associated with changes in serum paraoxonase activity compared with VitD sufficient controls [14]. These findings are in line with ours, since we found no effect of VitD supplementation on paraoxonase activity levels in MetS patients. We also found no alterations in arylesterase activity after VitD supplementation.

Observational studies also show conflicting results about the relationship between VitD and isoprostane levels. An analysis of the Framingham Offspring Study showed that plasma 25(OH)VitD concentration was inversely associated with urinary isoprostanes [26]. Similar results were obtained in a study in type 2 diabetic patients with hypovitaminosis D [25]. Another study though did not support an association of 25(OH)VitD levels with plasma isoprostanes in African-Americans [27]. Of note, data from interventional studies are lacking. In particular, one study in type 2 diabetic patients showed that treatment with 5000 IU VitD/day per os for 12 weeks versus placebo was not associated with improvement in plasma 8-isoprostanes [24]. In the present analysis in MetS subjects, urinary 8-iso-PGF_{2a} decreased by 22.7% ($p = 0.015$) in the Suppl group and by 14.4% (p = NS) in the No-Suppl group. The reduction in 8-iso-PGF_{2a} urine levels did not differ significantly between the 2 groups.

In order to explain our null findings we should take into consideration the following parameters. Compared with healthy controls participating in former studies of our group, MetS patients had increased ox-LDL levels [68.8 vs 45 U/L in controls ($n = 50$, M/F: 23/27, age 54 ± 11 years, BMI 25 ± 3 Kg/m^2)] [28], paraoxonase activity [80.3 vs 77.4 U/L in controls ($n = 30$, M/F: 16/14, age 33 ± 9 years, BMI 24 ± 3 Kg/m^2)] [29], arylesterase activity (88.1 vs 66.6 U/mL in controls) [29] and urine 8-iso-PGF_{2a} levels (48.0 vs 5.5 ng/mmol creatinine in controls]) [29], indicating high oxidative stress levels at baseline. It is probable that intervention with either dietary instructions alone or in combination with 2000 IU/day VitD for 3 months was not enough to reduce these increased oxidative stress markers. Indeed, subjects lost only 1–2 Kg and a higher dose of VitD may have been required. Moreover, the selected markers may not have been appropriate to evaluate changes in oxidative stress status.

4.1. Study limitations

This study has certain limitations. It is mainly a pre-specified analysis of a previously published pilot study with a small number of participants. Therefore, safe conclusions cannot be reached. Supplementation dose (2000 IU/day) and duration (3 months) may be inadequate to significantly reduce oxidative serum markers. According to previous suggestions, concentrations of at least 35–60 ng/mL would be necessary to treat VitD deficiency [30], while in our active treatment group mean VitD levels were 30.6 ng/mL. Moreover, the actual energy and nutrient intake as well as sunlight exposure of each participant were not precisely calculated. Also, we were unable to directly compare our findings with healthy matched controls, as such a group was not included in the original study.

5. Conclusion

VitD supplementation (2000 IU/day for 3 months) plus dietary intervention was associated with no meaningful changes in oxidative stress markers (ox-LDL, PON-1 activities, and urine 8-isoprostanes) compared with dietary intervention alone in MetS subjects.

Statement of authorship

- Conception and design of the study: Evangelos N. Liberopoulos.
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- Critical revision for important intellectual content: Evangelos N. Liberopoulos, Alexandros D. Tselepis.
- Final approval of the version to be submitted: Moses Elisaf, Alexandros D. Tselepis.

All authors have read and approved the final article.

Conflict of interest statement

The authors declare no conflict of interest.

Funding

None.

Acknowledgements

Authors would like to thank the Atherothrombosis Research Centre of the University of Ioannina for providing access to the laboratory equipments and facilities.

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