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Effects of a 1-year supplementation with cholecalciferol on interleukin-6, tumor necrosis factor-alpha and insulin resistance in overweight and obese subjects

Julia Beilfuss [a,b,*], Vivian Berg [c], Monica Sneve [d], Rolf Jorde [b], Elena Kamycheva [a,b]

[a] Department of Gastroenterology, Medical Clinic, University Hospital of North Norway, 9038 Tromsø, Norway
[b] Tromsø Endocrine Research Group, Institute of Clinical Medicine, University of Tromsø, 9038 Tromsø, Norway
[c] Department of Laboratory Medicine, University Hospital of North Norway, 9038 Tromsø, Norway
[d] Clinic of Surgery and Neurology, Ophthalmological Department, Oslo University Hospital, Ullevål, 0424 Oslo, Norway[1]

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ABSTRACT

Insufficient vitamin D status has been linked to autoimmune diseases, cancer and metabolic disorders, like obesity and insulin resistance. In vitro and animal studies suggest that vitamin D may play a crucial role in immune activation and inflammation. The relation between vitamin D and pro-inflammatory cytokines is not completely established. Furthermore, it is not known if the effect of vitamin D on entities of metabolic syndrome is mediated through its effect on cytokines or other biomarkers. The objectives of this study were to investigate if there is a relationship between vitamin D status and such pro-inflammatory cytokines as tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6) and high sensitive C-reactive protein (hs-CRP) in patients with overweigh and obesity. We also proposed that the intervention with high dose of cholecalciferol may have effect on the cytokine levels and result in corresponding changes in the measures of insulin resistance (HOMA-IR and QUICKI). Serum levels of IL-6, TNF-α and hs-CRP were measured in 332 overweight and obese subjects who completed a 1-year randomised intervention with either 40,000 IU vitamin D (cholecalciferol) per week or 20,000 IU vitamin D per week, or placebo. We found significant associations between IL-6, TNF-α, vitamin D and insulin resistance indices at baseline. One year intervention with vitamin D decreased serum IL-6 levels; however hs-CRP levels were significantly increased. Neither measures of insulin resistance, nor TNF-α were influenced by a 1-year vitamin D supplementation.

1. Introduction

Vitamin D is a fat soluble vitamin and its main role is maintenance of mineral homeostasis and bone health [1]. Epidemiological studies have linked vitamin D insufficiency to increased risk for cardiovascular disease, infections and even cancer [2–4]. Among other non-classical vitamin D effects, regulation of cell proliferation, innate and anti-bacterial immune responses and hormone control can be mentioned [5,6]. Patients with systemic connective tissue diseases like rheumatoid arthritis and systemic lupus erythematosus [7] are reported to have lower serum 25-hydroxyvitamin D (25(OH)D) levels, which is the metabolite used to evaluate a subject's vitamin D status [8] when compared to healthy individuals. In addition, both adult and pediatric patients with inflammatory bowel diseases (IBD) like Crohns' disease [9] and ulcerative colitis [10], which also have altered innate immunity, demonstrate the same pattern. It has been also suggested that decreased cutaneous synthesis of vitamin D due to low sun exposure might be an important contributing factor in the development of immunological alterations in these diseases [11,12].

The vitamin D receptor (VDR) has been found in human immune cells [13,14]. VDR binding activity was found elevated around the genes that were associated with a chain of immunological disturbances, inflammation and diseases like Crohns' disease, type I diabetes, multiple sclerosis, and chronic lymphocytic leukaemia [15]. In vitro, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) inhibits differentiation of dendritic cells and decreases T cell activation by augmenting the synthesis of interleukin 10 (IL-10) and inhibiting the IL-12 [16]. Moreover, in animal studies calcitriol inhibits the production of such cytokines as IL-17A and IL-17B, which are the markers of T helper cell 17 (TH17) function, linked to infections and some severe autoimmune diseases [17].

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic inflammatory cytokine [18] and is largely involved in regulation of immune system with several beneficial as well as pathological functions of both inflammatory and non-inflammatory origin [19,20]. As other

* Corresponding author at: Department of Gastroenterology, Medical Clinic, University Hospital of North Norway, 9038 Tromsø, Norway. Tel.: +47 45426488; fax: +47 77628060.
E-mail address: julia.beilfuss@unn.no (J. Beilfuss).
[1] Present address.

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acute-phase cytokines it initiates a cascade of immune cellular responses usually in interaction with IL-6 [21]. Interleukin 6 is also an important acute phase mediator with both pro-inflammatory and anti-inflammatory properties [22]. In addition, these cytokines have been linked to insulin resistance and obesity; and weight loss interventions in obese subjects have resulted in amelioration of IL-6 and TNF-α levels as well as the corresponding improvement of the metabolic profile was observed [23].

Vitamin D has also been linked to some pro-inflammatory conditions like metabolic syndrome [24] and atherosclerosis [25]. Low serum 25(OH)D levels are associated with insulin resistance and overweight [26,27]. Considering the strong association between vitamin D and the immune system, the relationship between vitamin D and metabolic syndrome could possibly be mediated through an effect of vitamin D on cytokines like IL-6 and TNF-α. To test this hypothesis we have measured these cytokines in sera from overweight and obese subjects who have participated in a 1-year intervention study with high dose vitamin D supplementation versus placebo, and also related cytokines to hallmarks of insulin resistance.

2. Materials and methods

2.1. Study design and subjects

The present work is the ancillary study to the 1-year intervention trial with high dose vitamin D supplementation versus placebo and was performed as previously described in detail [28]. In short, males and females 21–70 years old, with body mass index (BMI) between 28.0 and 47.0 kg/m^2, and without concomitant diseases, were recruited by advertisements in the local newspapers and from the Out-patient Department, University Hospital of North Norway, Tromsø. Subjects with serum calcium >2.55 mmol/L, males with serum creatinine >129 μmol/L and females with serum creatinine >104 μmol/L were excluded. If serum calcium was in the range 2.50–2.55 mmol/L, inclusion required a serum parathyroid hormone (PTH) below 5.0 pmol/L. In addition, since the primary focus of the study was weight loss, those with the weight loss of more than 10% of total body weight during last 6 months, those using weight reducing drugs were excluded.

Included subjects had to withdraw any current supplements with calcium and vitamin D (including cod liver oil), and instead they were all given supplementation with calcium 500 mg daily (Nycoplus Calcium, Nycomed, Norway) throughout the 12 months intervention period. The subjects were randomized into three groups: group DD were given two capsules of vitamin D (20,000 IU cholecalciferol per capsule) per week; group DP were given one capsule of vitamin D and one placebo capsule per week; and a PP group were given two placebo capsules per week. The subjects came to visits every third month for new supply and return of unused medication. At baseline and after 12 months fasting blood samples were drawn.

2.2. Measurements

Height, weight, plasma glucose, serum PTH, insulin and high sensitivity C-reactive protein (hs-CRP) were measured as previously described [28]. Serum levels of 25(OH)D were measured by radioimmunoassay (DiaSorin, Stillwater, MN, USA) with reference range 37–131 nmol/L. According to the manufacturer, this assay measures both 25(OH)D_2 and 25(OH)D_3, and the intra- and total assay coefficients (CVs) of analytical variation are 6% and 14%, respectively. Total assay variation includes intra- and inter-assay coefficient of variation. Inter-assay CV% = square root (total CV%^{2} – intra-assay CV%^{2}). The lower limit of detection was 4 nmol/L. Serum levels of IL-6 and TNF-α were measured using high sensitivity ELISAs (Cat. No. SS600B/IL-6 and SSTA00D/TNF-α, R&D systems, Abingdon, UK). The samples were analyzed in duplicates and read on a Multiscan EX (Thermo electron corporation, Vantaa, Finland) according to the instructions from the manufacturer. The intra- and total assay CVs are, according to the manufacturer, 7.4% and 7.8% for IL-6, and 5.3% and 8.4% for TNF-α. In addition the total CV was assessed by running one sample of known concentration in duplicates, together with the patient samples, in eighteen separate assays. The total CV was 11.3% for IL-6 and 10.2% for TNF-α. According to manufacturer the minimum detectable dose was 0.039 pg/ml and 0.106 pg/ml for IL-6 and TNF-α, respectively.

Estimates of insulin sensitivity in the fasting state were calculated with homeostasis model assessment (HOMA-IR) [(insulin (pmol/L) × glucose (mmol/L)/135] and the Quantitative Insulin Sensitivity Check Index (QUICKI) [1/(log(insulin, mU/mL) + log(glucose, mg/dL)]. To convert insulin and glucose from the SI units to conventional units, the values were divided by the coefficients 6.945 and 0.0555, respectively.

2.3. Statistical analysis

The distribution of each variable was determined by measuring skewness and kurtosis, and the values for skewness between −1 and 1 and for kurtosis between −2 and 2 were accepted for Gaussian distribution. Where skewness and kurtosis were unacceptable, three transformation methods (square root, logarithmic and negative reciprocal) were applied. The method which resulted in less skewness was used in the final transformation of each skewed variable. Since some variables contained several zero-values (“undetectable”), the data are presented as median with range in parenthesis, unless otherwise stated. Comparisons between groups and correlations were performed with the use of ANOVA and Pearson’s coefficient. A linear regression model was used to reveal possible predictors of insulin sensitivity parameters, with HOMA-IR, QUICKI and fasting insulin as the dependent variables and age, BMI, cytokines, 25(OH)D and PTH as independent variables, adjusted for gender and smoking status.

All tests were done two-sided, and a P-value <0.05 was considered statistically significant. The data were analyzed with IBM® SPSS® Statistics Version 19 (SPSS Inc., Chicago, IL, USA).

2.4. Ethics

The study was approved by the regional Ethics Committee. All subjects gave written informed consent before participating in the study. The trial was registered at ClinicalTrials.gov (NCT00243256).

3. Results

A total of 445 subjects met the inclusion criteria, and of these 332 fulfilled the intervention and had complete datasets, including IL-6 and TNF-α. The variables IL-6, TNF-α and hs-CRP were severely skewed, and before their inclusion in the statistic analysis the logarithmic, negative reciprocal and square root transformations were applied, respectively.

The baseline characteristics of the study population are shown in Table 1. Of the 62 smokers, 15 quit smoking during the study. The compliance rate for vitamin D/placebo capsules was 95% in all three groups, and for the calcium tablets 81%, 85% and 83% in the DD, DP and PP groups respectively. At baseline there was a significant and strong positive association between cytokines and metabolic syndrome parameters, but no significant correlations were found between cytokines and vitamin D status (Table 2).

Table 1
Baseline characteristics of the study population.

Variables	Values
Males/females	128/204
Smokers, %	18.7
Age, years	50 (23–70)
BMI, kg/m^2	33.9 (28.6–47.1)
Serum 25(OH)D, nmol/L	57.3 (15.4–111.5)
Serum PTH, pmol/L	5.0 (2.3–13.8)
Hs-CRP, mg/L	2.46 (0.2–66.0)
TNF-α, pg/ml	1.53 (0.5–29.6)
IL-6, pg/ml	1.14 (0.3–13.1)
Insulin, pmol/L	101 (21–422)
HOMA-IR	3.91 (0.8–17.4)
QUICKI	0.32 (0.26–0.41)

Table 2
Pearson's correlation coefficients r between 25(OH)D, cytokines and parameters of insulin sensitivity at baseline in the 332 subjects.

Variables	Insulin	HOMA-IR	QUICKI	25(OH)D
Age	0.09	0.14**	−0.15**	0.20***
BMI	0.40***	0.40***	−0.40***	−0.22***
25(OH)D	−0.15**	−0.14*	0.13*	NA
Hs-CRP	0.19***	0.19***	−0.19***	−0.04
TNF-α	0.15**	0.17**	−0.15**	0.06
IL-6	0.12*	0.13*	−0.10	−0.03
PTH	0.08	0.08	−0.11*	−0.33***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

In the linear regression model BMI and age were as expected the strongest predictors of the parameters of insulin sensitivity (Table 3). In addition, TNF-α was found to be a significant predictor of HOMA-IR, QUICKI and fasting insulin, whereas for IL-6 and hs-CRP the association was no longer significant after adjustment for BMI and smoking status.

Table 3
Standardized coefficients β from the linear regression model, adjusted for gender and smoking status in the 332 subjects at baseline.

Variables	HOMA-IR	QUICKI	Insulin	IL-6	TNF-α
Age	0.19***	−0.2***	0.13**	0.09	0.03
BMI	0.37***	−0.37***	0.37***	0.08	0.06
25(OH)D	−0.10*	0.10	−0.10*	−0.09	0.10
Hs-CRP	0.05	−0.07	0.04	0.12*	−0.03
TNF-α	0.14**	−0.13*	0.13*	0.24***	NA
IL-6	0.02	0.03	0.01	NA	0.25***
PTH	−0.03	−0.00	−0.02	−0.02	0.02

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Before the intervention, there were no significant differences between the subjects in the DD, DP and PP groups; neither there were significant differences when combining DD and DP groups in one group (Table 4). After the 12 months intervention there was a significant increase in 25(OH)D in the DD and DP groups as expected, with median and range serum levels of 138 (47–193) nmol/L, 97 (51–155) nmol/L, respectively. Whereas, the serum levels of 25(OH)D in the PP group after the 12 months intervention were similar to baseline (Table 4). Apart from a significant decrease in serum PTH in the first two groups, there were no significant differences in delta values (value at 12 months minus value at baseline) between the groups regarding cytokines and hallmarks of insulin resistance (data not shown). However, when combining the DD and DP groups to one vitamin D group, the latter had a pronounced, but not statistically significant ($P = 0.08$) decrease in IL-6 and an elevation of hs-CRP at the end of the study when compared to the placebo group (Table 4).

3.1. Adverse events

There were no significant differences between the treatment groups regarding adverse events [28]. Moreover, weekly supplementation with 20,000–40,000 IU cholecalciferol in our study was associated with low risk of adverse effects. However, the community of Tromsø in North Norway is located at latitude 70° North and the same findings of insignificant rate of adverse effects may not apply to the populations living in the southern latitudes.

4. Discussion

In the present study we have found a strong positive association between levels of the TNF-α and insulin resistance, and a negative association between serum 25(OH)D and insulin resistance. At baseline there were no significant associations between serum 25(OH)D and the cytokines, whereas supplementation with vitamin D appeared to lower the serum IL-6 levels and increase the hs-CRP levels. However, no corresponding effect on insulin resistance after vitamin D supplementation was seen.

There are a number of indications from in vitro studies for an association between cytokines and glucose metabolism. Thus, IL-6 is produced not only by T lymphocytes but also by adipocytes [29]. Furthermore, IL-6 is demonstrated in white fat tissue in high concentrations and IL-6 inhibits adiponectin gene expression in cultured adipocytes [30]. Adiponectin has pronounced anti-atherogenic and anti-inflammatory properties [31]. It is also considered to have beneficial effects on glucose metabolism and lower levels

Table 4
Effects of vitamin D supplementation on cytokines and insulin sensitivity parameters in the combined vitamin D group (DD + DP) and the placebo group (PP).

	Baseline values		12 months values		Delta values	
	DD + DP	PP	DD + DP	PP	DD + DP	PP
Number	220	112	220	112	220	112
BMI, kg/m^2	33.5 (28.7–46.1)	34.7 (28.6–47.1)	34.0 (27.0–45.6)	34.6 (27.4–46.9)	0.09 (−4.8 to 2.9)	0.23 (−5.75 to 3.88)
25(OH)D, nmol/L	54.3 (15.4–111.5)	52.4 (18.5–99.4)	99.0** (46.7–193.4)	50.0 (20.3–99.8)	57.0** (−5.1 to 59.3)	−0.12 (−45.6 to 43.6)
PTH, pmol/L	5.0 (2.3–13.8)	5.3 (2.3–11.0)	4.2** (1.5–12.8)	5.2 (1.7–10.8)	−0.7** (−5.7 to 4.1)	−0.2 (−4.80 to 4.40)
Hs-CRP, mg/L	2.42 (0.20–33.10)	2.49 (0.17–19.34)	2.62 (0.32–65.96)	2.57 (0.15–48.92)	0.11* (−9.91 to 59.28)	−0.08 (−18.89 to 37.04)
TNF-α, pg/ml	1.53 (0.51–29.60)	1.54 (0.59–9.28)	1.45 (0.38–33.05)	1.40 (0.49–3.85)	−0.1 (−9.98 to 27.85)	−0.13 (−5.43 to 2.23)
IL-6, pg/ml	1.13 (0.28–13.07)	1.20 (0.25–11.50)	1.21 (0.19–12.01)	1.23 (0.37–13.01)	−0.14 (−4.98 to 27.85)	0.02 (−8.08 to 7.72)
HOMA-IR	3.74 (0.8–17.4)	4.10 (1.19–16.76)	3.48 (0.54–42.98)	4.12 (1.16–31.38)	−0.30 (−10.0 to 39.3)	−0.15 (−7.3 to 19.5)
QUICKI	0.32 (0.26–0.41)	0.32 (0.27–0.38)	0.32 (0.25–0.40)	0.32 (0.25–0.41)	0.00 (−0.00 to 0.02)	0.00 (−0.00 to 0.01)

* $P < 0.05$ versus placebo group.
** $P < 0.001$ versus placebo group.

of adiponectin are associated with insulin resistance and metabolic syndrome [31]. Deranged levels of IL-6 are contributing to endothelial dysfunction, thus playing a predictive role for diabetes [32], future myocardial infarction [33], and are also associated with cardiovascular mortality [34]. Similar properties are described for TNF-α which has even been called an "adipokine" [35]. In addition to possible production of cytokines in the adipocytes, there is also an infiltration of adipose tissue with inflammatory cells like macrophages [36].

However, when levels of cytokines and measures of metabolic syndrome and insulin resistance have been evaluated in clinical studies, the results are conflicting [37–40]. Thus, in the large epidemiological study by Marques-Vidal et al. [39], subjects with diabetes and insulin resistance demonstrated elevated levels of several pro-inflammatory cytokines, including Il-6, TNF-α and hs-CRP, however, no significant associations were found between the cytokines and impaired glucose tolerance, based on the oral glucose tolerance testing. In a study by Abbatecola et al. [37] TNF-α was increased in patients within the highest tertile of insulin resistance, however in the linear regression analysis no significant predictive associations between TNF-α and either insulin resistance or metabolic syndrome were found. On the other hand, in that study the average age was 68 years and the average BMI 27 kg/m^2; whereas in our study the subjects were younger and also heavier.

Based on the demonstration that VDR is present in immunocompetent cells [13,14], and the influence of 1,25$(OH)_2$D on a number of cytokines in vitro [17,23]; a relation between vitamin D status and pro-inflammatory cytokines was proposed [41]. Human studies regarding the relation between vitamin D status and cytokines in vivo demonstrate inconsistent results. Opposite to our findings, Peterson and Heffernan revealed a significant negative association between TNF-α and 25(OH)D in 69 healthy women [41] even when corrected for body fat mass, menopausal age and other potential confounders. Interestingly, several researchers report significant favorable effect of vitamin D supplementation on pro-inflammatory cytokines like TNF-α, IL-6 and IL-10, but only in strictly selected groups of patients like type II diabetes patients [40], infants with congestive heart failure [42], patients with end stage kidney disease on hemodialysis [43] and patients with colorectal neoplasms [44]. Furthermore, dermatological studies where UV irradiation as well as topical treatment with vitamin D analogues has been used, indicate an effect by vitamin D on cytokines. Thus, a significant decline in epidermal IL-6 was demonstrated after topical treatment with vitamin D analogue of both healthy and affected skin in patients with psoriasis [45]. Our finding of lowering effects of vitamin D on IL-6 is therefore consistent with other studies. Regarding the lack of association between 25(OH)D and TNF-α, and no effect of vitamin D supplementation on TNF-α in our study, there are a number of plausible explanations. First of all, overweight and obesity are pro-inflammatory conditions; however, the immune system is not stimulated as in patients with IBD, multiple sclerosis [9–11] and chronic hepatitis C [46], where the vitamin D effects are more prominent. In addition, most of our study population had sufficient vitamin D levels at baseline, and it is reasonable to believe that vitamin D supplementation would have a more pronounced effect in subjects with vitamin D deficiency. Despite our finding of 1-year vitamin D supplementation decreases IL-6, we could not demonstrate any beneficial effect on insulin resistance. This may indicate that IL-6 involvement in glucose metabolism is clinically less significant. It is also consistent with the lack of associations between IL-6 and measures of insulin resistance in the linear regression model.

The present study has several short-comings. Since the study was originally designed to elucidate potential weight loss after vitamin D supplementation, only subjects with BMI > 28 kg/m^2 were included. Our results may therefore not apply to slimmer population. Furthermore, we used BMI as a measure of overweight and obesity, and not the direct assessment of body fat. Slight overestimate of overweight is therefore possible. Furthermore, we have only measurements at baseline and after 12 months at the end of the study, and transient effects may have been missed. The present study is the ancillary study to the intervention trial, where other cytokines with subtle clinical value were measured; however we measured TNF-α and IL-6, cytokines with the broad involvement in several autoimmune diseases and targets for the novel biologic drugs [47]. Finally, all study groups received calcium and we did not have a group receiving placebo for both vitamin D and calcium. However, we did include a large group of subjects, and our findings are confirmative to previous epidemiological studies.

In conclusion, our data is in consistency with the other recent studies on the relationship between increased cytokine levels, deranged vitamin D status and insulin resistance. Vitamin D supplementation over a 12 months period had a lowering effect on IL-6; however, the clinical implications are most likely small since potentially favorable effect of vitamin D had no corresponding impact on insulin sensitivity. However, our results should be treated with caution because of some limitations listed above. Further interventional studies on selected groups, like vitamin D deficient population and/or subjects with immunological disturbances, are needed.

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