Cytokine 50 (2010) 175–180

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

No effect of supplementation with cholecalciferol on cytokines and markers of inflammation in overweight and obese subjects

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ARTICLE INFO

Article history:
Received 12 September 2009
Received in revised form 29 November 2009
Accepted 12 December 2009

Keywords:
Autoimmune diseases
Cytokines
Interleukins
Vitamin D

ABSTRACT

Epidemiological studies indicate a relation between vitamin D status and autoimmune diseases, and in vitro studies demonstrate an effect of 1,25-dihydroxyvitamin D on immune activation. However, the relation between serum levels of 25-hydroxyvitamin D (25(OH)D) and the effect of vitamin D supplementation on serum levels of cytokines are not settled. In the present study interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, intercellular adhesion molecule-1, interferon-γ, monocyte chemotactic protein-1, and high sensitivity C-reactive protein, were measured in 437 overweight subjects and 324 completed a one year intervention with 40,000 IU vitamin D per week (group DD), 20,000 IU vitamin D per week (group DP), or placebo (group PP). No consistent relations between serum levels of the cytokines and 25(OH)D were found at baseline. In the intervention study, there was no difference in delta values (value at end of study minus value at inclusion) between the three groups regarding the individual cytokines measured, nor was there any indication of a polarization of the T cells towards a Th2 dominant type. In conclusion, we were not able to demonstrate with certainty any significant relation between serum levels of 25(OH)D levels and a number of cytokines and markers of inflammation.

1. Introduction

Vitamin D is of vital importance for bone health and also appears to have extra-skeletal effects [1]. In epidemiological studies low serum levels of 25-hydroxyvitamin D (25(OH)D), the storage form of the vitamin and the one used to evaluate a subject's vitamin D status, have been associated with cardiovascular diseases, cancer and autoimmune disorders [2]. Inflammatory bowel disease (IBD) [3] and multiple sclerosis (MS) [4] are both more prevalent in areas on higher latitudes (i.e. areas with less sunlight), and serum levels of 25(OH)D in subjects with IBD [5], MS [6], systemic lupus erythematosus (SLE) [7] and rheumatoid arthritis (RA) [8] are reported to be lower than in healthy controls.

These relations between vitamin D and autoimmunity were further substantiated when the vitamin D receptor (VDR) was found on human leukocytes [9], the antigen presenting dendritic cells [10] and also in $CD4^+$ T lymphocytes [11]. It was consequently shown in vitro that 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which is the active form of vitamin D, inhibits differentiation and maturation of the dendritic cells, decrease the production of interleukin (IL)-12 (IL-12), increase the production of IL-10, all of which results in decreased T cell activation [12]. This inhibition is particularly seen for the helper T cell 1 (Th1) [13] with subsequent decreased production of interferon-γ (IFN-γ) and IL-2 [14]. In the helper T cell 2 (Th2), vitamin D appears to increase the production of IL-4 and decrease the production of IL-5 [14]. Accordingly, vitamin D may have the potential to affect the immune system, in particular to suppress the Th1 response, and could be of importance in Th1 driven autoimmune diseases like MS, IBD, and type 1 diabetes mellitus (T1DM). Also, a recently discovered T helper cell type – the Th17 cell – plays a major role in immune response to some infections as well as several autoimmune diseases [15]. The IL17A and IL17F are the hallmark cytokines produced by these cells upon activation. Recent animal experiments demonstrate an inhibitory effect of calcitriol on Th17 functions [16,17].

This potential effect of vitamin D has been demonstrated in experimental autoimmunity, where vitamin D deficiency accelerates the development of experimental MS [18], IBD [19], and T1DM [20], whereas vitamin D supplementation halts the progression of disease in these models. It has also been shown in vivo that

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doi:10.1016/j.cyto.2009.12.006

supplementation with vitamin D decrease the risk of developing T1DM [21].

From these observations one would therefore, expect that supplementation with vitamin D in vivo would have an immunoregulatory effect by inhibiting dendritic cells and polarize T cells towards a more Th2 dominant type as well as suppress differentiation to Th17 cells and decrease their activity. This should be reflected in the pattern of serum cytokines and other markers of immune activation and inflammation. We had the opportunity to address this issue as we have recently performed a one year intervention study with vitamin D versus placebo in 437 overweight and obese subjects with weight loss as the primary endpoint [22], where cytokines and markers of inflammation were also measured.

2. Materials and methods

2.1. Subjects

Males and females 21–70 years old, with body mass index (BMI) between 28.0 and 47.0 kg/m^2, were recruited by advertisements in local newspapers and from our out-patient clinic at the University Hospital of North Norway, Tromsø. Subjects with a history of coronary infarction, angina pectoris, stroke, sarcoidosis or renal stone disease were excluded. Subjects using weight reducing drugs, or anti-depressive medication, pregnant or lactating women, and women below the age of 50 years without adequate contraception were not included. Subjects with serum calcium >2.55 mmol/L, males with serum creatinine >129 μmol/L and females with serum creatinine >104 μmol/L, were not included. If serum calcium was in the range 2.50–2.55 mmol/L, inclusion required a serum PTH below 5.0 pmol/L.

2.2. Study design

At inclusion, any current supplements with calcium and vitamin D (including cod liver oil) were discontinued, and all subjects were given supplementation with calcium 500 mg daily (Nycoplus Calcium®, Nycomed, Norway) throughout the one year intervention period. The subjects were randomized into three groups, stratified by gender and smoking status: group DD two capsules of vitamin D (20,000 IU cholecalciferol (vitamin D_3) per capsule (Decristol®, Jenapharm, Jena, Germany)) per week; group DP one capsule of vitamin D and one placebo capsule per week; and group PP two placebo capsules per week. All batches used in the present study were analyzed for content of vitamin D at the National Institute for Nutrition and Seafood Research, Bergen, Norway, and found to contain a median amount of 22,000 IU, range 21,600–22,800 IU. The placebo capsules, purchased from Hasco-lek, Wroclaw, Poland, had identical appearance as the vitamin D capsules. The subjects were supplied with new medication every third month. Unused calcium tablets and capsules were returned and counted. The subjects were classified as current smokers or current non-smokers.

2.3. Measurements

Height and weight were measured wearing light clothing and no shoes. BMI was calculated as weight (kg) divided by squared height (m^2). Total body fat was measured with dual-energy X-ray absorptiometry (DEXA) according to the manufacturer (GE Lunar Prodigy, LUNAR Corporation, Madison, WI, USA). The percentage total body fat was calculated by dividing the total fat mass by body weight and multiplying with hundred.

Blood samples for serum calcium were drawn every third month to detect any development of hypercalcaemia. If serum calcium increased >2.59 mmol/L, the subjects were asked to re-test, and if still >2.59 mmol/L, they were excluded from the study.

Serum calcium, creatinine and PTH were measured as previously described [23]. Reference ranges in our laboratory at the time of the study were for serum calcium 2.20–2.60 mmol/L; for serum PTH 1.1–6.8 pmol/L for those <50 years, and 1.1–7.5 pmol/L for those >50 years; for serum creatinine 70–120 μmol/L for men and 55–100 μmol/L for women.

Serum levels of 25-hydroxyvitamin D (25(OH)D) were measured by radioimmunoassay (DiaSorin, Stillwater, MN, USA) with reference range 37–131 nmol/L. This assay measures both 25(OH)D_3 and 25(OH)D_2, and the intra- and total assay coefficients of variation (CVs) are 6% and 14%, respectively [24].

For measuring IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IFN-γ, IL-17 (IL-17A), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1), the Th1/Th2–7 Ultra-Sensitive kit, IL-17 Ultra-Sensitive kit, ICAM-1 kit, and MCP-1 Ultra-Sensitive kit (Meso Scale Discovery, Gaithersburg, MD) were used and run on a Sector Imager 2400 (Meso Scale Discovery, Gaithersburg, MD). The analyses were performed according to the manufacturer's recommendations. High sensitivity C-reactive protein (HS-CRP) was assessed by an immunoturbidimetric assay (Roche Diagnostic®, Mannheim, Germany) using an automated clinical chemistry analyzer (Modular P, Roche Diagnostics®). All blood samples used in the present study were drawn in the fasting state at baseline and at the end of the study.

The trial was registered at ClinicalTrials.gov (NCT00243256).

2.4. Statistical analyses

A "composite" Th1 score was made by adding together the rank numbers for IL-2 and IFN-γ, and a "composite" Th2 score was made by adding together the rank numbers of IL-4, IL-5, IL-10, and IL-13. Of the main dependent variables IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, ICAM-1, IFN-γ, MCP-1, HS-CRP, and Th1 and Th2 scores, only ICAM-1 and the Th1 and Th2 scores were normally distributed. Furthermore, only IL-17, MCP-1 and HS-CRP were possible to log-transform, as the other variables contained several zero-values ("undetectable"). Therefore, the data are presented as median with range in parentheses, unless otherwise stated. Comparisons between groups and correlations were performed with non-parametric tests (the Mann–Whitney test and Spearman's rho coefficient). In addition, for comparing ICAM-1, IL-17, MCP-1, HS-CRP and the Th1 and Th2 scores between groups and for evaluating individual predictors of these four cytokines, a general linear model with ICAM-1, log IL-17, log MCP-1, log HS-CRP, Th1 and Th2 scores as dependent variables, gender and/or smoking status as factor(s), and age, BMI (or percentage total body fat), and 25(OH)D as covariates was also used.

In the intervention study, the data were analyzed with a per-protocol approach and comparisons were made for delta values (value at end of study minus value at baseline). The groups were also compared regarding delta Th1 and delta Th2 scores. The delta Th1 score was made by adding together the rank number for delta IL-2 and delta IFN-γ, and the delta Th2 score was made by adding together the rank numbers of delta IL-4, delta IL-5, delta IL-10, and delta IL-13.

All tests were done two-sided, but without a Bonferroni correction. A P-value <0.05 was considered statistically significant. The Statistical Package for Social Sciences version 14.0 was used for all statistical analyses (SPSS Inc., Chicago, Ill., USA).

2.5. Ethics

The study was approved by the Regional Ethics Committee. All participants gave written informed consent prior to the study.

3. Results

3.1. Cross-sectional study

A total of 496 subjects were screened, 445 met the inclusion criteria, and among these 437 had complete datasets. The inclusion period started in November 2005 and the last person was included in October 2006. Their baseline values are shown in Table 1. About 22.4% of the subjects were smokers, and smokers had significantly lower IL-2, IL-5, IL-17, and MCP-1 levels than the non-smokers. These differences were seen in both genders (data not shown). In the general linear model with log-transformed IL-17 and MCP-1 as dependent variables the statistically significant difference between smokers and non-smokers remained ($P < 0.05$), and there was also a highly significant interaction between gender and smoking status ($P < 0.001$). In addition to these differences in cytokine levels, the smokers were significantly younger and had lower serum 25(OH)D levels than non-smokers. Therefore, to avoid a confounding effect of smoking, the following cross-sectional analyses were done in non-smokers only.

Males and females had similar age, BMI, and serum 25(OH)D levels, whereas the percentage of total body fat was significantly higher in the females (46.0 (31.4–55.6)% versus 33.9 (21.4–45.5)%, (median (range)), $P < 0.001$). When using the Mann–Whitney test, the females had significantly higher serum IL-4, IL-10, IL-12, IL-13, IL-17, IFN-γ, MCP-1, ICAM-1, and HS-CRP values and Th1 and Th2 scores than the males (Table 1). These differences were also statistically significant for log IL-17, log MCP-1, ICAM-1, log HS-CRP, and Th1 and Th2 scores in the general linear model with age, BMI and 25(OH)D as covariates. However, when adjusting for percentage total body fat instead of BMI, only the difference in log HS-CRP, Th1 and Th2 scores remained statistically significant ($P < 0.05$).

In the correlation analyses, serum 25(OH)D was significantly and negatively associated with serum MCP-1 in the males, and positively associated in the females. In the females, serum 25(OH)D was significantly and negatively associated with IL-5, IL-10 and the Th2 score (Table 2). Regarding the association between 25(OH)D and MCP-1, this was still statistically significant in the linear regression model in both genders with BMI as well as percentage total body fat as covariates ($P < 0.05$). However, the relation between serum 25(OH)D and Th2 in the females did not remain significant in the regression model.

3.2. Intervention study

Three hundred and thirty-four subjects completed the intervention study, and 324 had complete datasets regarding the cytokines. Among these subjects, 15 smokers quitted smoking during the study and two non-smokers started smoking, leaving 307 subjects (47 smokers) with unchanged smoking status for analyses in the intervention study. The compliance rate for vitamin D/placebo capsules were 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 were no significant differences between the subjects in the DD, DP, and PP groups (data not shown). At the end of the study the median and range serum 25(OH)D in the DD, DP, and PP groups were 141 (40–231) nmol/L, 98 (67–176) nmol/L, and 57 (21–111) nmol/L, respectively. Serum PTH decreased significantly in the DD and DP groups (Table 3), whereas serum calcium was unchanged in all three groups. Regarding delta values for BMI and the cytokines, there were no statistically significant differences between the DD, DP and PP groups (Table 3), nor when comparing the combined vitamin D groups (DD + DP groups, $n = 202$) versus the PP group ($n = 105$), or when looking separately at those with serum 25(OH)D levels < 50 nmol/L at baseline (69 in the combined DD + DP group, 34 in the PP group) (data not shown). The composite delta Th1 and Th2 scores did not differ between the three groups, nor when comparing the combined vitamin D group (DD + DP) versus the placebo group (PP). Thus, for delta Th1 the mean ranks in the combined vitamin D group (DD + DP) and the PP group were 153.8 and 154.1, respectively; and for delta Th2 the mean ranks were 151.6 and 155.3, respectively.

Table 1
Baseline values in relation to smoking status and gender (median and range).

	All subjects	Non-smokers	Smokers	Non-smokers	
				Males	Females
Males/females	156/281	123/216	33/65	123	216
Age (years)	47.0 (21.0–70.0)	50.0 (21.0–70.0)	42.0 (23.0–68.0)***	48.0 (26.0–70.0)	50.5 (21.0–70.0)
BMI (kg/m^2)	34.3 (28.4–47.1)	34.0 (28.4–47.1)	34.9 (28.7–44.3)	33.8 (28.6–45.0)	34.2 (28.4–47.1)
Body fat (%)	43.2 (21.4–55.6)	42.9 (21.4–55.6)	43.8 (25.5–54.7)	33.9 (21.4–45.5)	46.0 (31.4–55.6)[c]
Serum 25(OH)D (nmol/L)	56 (16–136)	58 (16–136)	47.5 (17–93)***	57 (16–123)	59 (17–136)
Serum PTH (pmol/L)	5.0 (1.9–16.1)	5.0 (1.9–13.8)	4.8 (2.3–16.1)	5.2 (1.9–10.0)	5.0 (2.10–13.8)
Serum calcium (mmol/L)	2.31 (2.00–2.55)	2.31 (2.02–2.55)	2.32 (2.00–2.55)	2.31 (2.10–2.54)	2.31 (2.02–2.55)
Serum IL-2 (pg/ml)	0.0 (0.0–29.5)	0.0 (0.0–29.5)	0.0 (0.0–3.4)**	0.0 (0.0–8.8)	0.0 (0.0–29.5)
Serum IL-4 (pg/ml)	0.0 (0.0–4.3)	0.0 (0.0–4.3)	0.0 (0.0–2.0)	0.0 (0.0–0.5)	0.0 (0.0–4.3)[c]
Serum IL-5 (pg/ml)	0.3 (0.0–286.1)	0.3 (0.0–286.1)	0.3 (0.0–77.8)*	0.3 (0.0–19.6)	0.3 (0.0–286.1)
Serum IL-10 (pg/ml)	0.5 (0.0–3349.4)	0.5 (0.0–3349.4)	0.5 (0.0–1503.3)	0.0 (0.0–73.1)	0.7 (0.0–3349.4)[c]
Serum IL-12 (pg/ml)	0.0 (0.0–6283.9)	0.0 (0.0–2099.7)	0.0(0.0–6283.9)	0.0 (0.0–54.7)	0.0 (0.0–2099.7)[b]
Serum IL-13 (pg/ml)	0.0 (0.0–1897.2)	0.0 (0.0–863.0)	0.0 (0.0–1897.2)	0.0 (0.0–73.4)	0.0 (0.0–863.0)[a]
Serum IL-17 (pg/ml)	0.4 (0.0–10.0)	0.4 (0.0–10.0)	0.3 (0.0–4.1)***	0.3 (0.0–2.7)	0.5 (0.0–10.0)[c]
Serum IFN-γ (pg/ml)	0.5 (0.0–43.2)	0.4 (0.0–43.2)	0.6 (0.0–5.3)	0.2 (0.0–42.9)	0.8 (0.0–43.2)[c]
Serum MCP-1 (pg/ml)	602 (209–3177)	620 (209–3178)	551 (285–2590)**	587 (263–2387)	646 (209–3178)[b]
Serum ICAM-1 (ng/ml)	0.7 (0.0–5.4)	0.7 (0.0–5.4)	0.7 (0.0–1.6)	0.6 (0.0–5.4)	0.8 (0.0–1.8)[c]
Serum HS-CRP (mg/L)	2.5 (0.2–46.3)	2.4 (0.2–33.1)	3.0 (0.2–46.3)	1.8 (0.4–26.3)	3.0 (0.2–33.1)[b]
Th1 score (ranksum)	118 (2–376)	108 (2–376)	129 (2–349)	57 (2–376)	175 (2–374)[c]
Th2 score (ranksum)	257 (4–796)	252 (4–792)	269 (4–796)	156 (4–708)	280 (4–792)[c]

* $P < 0.05$ versus non-smokers (Mann–Whitney test).
** $P < 0.01$ versus non-smokers (Mann–Whitney test).
*** $P < 0.001$ versus non-smokers (Mann–Whitney test).
[a] $P < 0.05$ versus males (Mann–Whitney test).
[b] $P < 0.01$ versus males (Mann–Whitney test).
[c] $P < 0.001$ versus males (Mann–Whitney test).

Table 2
Spearman's rho correlation coefficients for serum 25(OH)D levels versus age, BMI, percentage total body fat, serum PTH and calcium, and cytokines at baseline in non-smoking males (n = 123) and females (n = 216).

	Males	Females
Age (years)	0.35***	0.28***
BMI (kg/m^2)	−0.20*	−0.11
Total body fat (%)	−0.26**	−0.06
Serum PTH (pmol/L)	−0.19*	−0.39***
Serum calcium (mmol/L)	0.00	0.21**
Serum IL-2 (pg/ml)	−0.09	−0.01
Serum IL-4 (pg/ml)	−0.10	0.00
Serum IL-5 (pg/ml)	−0.10	−0.16*
Serum IL-10 (pg/ml)	0.00	−0.14*
Serum IL-12 (pg/ml)	−0.06	0.01
Serum IL-13 (pg/ml)	−0.12	0.10
Serum IL-17 (pg/ml)	−0.05	−0.07
Serum IFN-γ (pg/ml)	0.10	0.03
Serum MCP-1 (pg/ml)	−0.28**	0.20**
Serum ICAM-1 (ng/ml)	0.05	0.05
Serum HS-CRP (mg/L)	−0.01	0.08
Th1 score (ranksum)	0.10	0.03
Th2 score (ranksum)	−0.09	−0.18**

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

3.3. Adverse events and withdrawal from the study

Two subjects (one in the DP group and one in the PP group) were diagnosed as having primary hyperparathyroidism during the study, one subject in the DP group had an increase in serum calcium to 2.62 mmol/L and all three were excluded from the study. Four subjects (three in the DD group and one in the PP group) had transient increases in serum calcium >2.59 mmol/L and remained in the study. In addition, 317 other adverse events were recorded, most of them related to gastro-intestinal discomfort. There were no significant differences between the treatment groups regarding adverse events.

4. Discussion

Based on epidemiological studies [2–8], the demonstration of the VDR on immunocompetent cells [9–11] and influence of 1,25(OH)$_2$D on a number of cytokines in vitro [12–14], a relation between serum levels of 25(OH)D and cytokines in vivo was to be expected. However, in the present study including a large group of overweight and obese subjects we could not convincingly demonstrate any significant cross-sectional association between serum levels of 25(OH)D and a panel of cytokines and markers of inflammation. An exception was MCP-1, but for this cytokine there was a negative relation for the males and a positive relation for the females, which is hard to explain. There was no dominance of Th2 cytokines in the subjects with higher serum levels of 25(OH)D, and serum levels of IL-17 were also unaffected. Nor did a one year intervention with large doses of vitamin D have any effect on the levels of these cytokines. On the other hand, smoking appeared to lower the levels of some of the measured cytokines, and females had strikingly higher cytokine levels than males.

There are but a few studies regarding the serum levels of 25(OH)D in relation to cytokines in vivo, and some of the results are conflicting. Thus, in a study by Peterson et al. no relation between IL-10 and serum 25(OH)D was found [25], whereas Schleithoff et al. found that supplementation with vitamin D increased the serum level of IL-10 in subjects with congestive heart failure [26]. Similar to our study, the Framingham Offspring study which included 1381 subjects found no relation between serum 25(OH)D and ICAM-1 and CRP levels [27]. However, in 171 healthy British Bangladeshi subjects Timms et al. found a significant inverse relation between vitamin D status and CRP, and in those who had vitamin D deficiency CRP fell after vitamin D supplementation [28].

There could be a number of explanations for the lack of association between serum 25(OH)D levels and cytokines in our study. Firstly, the study included only overweight and obese subjects in good health. It is possible that the effect of vitamin D is more clearly seen when the immune system is stimulated, and had we included subjects with inflammatory or autoimmune diseases, the results might have been different. Furthermore, it is reasonable to assume that an effect of vitamin D supplementation would be most pronounced in those with vitamin D deficiency, and most of our subjects had normal 25(OH)D levels. Although we measured a number of cytokines, including IFN-γ (the signature molecule for the Th1 cells) [26], IL-4 (the defining Th2 cytokine) [29], MCP-1 (the main cytokine responsible for the recruitment of monocytes to sites of active inflammation), ICAM-1 (thought to be a key factor when circulating monocytes adhere to the endothelium and transmigrate into the intima) [30], and HS-CRP as a pro-inflammatory biomarker, there are a number of other important cytokines like IL-6 and tumour necrosis factor-alpha (TNF-α) not measured in our study. Regarding the effects of 1,25(OH)$_2$D on cytokines in in vitro studies, it is possible that they reflect pharmacological and not physiological effects. However, because of the local

Table 3
Gender, smoking status and delta (Δ) values (value at end of study minus value at baseline) in the subjects with unchanged smoking status in relation to treatment group.

	DD group	DP group	PP group
Males/females	41/63	36/62	37/68
Smokers/non-smokers	16/88	17/81	14/91
Δ BMI (kg/m^2)	0.1 (−4.8–2.7)	0.1 (−3.4–2.9)	0.2 (−5.8–3.9)
Δ Serum 25(OH)D (nmol/L)	77 (15–168)*	44 (5–102)*	0 (−46–44)
Δ Serum PTH (pmol/L)	−0.8 (−5.7–4.1)*	−0.6 (−4.8–3.0)*	−0.2 (−4.8–4.4)
Δ Serum calcium (mmol/L)	0.0 (−0.4–0.3)	0.0 (−0.2–0.3)	0.0 (−0.3–0.3)
Δ Serum IL-2 (pg/ml)	0.0 (−7.1–1.6)	0.0 (−4.1–39.7)	0.0 (−10.4–56.0)
Δ Serum IL-4 (pg/ml)	0.0 (−1.3–0.5)	0.0 (−2.7–1.0)	0.0 (−1.2–6.6)
Δ Serum IL-5 (pg/ml)	0.0 (−12.0–1.2)	0.0 (−214.6–9.7)	0.0 (−123.9–2.5)
Δ Serum IL-10 (pg/ml)	0.0 (−23.1–68.9)	0.0 (−19.4–27.4)	0.0 (−371.9–21.7)
Δ Serum IL-12 (pg/ml)	0.0 (−15.3–3.6)	0.0 (−7.0–90.8)	0.0 (−1466.2–139.5)
Δ Serum IL-13 (pg/ml)	0.0 (−14.6–5.6)	0.0 (−9.1–84.3)	0.0 (−391.9–108.5)
Δ Serum IL-17 (pg/ml)	0.0 (−2.4–4.4)	0.0 (−1.1–1.6)	0.0 (−9.2–4.7)
Δ Serum IFN-γ (pg/ml)	0.0 (−11.4–12.2)	0.0 (−39.5–14.0)	0.0 (−42.9–11.8)
Δ Serum MCP-1 (pg/ml)	14 (−556–288)	−15 (−829–1206)	3 (−1526–615)
Δ Serum ICAM-1 (ng/ml)	0.0 (−2.4–4.7)	0.0 (−1.0–1.0)	0.0 (−1.3–0.9)
Δ Serum HS-CRP (mg/L)	0.1 (−9.9–8.8)	0.1 (−4.3–59.3)	−0.1 (−18.9–37.0)

* $P < 0.01$; versus the PP group (Mann–Whitney test).

production $1,25(OH)_2D$ we do not know what can be considered as physiological levels in the tissues. Similarly, the cross-sectional studies showing an association between low serum levels of 25(OH)D and disease will always be hampered by the fact that those with diseases are less active and spend less time outdoor, and the low serum 25(OH)D levels may therefore, be a reflection of disease and not a cause. Accordingly, the question whether the vitamin D status is of importance for immunological diseases can only be resolved by large scale intervention studies.

It is well know that smoking is associated with autoimmune diseases, but apparently with both promoting and inhibiting effects. Thus, smoking appears to increase the risk of RA, SLE, Grave's disease and MS, whereas the effect on ulcerative colitis may be protective [31]. In our study the smokers had significantly lower serum levels of IL-2, IL-5, IL-17, and MCP-1, and smoking could therefore, have pro- as well as anti-inflammatory effects. These differences versus non-smokers could not be explained by gender, and for IL-17 and MCP-1 the differences remained statistically significant also after adjustment for age, BMI (or percentage total body fat) and serum 25(OH)D levels. However, other studies have found higher levels of IL-5, IL-10, and ICAM-1 in smokers than non-smokers [32–34], and the true effects of smoking on cytokine levels are therefore still to be determined.

Compared to males, females may have better humoral and cell-mediated immunity, but apparently also an exaggerated response to auto-antigens that renders them more susceptible to autoimmune diseases [35]. In our study the most striking difference seen regarding cytokines were the significantly higher levels in women for almost all cytokines measured. Part of this difference can probably be ascribed to differences in fat mass. The females had considerably higher percentage total body fat, and adipose tissue is an important source of cytokines [36]. Thus, for IL-17, MCP-1, ICAM-1, HS-CRP, Th1, and Th2 scores where use of a linear regression model was possible, the differences between men and women were no longer significant after adjustment for percentage total body fat, except for HS-CRP and the Th1 and Th2 scores.

The present study has several limitations. The primary endpoint was weight loss, and we included only subjects with BMI > 28 kg/m^2. Therefore our results do not necessarily apply to a slimmer population. In particular, the resulting serum 25(OH)D levels might have been higher, and possible side-effects more pronounced, in subjects with lower weight. The drop-out rate was 24.9%, and most of the subjects who dropped out of the study gave no reason for their withdrawal, hence it is possible that not all side-effects were recorded. However, the drop-out rates were similar in all three groups. For several of the cytokines included, the levels were below the detection limit of the assay, and subtle differences between the groups may therefore, have been missed. We did not have a group that was given placebo preparations for both calcium and vitamin D. Although we find it unlikely, we cannot exclude that calcium supplementation may have masked an effect of vitamin D on the cytokine levels. And finally, there are a number of cytokines not included in our study. In particular, TNF-α would have been of interest to measure as supplementation with vitamin D has been reported to affect the serum level of this cytokine in subjects with congestive heart failure [26].

In conclusion, we have found no consistent relation between circulating levels of 25(OH)D and cytokines, but an association between cytokine levels and both smoking status and gender. The importance of vitamin D in clinical immunology remains to be determined.

Acknowledgements

The study was supported by a grant from The Northern Norway Regional Health Authority. The superb assistance by the staff at the Clinical Research Unit and by Inger Myrnes and Astrid Lindvall at the Department of Medical Biochemistry, University Hospital of North Norway, is gratefully acknowledged, as well as the help from laboratory technician Tone Berit Heng at the Research Laboratory at Stavanger University Hospital. We are grateful for the generous supply of calcium tablets from Nycomed Norway.

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