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## Vitamin D3 treatment of Crohn's disease patients increases stimulated T cell IL-6 production and proliferation

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**Vitamin D3 treatment of Crohn's disease patients increases  
stimulated T cell IL-6 production and proliferation**

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3 Vitamin D3 treatment of Crohn's disease patients increases  
4 stimulated T cell IL-6 production and proliferation

Comment [MB1]: R1

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25 *Short title:* Vitamin D3 treatment in Crohn's disease and T cell function

Comment [MB2]: R1

26 *Keywords:* Crohn disease, vitamin D3, CD4 positive T lymphocytes, interleukin-6, cell

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29 proliferation

Comment [MB3]: R1

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43 *Abbreviations:* CD, Crohn's disease; VDR, vitamin D receptor; 25-vitD, 25-  
44 hydroxyvitamin D3; 1.25-vitD3, 1.25-dihydroxyvitamin D3; IBD, inflammatory bowel  
45 disease; CDAI, Crohn's disease activity index; PBMC, Peripheral blood mononuclear cells.  
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## Summary:

**Introduction:** Vitamin D3 has shown immune-modulating effects in CD4+ T cells from Crohn's disease (CD) patients *in vitro*.

**Aim:** To investigate the effects of *in vivo* vitamin D3 treatment on T cells in CD patients.

Comment [MB4]: R1

**Methods:** Peripheral blood mononuclear cells (PBMC) were isolated at week 0 and

week 26 from 10 vitamin D3 and 10 placebo treated CD patients participating in a

Comment [MB5]: R1

randomized placebo-controlled clinical trial study. Monocyte-depleted PBMC were

stimulated with anti-CD3 and anti-CD28 and cultured for 7 days, to investigate CD4+ T

cell proliferation and T cell cytokine production.

Comment [MB6]: R1

**Results:** In vitamin D3 treated patients the median 25-hydroxyvitamin D3 levels

increased 70 nmol/l compared to -5 nmol/l in the placebo group. Vitamin D3 treatment

Comment [MB7]: R1

increased interleukin-6 production (delta = 188 pg/ml, range: -444 - 4071) compared to

a decrease in the placebo group (delta = -896 pg/ml, range: -3841 - 1323) ( $p < 0.02$ ,

Wilcoxon rank sum test). Interestingly vitamin D3 increased the amount of proliferating

stimulated CD4+ T cells from median 41 % (range: 10 - 75%), to 56 % (range: 26 -

77%) ( $p = 0.02$ , Wilcoxon rank sum test).

Comment [MB8]: R1

**Conclusions:** Vitamin D3 treatment of CD patients increased the IL-6 levels.

Interestingly vitamin D3 treatment enhanced the CD4+ T cell proliferation.

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## Introduction

Crohn's disease (CD) is characterized by transmural intestinal inflammation due to a dysregulated mucosal immune response against intra-luminal antigens in genetically predisposed individuals.<sup>1</sup> Epidemiological data suggest that sun exposure may influence the disease incidence and prevalence, with the highest prevalence of CD in northern latitudes.<sup>2</sup> In CD patients 25-hydroxyvitamin D3 (25-vitD) serum levels are lower compared to healthy controls and CD relapse rate shows seasonal variation with most cases in the winter, which is hypothesised to be explained by a seasonal drop in 25-vitD.<sup>3,4</sup> In addition only one in five recently diagnosed individuals with inflammatory bowel disease (IBD) have optimal 25-vitD plasma levels at 75 nmol/L or above.<sup>5</sup> Besides, a recent randomised placebo controlled study has demonstrated, that treatment with vitamin D3 restores reduced vitamin D3 levels during the winter and reduces the CD relapse rate during an observation period of one year.<sup>6</sup>

Inflammation in CD is dominated by a pro-inflammatory CD4+ T-helper cell response, which is characterized by an increased tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon  $\gamma$  (INF- $\gamma$ ) and interleukin 2 (IL-2) production<sup>7</sup> due to a broken tolerance to the commensal intestinal flora.<sup>1</sup> The tolerance to non-pathogenic bacteria is usually maintained by regulatory mechanisms, in which regulatory T cells might be important.<sup>8</sup> Recent studies have identified a new type of effector helper T cells, known as Th17, characterized by IL-17 production and thought to play an important role in CD development.<sup>9,10</sup> IL-6 facilitates the Th17 differentiation from naïve T cells in the presence of TGF $\beta$ .<sup>11</sup> IL-23 is important for the expansion and survival of Th17 cells<sup>12,13</sup> and polymorphisms in the IL-



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3 23R have been identified to be associated with development of CD.<sup>14</sup> The interplay  
4  
5 between IL-6, TGF $\beta$ , IL-17A and IL-23 is important for the immune regulation. Recently  
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7 it has been suggested that IL-17A has anti-inflammatory effects<sup>15</sup> and TH-17 cells could  
8  
9 be a target for vitamin D3 modulation of inflammation.

Comment [MB9]: R1

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12 Vitamin D3 has immune-modulating impact beside the well-known effects on calcium  
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14 metabolism.<sup>16</sup> Vitamin D3 is hydroxylated twice; first in the liver and then in the  
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16 kidneys, into the biologically active metabolite called 1.25-dihydroxyvitamin D3 (1.25-  
17  
18 vitD3). 1.25-vitD3 binds to the nuclear vitamin D receptor (VDR), which is localised in  
19  
20 most body tissues.<sup>17</sup> VDR knock out (KO) mice develop symptoms of colitis which is  
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22 associated with increased IFN- $\gamma$  production along with a decreased IL-4 and IL-5  
23  
24 production and defect homing of CD4/CD8 $\alpha\alpha$  intraepithelial T cells.<sup>18</sup> Treatment with  
25  
26 1.25-vitD3 ameliorates the colitis symptoms and increases the production of IL-4.<sup>19</sup>

27  
28 Vitamin D3 has also been shown to reduce colitis symptoms in animal T cell transfer  
29  
30 models of IBD.<sup>20, 21</sup> In CD-*in vitro* CD4+ T cell studies vitamin D3 inhibits the production  
31  
32 of the pro-inflammatory cytokines IL-2<sup>22</sup>, TNF- $\alpha$ <sup>23</sup> and IFN- $\gamma$ .<sup>22, 24</sup> In addition vitamin D3  
33  
34 enhances the IL-4<sup>24, 25</sup>, IL-6<sup>24</sup> and IL-10<sup>24, 26, 27</sup> production and elevates the CD4+ CD25+  
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36 Treg levels expressing forkhead box protein 3 (FoxP3).<sup>28</sup> Beside these effects some  
37  
38 studies have demonstrated that stimulated CD4+ T cells cultured in the presence of  
39  
40 1.25-vitD3 decreases the proliferation rate.<sup>22, 23, 29, 30</sup> However, this finding is debatable  
41  
42 since a recent study has demonstrated reversible reduced proliferative capacity in T  
43  
44 cells isolated from patients with low serum concentrations of vitamin D3.<sup>31</sup>

Comment [MB10]: R1

45  
46 It is also known that 1.25-vitD3 inhibit the differentiation, maturation and immune-  
47  
48 stimulatory effects of dendritic cells (DC) by decreasing the expression of MHC class II  
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3 molecules and of the maturation markers CD40, CD80 and CD86.<sup>28, 32, 33</sup> Vitamin D3  
4  
5 stimulate the DC IL-10 production, which probably reduces the pro-inflammatory T cell  
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7 response and enhance the amount of IL-10 producing Treg.<sup>34</sup>  
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9 However, how 25-vitD levels influences the incidence and cause of human autoimmune  
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11 diseases has yet to be elucidated. Currently two hypotheses have been presented; one  
12  
13 suggesting a reduced adaptive T-cell mediated immune response<sup>25, 26</sup> and another study  
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15 with sepsis patients in intensive care units suggesting enhanced innate immunity  
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17 through cathelicidin producing macrophages or DC.<sup>35, 36</sup> In the present study we  
18  
19 investigate stimulated T cell mediated immune function during *in vivo* treatment of CD  
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21 patients with 1200 IU vitamin D3 per day for 26 weeks monitoring CD4+ T cell cytokine  
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23 production, proliferation and phenotype.  
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## Materials and Methods

### Patients

In a randomized placebo-controlled clinical study, 108 patients were randomized to supplementation with 1200 international units (IU) vitamin D3 and 1200 mg calcium per day for one year or placebo and calcium. From each patient serum and plasma samples were collected and peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation (Ficoll-Paque, Amersham Biosciences, Upssala, Sweden) week 0, 26 and 52 and frozen at -140°C.<sup>6</sup>

Comment [MB11]: R1

In the present study 10 patients receiving treatment with 1200 IU vitamin D3 were selected from the clinical study mentioned above, on the basis of largest 25-vitD increment from week 0 to 26, and seasonally matched by inclusion date with 10 placebo patients. Table 1 shows the characteristics of the patients regarding gender, age, inclusion time, Crohn's disease activity index (CDAI), number of relapses and medical treatment with mesalazine, azathioprine and biologics (infliximab or natalizumab) prior to inclusion.

Comment [MB12]: R1

The study was approved by The Central Denmark Region Committees on Biomedical Research (j. no. 2004/0149) and the Danish Medicines Agency (EudraCT no. 2005/001216/50) and was registered at ClinicalTrial.gov (NCT 0012 2184).<sup>6</sup>

Comment [MB13]: R1

### Vitamin D measurement

Serum 25-vitD levels were estimated by the addition of serum 25-hydroxyvitamin D2 level (25-vitD2) and serum 25-hydroxyvitamin D3 level (25-vitD3). Serum 25-vitD2 and 25-vitD3 were analysed by isotope-dilution liquid chromatography-tandem mass

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3 spectrometry on an API300 TM mass spectrometer (Applied Biosystems, Lincoln, USA)  
4  
5 applying a method adapted from Maunsell et al<sup>37</sup> as previously described.<sup>6</sup>  
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#### 8 9 **Preparation of PBMC for cytokine measurements and proliferation**

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11 In each patient PBMC from week 0 and week 26 were thawed and diluted in RPMI (25  
12 mM HEPES, and 2 mM L-glutamine) and washed twice. Cells ( $2 \times 10^6$ ) were separated  
13  
14 and stained for expression of FoxP3 and cell phenotypes (described later). Remaining  
15  
16 PBMC (10-17 million) were suspended in cytokine free medium (RPMI 1640, penicillin  
17  
18 and streptomycin, 2% human AB serum) in 6-well plates (NUNC, Roskilde, DK) for 3  
19  
20 hours at 37°C and at a cell density of  $2 \times 10^6$  cells/ml (2.5 ml) to make the monocytes  
21  
22 adhere to the bottom of the wells. After 3 hours incubation wells were washed twice and  
23  
24 the wash off (containing PBMC deprived from monocytes, (PBMCm-)) were divided into  
25  
26 analysis of; 1) cytokine production ( $2 \times 10^6$  cells), 2) proliferation of CD4+ T cells ( $5 \times$   
27  
28  $10^6$  cells) and 3) viability. The adherent monocytes were spared for later studies.  
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#### 31 32 33 *Cytokines*

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35 PBMCm- ( $2 \times 10^6$  cells/ml) were divided into 96 well plates (TPP, Switzerland) and  
36  
37 suspended in 200  $\mu$ l of IL-2 medium (RPMI with 10% pooled human AB serum, IL-2 50  
38  
39 U/ml (proleukin®, Chiron Therapeutics, Emeryville, CA) penicillin and streptomycin).  
40  
41 PBMCm- were stimulated with 1  $\mu$ g/ml immobilized anti-CD3 (Orthoclone OKT3, Cilag  
42  
43 AG international, Switzerland) and 1  $\mu$ g/ml soluble anti-CD28 (BD Biosciences, San  
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45 Diego, CA). PBMCm- were cultured for 7 days and supernatants were harvested and  
46  
47 frozen at -20°C up to 6 weeks until examination for their production of the following  
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3 cytokines: IL-4, IL-6, IL-10, TNF- $\alpha$  and IFN- $\gamma$  by **Cytometric Be**  **ray** (CBA, BD  
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5 Biosciences, San Diego, CA). Detection limit was 10 pg/ml.

Comment [MB14]: R1

#### 9 **CD4+ T cell proliferation**

10 Proliferation was measured by carboxyfluorescein succinimidyl ester (CFSE) **labelling**  
11 (Molecular probes Europe BV, Netherlands).  $5 \times 10^6$  PBMCm- were **coloured** with  $5 \mu\text{M}$   
12 CFSE diluted in Dubecco's phosphate buffered saline (DPBS) with 0.1 % human AB  
13 serum. The CFSE **labelled** PBMCm- suspension was cultured in 96-well plates in a  $200 \mu\text{l}$   
14 of IL-2 medium ( $2 \times 10^6$  cells/ml). Wells were stimulated with  $1 \mu\text{g/ml}$  immobilized  
15 anti-CD3 and  $1 \mu\text{g/ml}$  soluble anti-CD28.  $20\text{-}25 \mu\text{l}$  of IL-2 medium was added each well  
16 at day 4.  
17

  
Comment [MB15]: R1

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19 PBMCm- were cultured for 7 days. CD4 T cells were marked with  $1 \mu\text{l}$  CD4 APC-Cy7 (BD  
20 Biosciences, San Diego, CA) and  $2.5 \mu\text{l}$  CD3 APC (BD Biosciences, San Diego, CA) before  
21 flow cytometric evaluation. Wells without stimulation served as spontaneous  
22 proliferation controls and the proliferation in un-stimulated wells was subtracted from  
23 the proliferation in stimulated wells.  
24

#### 25 **Viability (7-AAD)**

26  
27 **The CFSE coloured lymphocyte viability was estimated by flow cytometry by the use of**  
28 **7-AAD.** After 7 days of culture, stimulated with  $1 \mu\text{g/ml}$  immobilized anti-CD3 and  $1$   
29  $\mu\text{g/ml}$  soluble anti-CD28, PBMCm- ( $2.0 \times 10^5$ ) were **coloured** with  $5.0 \mu\text{l}$  7-  
30 AAD/viaprobe (BD Biosciences, San Diego, CA) according to manufactures description.  
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32 Lym  cytes were gated based on their forward and side scatter appearance.  
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Comment [MB17]: R1



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### Forkhead box protein 3 (FoxP3) expression

Expression of intracellular FoxP3 was measured by flow cytometry (alexa flour 488 clone 206D, Bio Legend, San Diego, CA) according to manufacturer's instructions. Cells were stained for surface-markers with CD3 PerCP (BD Biosciences, San Diego, CA), CD4 APC (Beckman Coulter, Marseille, France), CD25 Pe-Cy7 (BD Biosciences, San Diego, CA) and CD127 PE (BD Biosciences, San Diego, CA).

### Cell phenotype distribution

To investigate whether *in vivo* treatment with vitamin D3 influenced the composition of cell types, PBMC were stained with CD3 PerCP (BD Biosciences, San Diego, CA), CD4 FITC (BD Biosciences, San Diego, CA), CD14 PE (BD Biosciences, San Diego, CA), CD19 APC-Cy7 (BD Biosciences, San Diego, CA), CD56 PE-Cy7 (BD Biosciences, San Diego, CA) and CD86 APC (BD Biosciences, San Diego, CA). The distribution of cell types was examined by flow cytometric analysis.

### Statistical analysis

Descriptive statistics are given by absolute values and median values. The non-parametric Wilcoxon signed rank test was used to compare paired data. We used the non-parametric Wilcoxon rank sum test to compare unpaired data. An association between two variables was estimated by the Spearman rank correlation coefficient (Spearman's rho). Correlations comprised all single measurements (both week 0 and week 26 data) from each variable. A *p* value below 0.05 was considered statistically significant. All statistical analyses were carried out using Statistix 9.0 software

Comment [MB18]: R1

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3 (Analytical Software, Tallahassee, USA). Graphs were performed using Sigma Plot 10.0  
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5 software (Systat Software Inc, San Jose, USA).  
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## Results

### The vitamin D3 and placebo group did not differ in patient characteristics

There were no significant differences between the vitamin D3 treatment- and placebo group regarding age, gender, time of inclusion, CDAI score, number of patients with relapse of CD or medical treatment with mesalazine, azathioprine, or biologics prior to inclusion.

Comment [MB20]: R1

Comment [MB21]: R1

### 25-hydroxyvitamin D3 levels increased with vitamin D3 treatment

The values for 25-vitD serum levels within the vitamin D3 and placebo groups at week 0 and 26 are seen in figure 1. In the vitamin D3 substituted group the median 25-vitD level increased from week 0 (33 nmol/L, range: 16 – 66) to week 26 (118 nmol/L, range: 62 – 154) as expected. Median 25-vitD levels in the placebo group slightly decreased insignificantly from week 0 (61 nmol/L, range: 27 – 105) to week 26 (46 nmol/L, range: 27 – 97). Median change in 25-vitD serum levels from week 0 to week 26 was 70nmol/l (range: 32 – 135) in the vitamin D3 group, and -5 nmol/l (range: -67 – 33) in the placebo group. The vitamin D3 group at week 0 did not differ from the placebo group week 0.

Comment [MB22]: R1

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Comment [MB25]: R1

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## Cytokines

### IL-6 production increased with vitamin D3 treatment

The increase in IL-6 production (delta = week 26 – week 0) in T cells from the vitamin D3 treated patients (delta = 188 pg/ml, range: -444 – 4071) was significantly different compared to the IL-6 production observed in the placebo group (delta = -896 pg/ml, range: -3841 – 1323) ( $p < 0.02$ ) (figure 2). Although absolute IL-6 production increased non-significantly in the vitamin D3 group from week 0 to week 26, the IL-6 production

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3 significantly decreased in the placebo group from week 0 to week 26 ( $p = 0.05$ )(figure  
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5 2). IL-6 levels were significantly correlated to the 25-vitD serum levels ( $p = 0.02$ ) (see  
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7 figure 1 in supplementary materials). |

Comment [MB28]: R1

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11 *Vitamin D3's non-significant effects on IL-4, TNF- $\alpha$  and IFN- $\gamma$  productions*

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13  Vitamin D3 treatment tended to increase the IL-4 production in T cells ( $\Delta = 185$  pg/ml,  
14  
15 range: -543 – 777) compared to a decrease in the placebo group ( $\Delta = -111$  pg/ml, range:  
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17 -245 – 386)( $p = 0.076$ )(see figure 2 in supplementary figures). Vitamin D3 did not affect  
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19 the TNF- $\alpha$  and IFN- $\gamma$  productions after 26 weeks treatment. |

Comment [MB29]: R1

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23 *IL-10 production and amount of Treg in vitamin D3 treated patients*

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25 There was no significant change in IL-10 production in T cells in the vitamin D3 group  
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27 from  week 0 (median: 754 pg/ml, range: 313 – 1919) to week 26 (median: 601 pg/ml,  
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29 range: 257 – 1509). The amount of CD4+CD25+FoxP3+ T cells did not increase by  
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31 vitamin D3 treatment for 26 weeks nor in the placebo group (data not shown).  
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Comment [MB30]: R1

### 35 Proliferation

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37 The amount of proliferating CD4+ T cells was determined in percent of all counted CD4+  
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39 T cells from the configuration of the flow histogram. The gate containing the amount of  
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41 non-proliferating cells in the experiments was fixed on the basis of the non-proliferating  
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43 cells in the un-stimulated control. 7AAD staining showed a viability > 95 %.

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45 The vitamin D3 substituted group significantly increased the amount of proliferating  
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47 CD4+ T cells from week 0 (median: 41 %, range: 10 – 75%) to week 26 (median: 56 %,  
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3 range: 26 - 77%)( $p = 0.02$ ), a finding not observed in the placebo group ( $p =$   
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5 0.13)(figure 3).

Comment [MB31]: R1

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7 Figure 4 demonstrates a strong correlation between decreasing IL-10 levels and  
8  
9 increasing proliferation of CD4+T cells ( $n = 40$ ) (Spearman's rho:  $-0.45$ ,  $p = 0.006$ ).

Comment [MB32]: R1

### 13 Phenotype distribution

14 We observed no significant change in the distribution of cell types in patients receiving  
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16 vitamin D3 treatment nor when compared to patients receiving placebo.  
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For Peer Review

## Discussion

In the present study we investigated the effects of vitamin D3 treatment on T cell function in CD patients. The increased 25-vitD serum levels were associated with a significant increase in the T cell IL-6 production and a tendency to increase their IL-4 production compared to T cells obtained from patients receiving placebo treatment. The observed increase in the IL-6 level was correlated to the increase in 25-vitD serum levels. Interestingly, we found an increased proliferation in CD4+ T cells from vitamin D3 treated patients that inversely correlated to a decrease in the IL-10 production.

Interleukin-6 can induce T cell IL-4 production, and consequently the observed increase in IL-4 production could be a result of the increased IL-6 levels.<sup>38</sup> We demonstrated that T cell IL-6 production increased after *in vivo* vitamin D3 treatment and the IL-6 levels were correlated to 25-vitD serum levels. *In vitro* cellular studies have shown that 1.25-vitD3 substituted T cells increase their production of IL-6.<sup>24, 39</sup> The role of the multifunctional cytokine IL-6 is not clear and the presence of IL-6 has been associated with both pro- and anti-inflammatory effects.<sup>40</sup> Some of the anti-inflammatory effects of IL-6 have been shown by Steensberg et al.<sup>41</sup> Infusion of recombinant human IL-6 to healthy volunteers increased the IL-10 plasma levels, IL-1 receptor antagonist and cortisol. In our study IL-6 production was not correlated with increased IL-10 production (IL-1receptor antagonist and cortisol levels were not measured). Data supporting the statement that IL-6 may have immune regulating properties is the interesting observation that IL-6 KO mice develop type 2 diabetes symptoms, a disease with low-grade chronic inflammation.<sup>42</sup> Several studies have shown that an increased

Comment [MB33]: R1



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3 amount of Th17 cells in CD patients and increased IL-17 and IL-23 production in  
4 intestinal mucosa biopsies compared to healthy controls. These findings are not  
5 significant in peripheral blood in CD patients in remission.<sup>9, 43</sup> IL-6 is involved in the  
6 differentiation of Th17 cells in the presence of TGFβ.<sup>11</sup> A recent study by O'Connor et al  
7 has hypothesised that IL-17A may have protective role in inflammatory bowel disease<sup>15</sup>.  
8 Therefore the effects of vitamin D3 treatment could be mediated through IL-6 and  
9 changes in the Th17 axis, however, a complex hypothesis that needs to be further  
10 investigated.  
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Comment [MB34]: R1

21 We controversially observed an increased T cell proliferation and a non-significant  
22 reduced IL-10 production in the active treatment group. We found that the increased T  
23 cell proliferation was highly correlated to decreasing IL-10 levels. The obvious  
24 differences between the *in vitro* and the *in vivo* effects of vitamin D3 on T cell function  
25 may be caused by vitamin D3 stimulation of DC<sup>44</sup> which indirectly changes the cytokine  
26 production and function of the T cells. Our cell populations were PBMC separated from  
27 most monocytes and not isolated CD4+ T cells, which were previously used by Bartels et  
28 al.<sup>24</sup> However the stimulating antibodies were directed against the T cells (CD3+,  
29 CD28+). Our findings are controversial compared to previous *in vitro* studies where  
30 1,25-vitD3 decreased proliferation in stimulated CD4+ T cells.<sup>23, 29</sup> However, a recent  
31 publication from von Essen<sup>31</sup> demonstrated that the proliferation of T cells was related  
32 to serum vitamin D levels. Patients with renal disease and low vitamin D3 levels showed  
33 reduced T cell proliferation compared to healthy subjects, a defect that could be  
34 reversed by exogenous addition of 1,25-vitD3. Vitamin D3 influence the phospholipase  
35 C (PLC) levels and signalling via the T cell receptor. Vitamin D3 upregulates the  
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3 expression of PLC- $\gamma$ 1 protein expression thereby changing the adaptive immune  
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5 response in T cells. Consequently this mechanism may be involved in the increased  
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7 proliferative response observed in our study, although the findings may be a secondary  
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9 phenomenon to changes in the cytokine milieu produced by DC.

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13 Vitamin D3 treatment of patients with Crohn's disease tended to increase ex vivo T cell  
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15 IL-4 production as it has been demonstrated *in vitro* where 1.25-vitD3 stimulated T cells  
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17 increase the IL-4 production.<sup>24,25</sup> *In vitro* IL-4 facilitates the development of IL-4, IL-5  
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19 and IL-10 producing T cells inducing the expression of FoxP3 in a CD4+ CD25+  
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21 phenotype.<sup>45</sup> However, in the present *in vivo* study T cell IL-10 production decreased  
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23 non-significantly after 26 weeks of vitamin D3 treatment in CD patients and we did not  
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25 observe increased expression of CD4+ CD25+ FoxP3+ T cells as it previously has been  
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27 shown *in vitro*.<sup>28</sup> These differences may indicate that the *in vivo* effects of vitamin D3  
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29 treatment may be associated with different immunological pathways and not a simple  
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31 direct modulation of the T cell response. A recent study demonstrated that topical  
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33 application with the vitamin D3 analogue calcipotriol inhibited immunization with  
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35 ovalbumin (OVA) protein and prevented the antigen (Ag)-specific CD8+ T cell priming  
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37 coincident with depletion of Langerhans cells.<sup>46</sup> Concomitantly the authors observed an  
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39 up-regulation of CD4+CD25+ regulatory T cells that prevented the proliferation of Ag-  
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41 specific CD8+ T cells and IFN- $\gamma$  production. However, in that study, the changes were  
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43 observed in the target organ (the skin). Therefore mucosal bowel biopsies might reveal  
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45 similar changes. It is known that vitamin D3 may modulate macrophage function via  
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47 cathelicidin or change the function of DC.<sup>44</sup> These changes in innate immunity may alter  
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49 the "bacterial drive" on the intestinal mucosal immune system and thereby modify the  
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3 broken tolerance to intestinal bacteria. As a consequence the observed changes in the  
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5 adaptive immunity may be a secondary result of changes in innate immunity at the  
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7 mucosal level.  
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11 In conclusion we have demonstrated that *in vivo* vitamin D3 treatment changes T cell  
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13 function with increased IL-6 production and a tendency to increase the IL-4 production.  
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15 The change in IL-6 production was correlated to the serum 25-vitD levels. However the  
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17 increased IL-6 production may also influence Th17 cells and IL-17A production, and  
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19 need further investigation. CD4+ T cell proliferation increased and was inversely related  
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21 IL-10 production. Further studies are needed to elucidate the *in vivo* effects of vitamin  
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23 D3 treatment and possible targets for therapy with vitamin D3 and vitamin D3  
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25 analogues.  
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## 29 Conflict of interests

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32 None.  
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## 35 Acknowledgements

36  
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45 wife Asmine Colind foundation.  
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Comment [MB35]: R1

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## Legends

### Figure 1

25-vitD serum levels (25-hydroxyvitamin D2 and D3) (absolute values, bars indicate medians) at week 0 and week 26 in 10 vitamin D3 treated CD patients and 10 placebo treated CD patients. 25-vitD serum levels increased in the vitamin D3 treated CD patients as expected (25-vitD median: from 33 nmol/l to 118 nmol/l). 25-vitD serum levels decreased slightly and insignificantly in the placebo group (25-vitD median: from 61 nmol/l to 46 nmol/l). No differences in 25-vitD serum levels were observed between the vitamin D3 and placebo group week 0 ( $p = 0.17$ ). VitD; vitamin D3 group, plac.; placebo group.

Comment [MB38]: R1

### Figure 2

IL-6 production from stimulated T cells at week 0 and week 26 in 10 vitamin D3 treated CD patients and 10 placebo treated CD patients. Values are shown in absolute values (bars indicate medians) and delta values (week 26 - week 0). IL-6 production increased insignificantly within 26 weeks of vitamin D3 treatment ( $p = 0.12$ , Wilcoxon signed rank test) and decreased significantly with placebo treatment ( $p = 0.05$ , Wilcoxon signed rank test). The difference (week 26 - week 0) in IL-6 production from CD patients substituted with vitamin D3 (Delta vitD) was significantly higher compared to placebo (Delta plac.) (median IL-6 levels: Delta vitD = 188 pg/ml, Delta plac. = -896 pg/ml,  $p < 0.02$ , Wilcoxon rank sum test).

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Figure 3

CD4 cell proliferation showed in percent (absolute values, bars indicate medians) of all counted CD4+ T cells at week 0 and 26 in 8 vitamin D3 treated CD patients and 10 placebo treated CD patients. The vitamin D3 substituted group significantly increased the amount of proliferating CD4+ T cells from week 0 (median: 41 %, range: 10 - 75%) to week 26 (median: 56 %, range: 26 - 77%) ( $p = 0.02$ , Wilcoxon signed rank test), a finding not observed in the placebo group ( $p = 0.13$ , Wilcoxon signed rank test).

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Figure 4

Correlation between IL-10 levels from stimulated T cells and percentage of proliferating CD4+ T cells from vitamin D3-substituted CD patients week 0 and week 26 ( $n = 8 + 8$ ) and from placebo substituted patients week 0 and week 26 ( $n = 10 + 10$ ). Increase in percentage of proliferating CD4+ T cells was significantly correlated to a decrease in the T cell IL-10 production (Spearman's rho = -0.45,  $p < 0.01$ ).

Comment [MB44]: R1

Preprint Review

## Figures and tables



	Vitamin D3	Placebo	<i>p</i> -value
N	10	10	1.0
Female patients, n	7	6	1.0
Age (years), median, (range)	37, (28 – 65)	45, (23 – 65)	0.45
Included at winter*, n	9	9	1.0
CDAI week 0, median, (range)	37, (8 – 111)	23, (0 – 187)	0.20
CDAI week 26, median, (range)	34**, (23 – 53)	45, (0 – 273)	0.83
Relapse during 26 weeks, n	0	1	1.0
Mesalazin users, n,	3	3	1.0
Azathioprine users, n,	7	3	0.18
Biologics (infliximab or natalizumab) prior to inclusion, n	1	1	1.0

Table 1: Characteristics of the vitamin D3 and placebo treated CD patients. Relapse was defined as an increase in Crohn's disease activity index (CDAI) > 70 and a total CDAI score > 150. \* Winter inclusion was from 1<sup>st</sup> of November to 30<sup>th</sup> of April. \*\*N = 9. CDAI; Crohn's disease activity index.

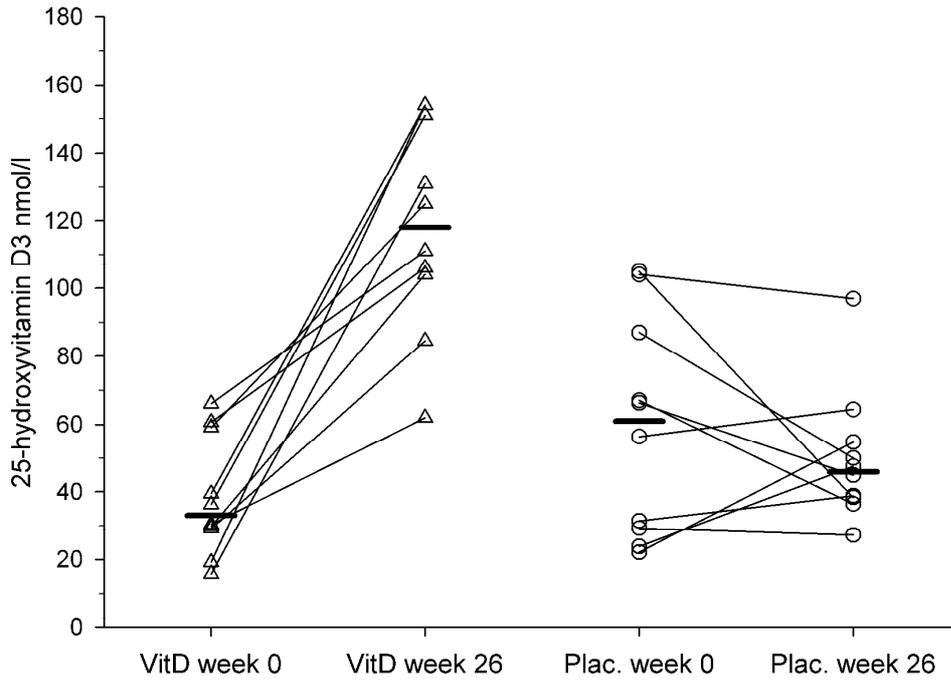
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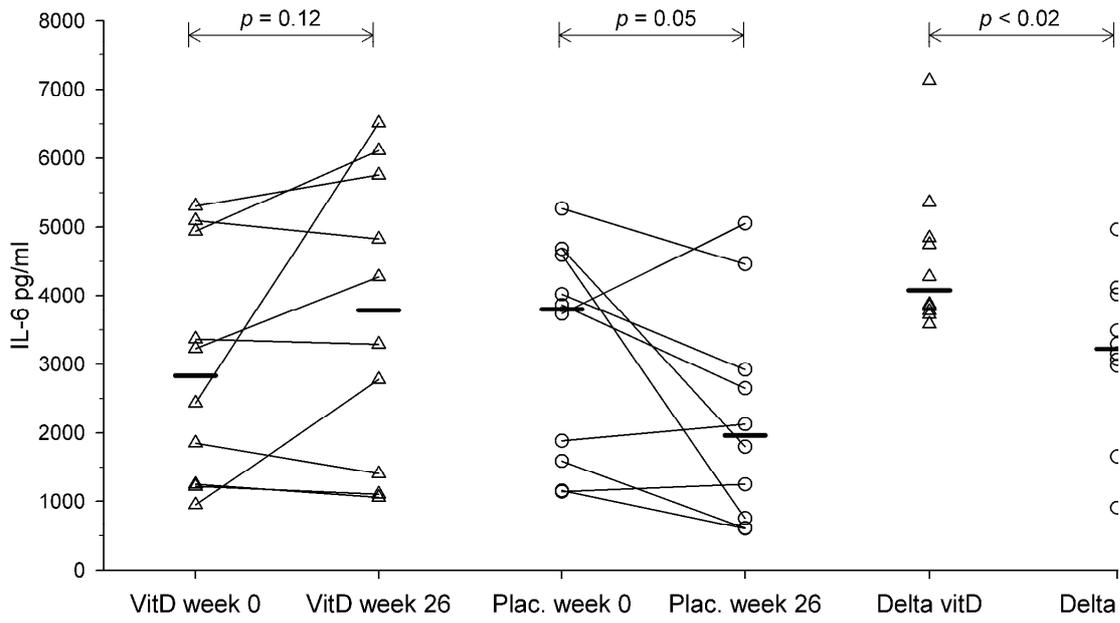


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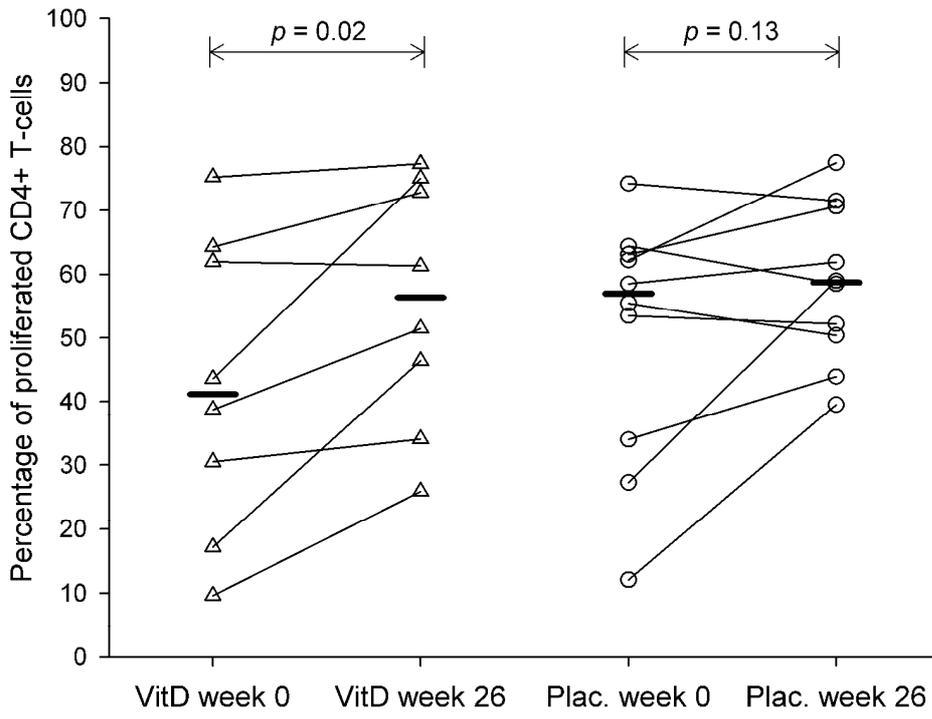


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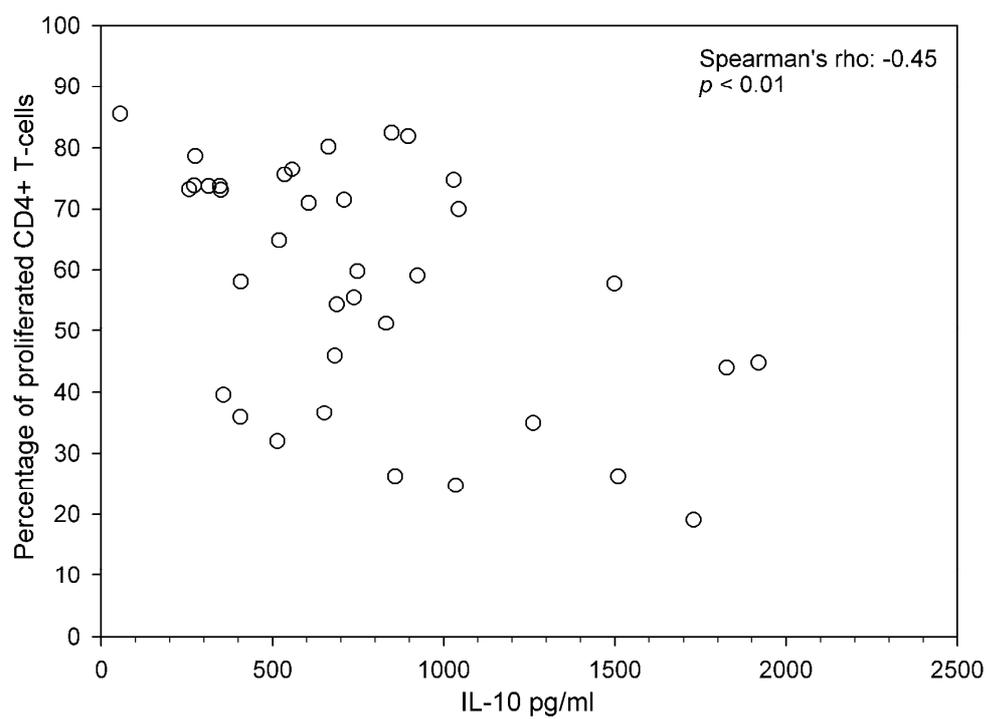


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Fig1

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## Supplementary figures

Figure 1s

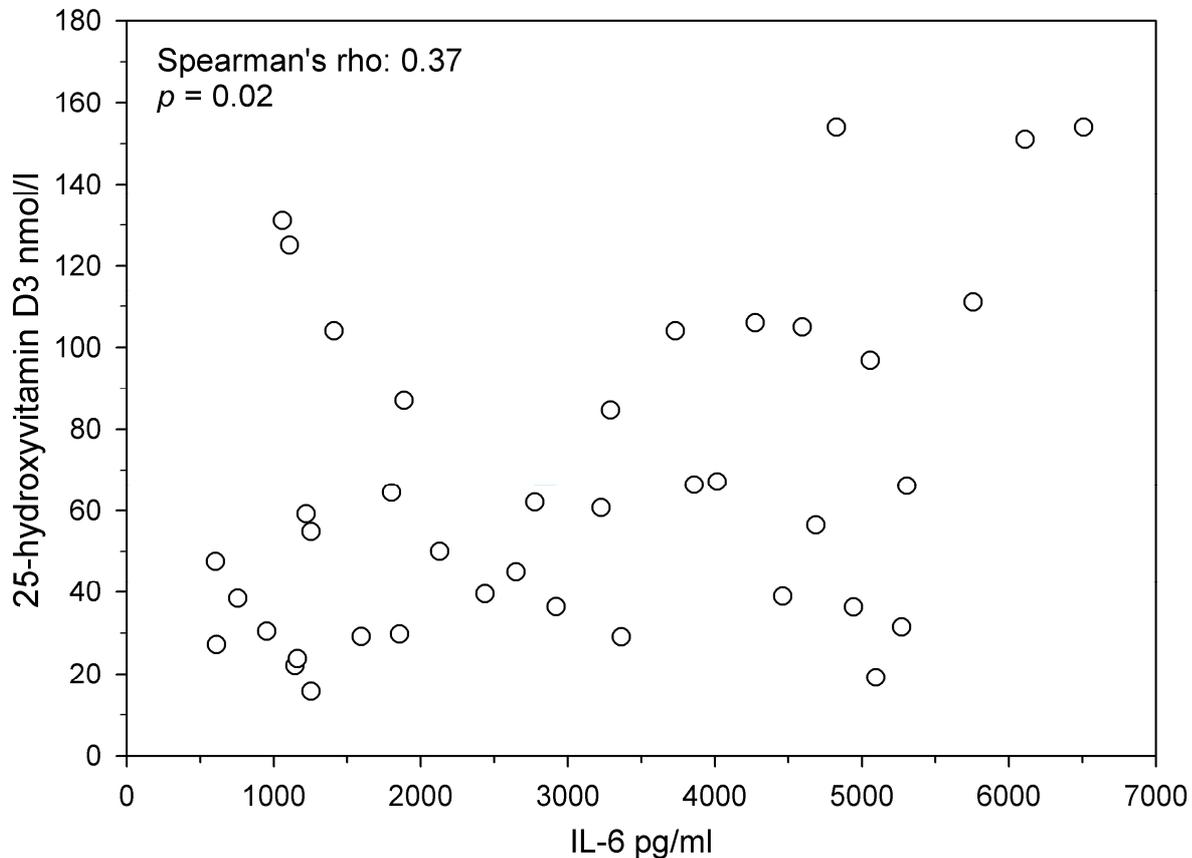


Figure 1s:

Correlation between 25-hydroxyvitamin D3 serum levels and IL-6 production in stimulated T cells from vitamin D3 treated CD patients week 0 and week 26 ( $n = 10 + 10$ ) and from placebo CD patients week 0 and week 26 ( $n = 10 + 10$ ). 25-hydroxyvitamin D3 levels were correlated to IL-6 production (Spearman's  $\rho = 0.37$ ,  $p = 0.02$ ).

Figure 2s

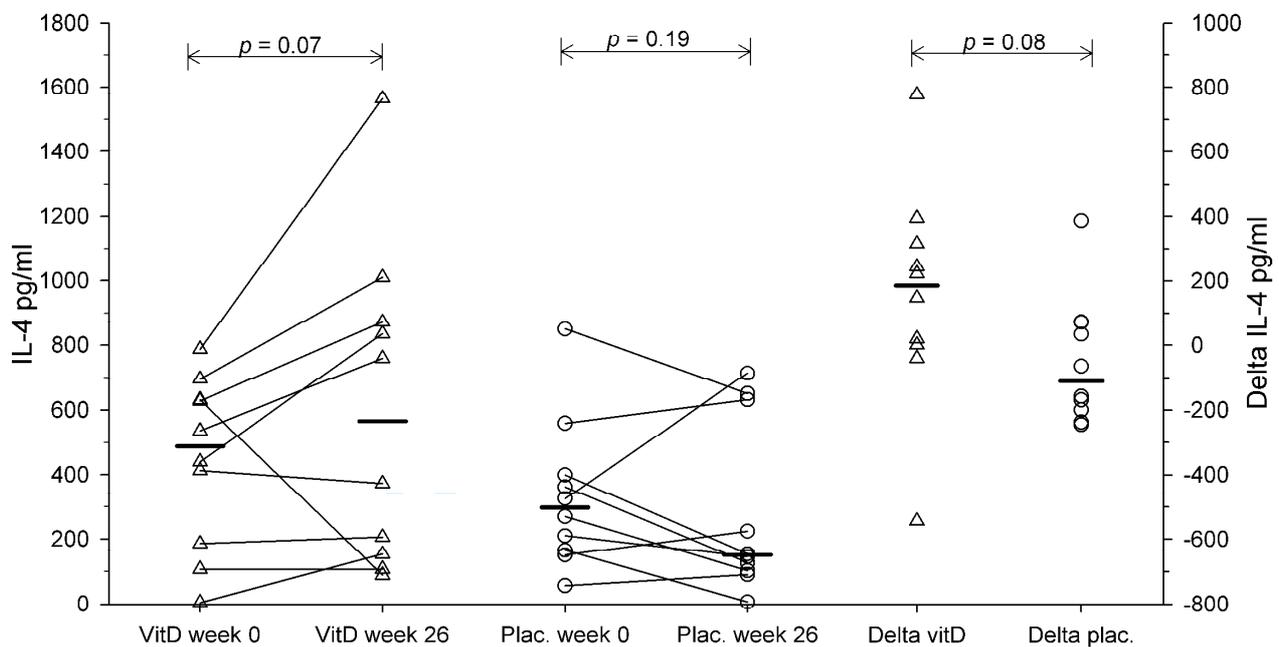


Figure 2s

IL-4 production from stimulated T cells at week 0 and week 26 in 10 vitamin D3 treated CD patients and 10 placebo treated CD patients. Values are shown in absolute values and delta values (week 26 - week 0) (bars indicate medians). IL-4 production tended to increase within 26 weeks of vitamin D3 treatment ( $p = 0.07$ , Wilcoxon signed rank test) and decreased non-significantly with placebo treatment ( $p = 0.19$ , Wilcoxon signed rank test). Changes (week 26 - week 0) in IL-4 production from vitamin D3 treated CD patients (Delta vitD) tended to increase compared to placebo (Delta plac.) (median IL-4 levels: Delta vitD = 185 pg/ml, Delta plac. = -111 pg/ml,  $p = 0.08$ , Wilcoxon rank sum test).