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Complete List of Authors:	Bergman, Peter; Karolinska Institutet, Dept of Laboratory Medicine, Div of Clinical Microbiology Norlin, Anna-Carin; Karolinska Institutet, Dept of Laboratory Medicine, Div of Clinical Immunology Hansen, Susanne; Karolinska University Hospital, Immunodeficiency Unit Rekha, Rokeya; Karolinska Institutet, Dept of Medical Biochemisrty and Biophysics Agerberth, Birgitta; Karolinska Institutet, Dept of Medical Biochemisrty and Biophysics Bjorkhem-Bergman, Linda; Karolinska Institutet, Dept of Laboratory Medicine, Div of Clinical Pharmacology Ekstrom, Lena; Karolinska Institutet, Dept of Laboratory Medicine, Div of Clinical Pharmacology Lindh, Jonatan; Karolinska Institutet, Dept of Laboratory Medicine, Div of Clinical Pharmacology Andersson, Jan; Karolinska Institutet, Dept of Medicine, Center for Infectious Medicine
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Vitamin D_3 supplementation reduces infectious symptoms and antibiotic consumption among patients with antibody deficiencies and frequent respiratory tract infections - a randomized controlled trial.

Peter Bergman*[1,3,6#], Anna-Carin Norlin*[2,3], Susanne Hansen[3], Rokeya Sultana Rekha[4], Birgitta Agerberth[4], Linda Björkhem-Bergman[5], Lena Ekström[5], Jonatan D Lindh[5], Jan Andersson[6]

1. Department of Laboratory Medicine, Clinical Microbiology, KI
2. Department of Laboratory Medicine, Clinical Immunology, KI
3. Karolinska University Hospital, Clinical Immunology, Immunodeficiency Unit
4. Department of Medical Biochemistry and Biophysics, KI
5. Department of Laboratory Medicine, Clinical Pharmacology, KI
6. Department of Medicine, Center for Infectious Medicine (CIM), KI

*Equal contribution

Positions:

Peter Bergman, MD, PhD, Resident Physician, Clinical Microbiology

Anna-Carin Norlin, MD, Specialist in Clinical Immunology

Susanne Hansen, RN, Head of Immunedeficiency Unit

Rokeya Sultana Rekha, MSc, PhD-student

Birgitta Agerberth, PhD, Professor, Medical Microbial Pathogenesis

Linda Bjorkhem-Bergman, MD, PhD, Resident Physician, Clinical Pharmacology

Lena Ekström, PhD, Associate Professor, Clinical Pharmacology

Jonatan D Lindh, MD, PhD, Specialist in Clinical Pharmacology

Jan Andersson, MD, PhD, Professor, Infectious Diseases

Running title: Vitamin D_3 supplementation in patients with frequent respiratory tract infections

#Corresponding author:

Peter Bergman, MD, PhD

Department of Laboratory Medicine, Clinical Microbiology F68

Karolinska Institutet and Karolinska University Hospital, Huddinge

SE-141 86 Stockholm, Sweden

peter.bergman@ki.se

Phone: +46858587942

Fax: +46858581305

Competing interest statement

All authors have completed the Unified Competing Interest form (available on request from the corresponding author) and declare no support from any organisation for the submitted work, no financial relationships with any organisations that might have an interest in the submitted work in the previous three years and no other relationships or activities that could appear to have influenced the submitted work.

Article Summary

Article focus

• Recent evidence suggests that vitamin D_3 has potent extra-skeletal effects, such as suppression of inflammation and strengthening of mucosal immunity by induction of antimicrobial peptides.

• Data from observational studies suggest that low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections.

• Results from a limited number of randomized controlled trials on the protective role of vitamin D_3 against respiratory tract infections are inconclusive and thus additional studies are warranted.

Key Messages

• Therefore we designed and carried out a randomized controlled trial where a large dose (4000 IU) of vitamin D_3 was given to patients with an increased susceptibility to infections for one year.

• The main conclusion is that vitamin D_3 supplementation reduces symptoms and antibiotic consumption among patients with an increased frequency of respiratory tract infections. Thus, vitamin D_3 supplementation may be an alternative strategy to reduce antibiotic use among patients with recurrent respiratory tract infections.

Strengths and limitations

• Strengths: A high daily dose of vitamin D3 was used, the study time was a full year covering all seasons and patient with an increased frequency of respiratory tract infections were studied.

• Limitations: A single study center, small sample size (n=140) and a selected group of patients.

Abstract

Background: Low serum levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections (RTIs). Clinical trials with vitamin D_3 against various infections have been carried out but data are so far not conclusive. Thus, there is a need for additional randomized controlled trials of effects of vitamin D_3 on infections.

Objective: To investigate if supplementation with vitamin D_3 could reduce infectious symptoms and antibiotic consumption among patients with antibody deficiency or frequent RTIs.

Design: A double-blind randomized controlled trial.

Setting: Karolinska University Hospital, Huddinge

Participants: 140 patients with antibody deficiency (sIgA-, IgG subclass deficiency, CVID) and patients with increased susceptibility to RTIs but without immunological diagnosis.

Intervention: vitamin D3 (4000 IU) or placebo was given daily for one year

Primary and secondary outcome measures: The primary endpoint was an infectious score based on five parameters: symptoms from respiratory tract, ears and sinuses, malaise and antibiotic consumption. Secondary endpoints were serum levels of 25-hydroxyvitamin D_3, microbiological findings and levels of antimicrobial peptides (LL-37, HNP1-3) in nasal fluid.

Results: The overall infectious score was significantly reduced for patients allocated to the vitamin D-group (202 points) compared with the placebo group (249 points) (adjusted relative score 0.771, 95% CI 0.604-0.985, p=0.04). The probability of receiving antibiotics during the study period was reduced by 63.5% (OR 0.365, 95% CI 0.153-0.872, p=0.023) for the vitamin D group.

Limitations: A single study center, small sample size and a selected group of patients.

Conclusions: Supplementation with vitamin D_3 may reduce disease burden and antibiotic consumption in patients with frequent respiratory tract infections.

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The study was registered at www.clinicaltrials.gov (NCT01131858)

273/275 words

Introduction

Vitamin D was discovered when it was noted that rachitic children were improved by exposure to sunlight [1]. It was later shown by Holick *et al* that vitamin D_3 is synthesized in the skin under the influence of UVB-light [2]. Vitamin D_3 is further hydroxylated in the liver to 25-hydroxyvitamin D_3, which is considered to reflect the vitamin D status of an individual patient [3]. The final activation to the active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)D_3$) requires 1-α-hydroxylase activity. This enzyme (also designated CYP27B1) is expressed in the kidney but also in many other cell-types, including epithelial- and immune-cells [4]. The active vitamin D_3 ($1,25(OH)D_3$) binds to the vitamin D receptor (VDR), which belongs to the nuclear receptor family. Active vitamin D_3 is only present in minute amounts in the circulation and local activation in target cells is crucial for vitamin D-mediated effects on the immune system [5].

Low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of tuberculosis [6-8] and respiratory tract infections [9]. The mechanism is not fully elucidated but vitamin D_3 has been shown to induce antimicrobial peptides in immunecells [10]. In addition, active vitamin D_3 ($1,25(OH)D_3$) has broad anti-inflammatory effects on the adaptive immune system by shifting the T-helper cell pool from a Th1/Th17-response to a Th2/Treg-dominated response [11 12]. Vitamin D_3 has also been shown to suppress the Th2-response in allergic bronchopulmonary aspergillosis [13]. Thus, vitamin D_3 modulates both the adaptive and innate immune system [14]. The bulk of data on vitamin D_3 and infections stems from *in vitro* experiments and retrospective observational studies. Results from randomized controlled trials where the effects of vitamin D_3 on infections have been investigated (reviewed in Yamshchikov et al. [15]) are not conclusive and larger clinical trials are therefore warranted.

We designed a study to test the hypothesis that 4000 IU of vitamin D_3 given daily to patients with antibody deficiency and frequent respiratory tract infections for one year could prevent or ameliorate infections. In addition, we investigated whether genetic polymorphisms in genes involved in the effect and/or metabolism of vitamin D_3 have an influence on the outcome of vitamin D_3 supplementation.

Methods

Study design

A prospective, randomized, double-blind placebo-controlled study of vitamin D_3 supplementation in patients with an increased susceptibility to respiratory tract infections. The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants. The study was registered at www.clinicaltrials.gov prior to inclusion of the first patient (NCT01131858). The EudraCT number is 2009-011758-16. The full protocol is available from the corresponding author upon request.

Participants

Patients at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden, were included between March 2010 and June 2010 by the study nurses (SH, ML, KJ). Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infections; i.e. > 42 days with symptoms from the respiratory tract during a 12 months period prior to study inclusion. Patients registered at the Immunodeficiency Unit are closely followed with a diary of symptoms and antibiotic consumption. Thus, this dairy (similar, but not identical to the one used in the current study) was used as an instrument in order to assess patients for eligibility, both via telephone and by the responsible physician (PB, ACN) prior to inclusion. Patients with selective IgA-deficiency (**D80.2**), IgG-subclass deficiency (**D80.3**) and common variable immune disorder (CVID, **D83.0**) as well as patients without a defined immunological diagnosis (**D89.9**) were included. Exclusion criteria were prophylactic

treatment with antibiotics, history of hypercalcemia or stones in the urinary tract, sarcoidosis, ongoing supplementation with vitamin D_3 exceeding 400 IU/day and pregnancy.

Interventions

Patients were randomized to 12 months' treatment with Vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. One drop contained 500 IU vitamin D_3 or placebo oil (Miglyol oil®) and the participants were asked to take 8 drops daily. The participants had to mark their daily symptoms of infection in a diary, which was sent via regular mail to the study site every month. The following data was recorded: symptoms from the respiratory tract, ears and sinuses, treatment with antibiotics, numbers of bacterial cultures, times and reasons of visits to hospitals, frequency of travelling abroad and adherence to study drug.

Outcomes

The primary outcome was a composite infectious score, based on a daily patient-reported questionnaire, and included five parameters: symptoms from the respiratory tract, ears and sinuses, malaise and use of antibiotics (Figure S1), each parameter gave 1 point/day. The occurrence of X-ray verified pneumonia gave 3 additional points per day. Thus, the maximum score per day was 8 points (Suppl. figure S1). Patients were specifically instructed to record only symptoms related to ongoing respiratory tract infections. Symptoms related to infections at other sites (urinary tract, wounds etc) as well as non-infectious symptoms were reported as adverse events. Secondary outcomes were serum levels of 25-hydroxyvitamin D_3 (at baseline and after 3, 6, 9 and 12 months), numbers of bacterial cultures, microbiological findings and levels of antimicrobial peptides (LL-37 and HNP1-3) in nasal fluid (at baseline and after 6 and 12 months). In addition, 6 *post hoc* genotype analyses were performed in all participants.

Analysis of single nucleotide polymorphisms (SNPs) were carried out for VDR (Taq1 and Foq1), CYP27B1, CYP24A1, CYP2R1 and Vitamin D binding protein (GC). Safety tests included plasma levels of creatinine, calcium, phosphate and albumin, measured at baseline and after 3, 6, 9 and 12 months. At inclusion, urine-HCG (human chorionic gonadotropin) in women was measured and p-parathyroid hormone in both genders. The results of the safety tests were reviewed by an independent and un-blinded consultant physician. Two physicians (PB and ACN) were responsible for inclusion and all medical visits to the study site (Immunodeficiency Unit, Karolinska University Hospital, Huddinge).

Randomisation and statistical analysis

Participants were randomised to 12 months' treatment with vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. Block randomization with a block size of ten was used to ascertain equal group sizes. Staff at Karolinska Trial Alliance (KTA) was responsible for randomization procedures. In the statistical analysis, continuous variables were compared using Mann-Whitney U test or multiple linear regression and dichotomous variables by Fisher's exact test or multiple logistic regression. In the regression models, adjustment was made for age, gender, smoking status, type of immune deficiency and co-morbidities.

Detailed descriptions of randomisation and blinding, sampling of nasal fluid, measurement of antimicrobial peptides, measurement of 25-hydroxyvitamin D_3, genotyping and statistical analyses including sample size calculations are presented in the Supplementary Method Section.

Results

Baseline data

A total of 286 patients were first assessed for eligibility but 144 were not included because they did not fulfill all inclusion criteria; <42 days with infection/year (n=35), lacked other inclusion criteria (n=42), or declined to participate (n=67). The remaining 142 patients were further screened and 140 patients were included in the study. Of these, 70 were randomized to vitamin D_3 supplementation and 70 to placebo (Figure 1). The groups did not differ with regards to gender, IgG replacement therapy, smoking, baseline 25-hydroxyvitamin D_3 levels, type of immune defect or co-morbidities. Patients allocated to the placebo group were slightly younger than patients in the treatment group (p=0.025, table 1). However, this difference was not evident in patients with assessable study data and according to the adjusted analysis, age had no influence on the primary outcome (p=0.36, data not shown).

Primary endpoint: Infectious score

One year of vitamin D_3 treatment was associated with a significantly reduced total infectious score both in the unadjusted (p=0.023, Table 2) and the adjusted analyses (p=0.040) (Table 2, Figure 2A and Table S1). The adjusted relative score in the intervention group was 0.771, corresponding to a 23% reduction and the unadjusted absolute effect was a reduction by 47 points (249 vs 202 points), (Table 2). According to the temporal analysis, the effect of vitamin D_3 supplementation tended to improve with time (Figure 2A).

When the individual items of the infectious score were analyzed separately, all point estimates indicated a reduction in the treatment group (Table 2, figure S2), although only antibiotic consumption reached statistical significance (Figure 2B and S2, panel E). The OR for antibiotic use was 0.365, i.e. a 63.5% reduction in the intervention group (p=0.023, Table 2).

The absolute values were 33 days on antibiotics for the placebo group and 16 days for the vitamin D_3 group, i.e. a reduction of 17 days in the vitamin D_3 group (p=0.024, Table 2). The temporal trends for specific symptoms and antibiotic consumption were similar to the total score and reached statistical significance for 'ear'-symptoms (p=0.041) and for 'malaise' (p=0.053) in the final quarter of the study (Figure 2S, panels B and C).

When analyzing according to intention-to-treat, the between-group difference in total score was even larger (51 points compared to 47 points in the per-protocol analysis) and statistically significant (p<0.01, Mann-Whitney U test) (Suppl. Methods for details).

Serum levels of 25-OH vitamin D_3

Serum 25-hydroxyvitamin D_3 levels did not differ between the groups at baseline (Table 1) but already after 3 months the intervention group had a significantly higher level of 25-hydroxyvitamin D_3 (133.4 nmol/L versus 66.6 nmol/L, p<0.001, Figure 3). This increase remained throughout the study (Figure 3).

Bacterial cultures and microbiology

During the course of the study, 173 microbiological samples were obtained in the vitamin D_3 group (2.79/patient) and 301 in the placebo group (4.85/patient) (p=0·010, Table 3). The number of samples with at least one positive finding was higher in the placebo group, with close to statistical significance (p=0.052), while the fraction of positive samples was similar for both groups (Table 3). Significantly more patients had a microbiological sample taken from the respiratory tract during the study period in the placebo group; OR 2.63 (95% CI 1.17-5.92), (Table 3).

In total, the vitamin D_3 group generated 76 positive microbiological findings (bacteria or fungi), compared to 159 in the placebo group (p=0.023). There was no difference between the groups for the traditional respiratory pathogens (*H. influenza, M. catharralis and S. pneumonia*), but there were significantly fewer findings of *S. aureus* (p=0.019) and fungi (p=0.028, *Candida* spp. and *Aspergillus* spp.) in the treatment group (Table 4). Likewise, significantly fewer vitamin D_3-treated patients had a bacterial culture positive for *S. aureus* (p=0.019) or fungal species (p=0.058), although the latter difference did not reach statistical significance (Table 4).

Levels of antimicrobial peptides (AMPs) in nasal fluid

There was no statistically significant difference between the vitamin D_3 or placebo groups when nasal fluids were analyzed for the presence of AMPs. Initially, levels of both LL-37 and HNP1-3 tended to be higher in the placebo group (Figure S3, panels A and B). However, after 12 months the microbiological pattern was reversed and no primary pathogens could be detected in nasal swabs from vitamin D_3-treated patients (p=0.039) (Figure S3, panel C). The placebo-treated patients exhibited the same mix between normal flora and primary pathogens at all three sampling points (0, 6 and 12 months) (Figure S3, panel C).

SNP variants and treatment effect

Most genetic variants did not affect the primary endpoint. However, patients carrying the 'AA' genotype in the CYP2R1-gene, encoding the 25-hydroxylase enzyme, had a larger benefit of vitamin D_3-supplementation (-55%) compared to AG or GG carriers (-6%) (p=0.046 for interaction, Table S2).

Adverse events

In total, the vitamin D_3 group reported 38 adverse events (AEs) versus 56 AEs in the placebo group. The most common symptoms in the treatment group were headache (n=5) and lumbago (n=5), whereas placebo-treated patients reported paresthesias (n=8), diverticulitis (n=4) and urinary tract infection (n=4) as most frequent AEs (Table 5, Table S3). There was a clear trend towards the number of adverse events being higher in the placebo group. Significantly more patients in the placebo group reported cardiovascular problems, such as heart failure, hypertonia and thrombosis (p= 0.028). For gastrointestinal and other (non-respiratory) infections there was also a trend favoring the vitamin D_3 group (p=0·058 and p=0·09, respectively). No clinically relevant changes in serum levels of calcium, phosphate, creatinine or albumin could be observed (Figure S4). There was one severe adverse event (SAE) in each group (rabdomyosarcoma in the vitamin D_3 group and lung bleeding in the placebo group), both judged as being unrelated to the study drug.

Discussion

The main conclusion from this long-term randomized controlled trial (RCT) is that vitamin D_3 supplementation reduces the total burden of respiratory tract infections. The primary endpoint was composed of five different parameters that patients recorded daily throughout the study year. All point estimates favoured the vitamin D_3 group and adjusted analyses showed a statistically significant effect on the total score and on the probability of receiving antibiotics. In addition, the number of bacterial cultures and microbiological findings was significantly reduced in the intervention group. These findings are potentially important and support that Vitamin D_3 supplementation may prevent respiratory tract infections and reduce antibiotic consumption.

However, our study has several limitations: Firstly, the choice of primary endpoint may be questioned since it relies solely on patient-reported information. To compensate for inherent problems with patient-reported data, the evaluation instrument was designed to cover many aspects of an infectious episode, including various symptoms as well as antibiotic consumption. Together the reported data formed an "infectious score", which constituted the primary endpoint of the study. Similar composite scores have successfully been applied to different diseases, such as tuberculosis (TB-score [16]), pneumonia (CURB-65 [17]) and bacterial meningitis (BMS-score [18]). Interestingly, the number of days on antibiotics was reduced by 50%, statistically significant both in the adjusted and unadjusted analyses. Despite a small reduction for the other components of the primary endpoint the overall infectious score was significantly reduced both in the unadjusted and in the adjusted analyses. Another potential problem was that the patient population was very heterogeneous with regards to immune deficiency and concomitant diseases. We adjusted for these factors in the multivariable analyses, but the sample sizes in each subgroup were too small to draw any conclusions of effects in specific disease groups.

Nevertheless, our double-blind RCT has several strengths. For example, we chose a high daily dose of vitamin D_3 based on published calculations on metabolism and effects on immunity [14 19]. Other RCTs using lower doses of vitamin D_3, 400-2000 IU/day, have mainly been negative with regards to prevention of infections [20 21]. However, one study using 1200 IU/day showed a significant reduction of influenza among school children in Japan [22]. Notably, also studies using higher doses of vitamin D_3 have been negative. Martineau *et al* used 400,000 IU vitamin D_3 during 42 days (9523 IU/day) with the aim of shortening time to sputum conversion in tuberculosis. No significant effect on the primary endpoint could be observed in that study, except in a subgroup with the *tt* genotype in the vitamin D receptor gene [23]. A recent study investigated whether 100,000 IU vitamin D_3/month (3333 IU/day) could reduce the incidence of chronic obstructive pulmonary disease (COPD) exacerbations. There was no significant effect on the primary endpoint, although a *post hoc* analysis revealed that patients with a low vitamin D_3 level at baseline had a significant effect of Vitamin D_3 supplementation [24].

Importantly, this study is the first to utilize high daily doses for an extended period of one full year. Thus, we covered all four seasons, which was important in Sweden with a known seasonal variation in 25-hydroxyvitamin D_3 levels [25]. Two previous RCTs were performed during the winter season – when vitamin D levels are low – but only during 4 [22] and 6 months [20], respectively. Previous RCTs have been conducted during shorter periods; 42 days [23], 6 weeks [26] and 12 weeks [21], respectively. To expand on the results of a previous study in healthy individuals where no difference between the intervention and placebo groups was observed [21], we chose a study population with frequent RTIs and at least 42 days with infection during the year prior to inclusion. Notably, patients in the study represent a selected group of individuals with frequent RTI, although the immune disorders that they represent (IgA-, IgG-subclass and patients with no defined immune disorder) are generally mild in character and dominated by

mucosal RTIs. We also included a small number of CVID-patients, which can be considered to be a more severe immune disorder, but all these patients are treated with IgG replacement therapy and thus well controlled. Hence, the results from this study cannot directly be applied to the general healthy population. Nevertheless, the results provide solid support for additional interventional studies of vitamin D_3, especially in groups consuming large amounts of antibiotics.

The mechanism of the observed effects remains largely unknown. Vitamin D_3 modulates the immune response at many levels, such as induction of AMPs, skewing of T-cells from Th1/Th17 to Tregs as well as general anti-inflammatory effects [14]. Here, we investigated the role of AMPs in nasal fluid. However, we could not detect any significant changes of LL-37 or HNP1-3 during the study period, but noted that placebo-treated patients tended to have higher levels of AMPs after one year of treatment. This was paralleled by a shift of the microflora in the nasal compartment that could explain the unexpected finding of higher AMP-levels in the placebo group. Recently, it was shown that 1,25 $(OH)_2$-vitamin D_3 induces both HNP1-3 and LL-37 in nasal fluid of healthy volunteers (Hiemstra et al, abstract, European Respiratory Society, 2011), supporting that LL-37 may indeed be induced *in vivo*. However, our study design did not allow such conclusions but rather support that vitamin D_3 affect mucosal immunity, leading to a shift of the microflora. Recently, we showed that the bacterial composition in nasal swabs is an important determinant of AMP-levels in nasal fluid [27].

Given that vitamin D_3 induces LL-37 in epithelial cells and that LL-37 kills bacteria *in vitro,* we expected a reduction of the classical bacterial pathogens *H. influenza*, *M. catharralis* and *S. pneumonia* in the intervention group. However, the frequency of these bacteria was not reduced but a reduction of *S. aureus* and fungal species that often colonise the airways was observed. This could be explained by specific effects by vitamin D_3 on immunity against *S.*

aureus. In fact, vitamin D induces human beta defensin-2 (HBD-2) with bactericidal activity against *S. aureus* [28]. A recent study showed that low vitamin D levels were associated with an increased risk of being colonised by this bacterium [29]. Further, vitamin D affects immunity against *C. albicans*, which indicates direct effects of vitamin D on human immunity [30]. Alternatively, it is possible that vitamin D_3 may have prevented symptomatic viral infections, which prompted patients to leave a bacterial sample from the airways. Interestingly, there is both mechanistic and clinical evidence that vitamin D_3 can prevent viral infections [31-33], although we did not address this in the current study.

Notably, we observed a prominent increase in the serum concentration of 25-hydroxyvitamin D_3, which indicated good compliance and tolerability of the study drug. In fact, there was a trend towards adverse events being reported more often in the placebo group, suggesting that vitamin D_3 possibly could be efficient against other diseases, but this observation requires further studies. No clinically relevant changes of blood chemistry (calcium, phosphate, albumin or creatinine) were observed. Despite few adverse events and high tolerability, 16 exclusions occurred during the study year. The main reason was problems to adhere to the protocol and 6/16 patients dropped out of the study after a few weeks. The rest failed to send in the diaries, did not leave blood for monitoring of safety parameters or did not take the study drug. One patient was excluded based on symptoms that could be attributed to vitamin D_3 (facial paraesthesia). However, this patient was later confirmed to have been allocated to placebo.

In summary, we found that supplementation with vitamin D_3 reduced the total infectious score with 47 points per patient during the study year. A clinically meaningful translation of this effect could be e.g. 47 days with cough (47 points), 23 days with ear and sinus symptoms (23×2=46 points) or 9 days with cough, sinus and ear symptoms together with malaise and antibiotics (9×5=45 points). In addition, our data indicate that vitamin D_3 supplementation

reduces antibiotic consumption with approximately 60% in patients with frequent respiratory tract infections. Thus, supplementation with vitamin D_3 could provide a novel strategy to reduce antibiotic use among high consumers and indirectly prevent the emerging epidemic of bacterial resistance.

Acknowledgment

The study was registered at www.clinicaltrials.gov (NCT01131858), prior to the start of the study. The entire process of study design and protocol, data monitoring and analyses was performed by academic authors; there was no industry involvement in the study except that vitamin D_3 (Vigantol®) and placebo oil (Miglyol®) were provided by Merck KGaA (Darmstadt, Germany). Merck did not have any influence on study design, analysis of data, writing or decision to publish. We extend our gratitude to Ilona Skilving, Karolinska Trial Alliance for invaluable help with the protocol. Further, we thank registered nurses Maria Lindén and Kristina Johansson for skillful work with patients. Thanks also to Jenny Lindén and Alicia Hansson for registration of data and to professor Mats Remberger for discussions on statistical methods. PB, LBB and JDL are holding PostDoc-positions financed by Karolinska Institutet and Stockholm County Council (KI/SLL).

Data sharing statement:

There is no additional data available.

Statements

Author contributions:

Peter Bergman, designed the study, collected, analysed and interpreted data, wrote the paper.

Anna-Carin Norlin, designed the study, collected and interpreted data, wrote the paper.

Susanne Hansen, designed and coordinated the study, collected and interpreted data.

Rokeya Sultana Rekha, carried out experimental work, analysed data

Birgitta Agerberth, analysed and interpreted data, wrote the paper.

Linda Björkhem-Bergman, analysed and interpreted data, wrote the paper.

Lena Ekström, analysed and interpreted data

Jonatan Lindh, analysed and interpreted data, wrote the paper.

Jan Andersson, designed the study, interpreted data, wrote the paper.

Role of the funding source

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Merck GmbH provided the study drug (Vigantol) but did not have any influence on study design, analysis of data, writing or decision to publish.

Ethics statement: The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants.

Conflicts of interest: There are no conflicts of interest.

Legends to figures

Figure 1. Study outline.

Figure 2. Primary endpoint. The adjusted total relative infectious score (A) is expressed 'per quarter' (3 month periods). The adjusted one-year scores (total score, airway, malaise, ear, sinus and antibiotics) are depicted in a Forrest-plot (B) together with 95% confidence intervals. Effects are presented as relative scores (total score, airway, malaise) or odds ratios (ear, sinus, antibiotics, indicated with asterisks).

Figure 3. Secondary endpoint. Vitamin D-levels. Serum was collected at day 0, 3, 6, 9 and 12 months and levels of 25-hydroxyvitamin D_3 were measured. Values are expressed as mean +/- 95% confidence interval.

Tables

Table 1. Baseline data. Mann Whitney U-test was used for comparisons of age and 25-OH vitamin D_3. Fisher's exact test was used for all other comparisons. 1) "Lung disease" comprises asthma, bronchiectasis and chronic obstructive pulmonary disease (COPD); 2) "other disease" includes hypertension, body pain, hypothyroidism and gastritis as most common diagnoses. CVID, common variable immuno deficiency; ND, increased susceptibility to infections without a defined immunological disorder.

	Vitamin D_3	Placebo	p-value
Number	**70**	**70**	
Age (mean)	**55,4**	**50,8**	0.025
Female	**52/70**	**50/70**	
Male	**18/70**	**20/70**	ns
IgG-replacem.	**39/70**	**42/70**	ns
Smoking	**4/70**	**6/70**	ns
25-OH levels (mean)	**51,5 nmol/L**	**46,9 nmol/L**	ns
Immunological diagnosis			
sIgA-deficiency	**9/70**	**9/70**	ns
IgG subclass	**26/70**	**30/70**	ns
CVID	**6/70**	**4/70**	ns
ND	**28/70**	**27/70**	ns
Other imm. diagnosis	**1/70**	**0/70**	ns
Concomitant disease			
No other disease	**16/70**	**18/70**	ns
Lung disease[1]	**35/70**	**38/70**	ns
Other disease[2]	**19/70**	**14/70**	ns

Table 2. Primary Endpoint. Absolute effects expressed as infectious score points (mean) per patient and study period (360 days). The unadjusted values were compared between vitamin D_3 and placebo groups using Mann-Whitney U test. The relative effects are described as adjusted relative score (continuous variables) or adjusted odds ratios (asterisk, dichotomous variables) with 95% CI and p-values derived from multiple regression models.

	Mean score / patient / year			MWU (unadjusted)	Multiple Regression model (adjusted values)		
Endpoint	**Vitamin D**	**Placebo**	**Diff.**	**p-value**	**Effect**	**95% CI**	**p-value**
Total score	202	249	-47	0,023	0,771	0,604-0,985	0,040
Airway	94	101	-7	0,302	0,871	0,706-1,074	0,200
Ear*	16	25	-9	0,225	0,695	0,320-1,501	0,357
Sinus*	18	21	-3	0,126	0,594	0,265-1,328	0,204
Malaise	56	66	-10	0,041	0,845	0,689-1,036	0,108
Antibiotics*	16	33	-17	0,024	0,365	0,153-0,872	0,023

Table 3. Bacterial cultures. [1]Mann-Whitney U-test, [2]Fisher's exact test.

	Vitamin D_3	Placebo	Significance
Number of samples per patient (mean)	2,79	4,85	p=0.010[1]
Number of positive samples per patient (mean)	1,01	2,02	p=0.052[1]
Fraction positive cultures (%)	63/173 (36%)	125/301 (41%)	n.s.[2]
Patients with ≥ 1 sample taken	38/62 (61%)	50/62 (81%)	p=0.029[2]

Table 4. Microbiological findings. Mann-Whitney U-test was used to analyze the total number of findings, whereas Fisher's exact test was used for analysis of the number of patients (fraction) with a specific finding.

	Number of findings (total)			Number of patients		
Microorganism	Vitamin D_3	Placebo	MW-U	Vitamin D_3	Placebo	Fisher
H. influenzae	28	27	ns	10/62	13/62	ns
M. catharralis	8	17	ns	7/62	10/62	ns
S. pneumoniae	7	6	ns	4/62	5/62	ns
S. aureus	6	33	P=0.010	4/62	14/62	p=0.019
Enterobacteriacae	8	8	ns	4/62	7/62	ns
P. aeruginosa	8	15	ns	3/62	4/62	ns
Fungal infection	11	53	P=0.028	4/62	12/62	p=0.058
Total	76	159	P=0.023			

Table 5. Adverse events. Mann-Whitney U-test was used as statistical method.

Organ	Vitamin D_3 n (%)	Placebo n (%)	P-value
CNS	11 (29)	10 (18)	n.s.
Gastrointestinal	4 (11)	12 (21)	**0.058**
Cardiovascular	0 (0)	6 (11)	**0.028**
Infections (other than RTI)	2 (5)	8 (14)	**0.09**
Musculoskeletal	10 (26)	10 (18)	n.s.
Respiratory (non-infectious)	2 (5)	4 (7)	n.s.
Skin	5 (13)	2 (4)	n.s.
Other	4 (10)	4 (7)	n.s.
Total	**38**	**56**	

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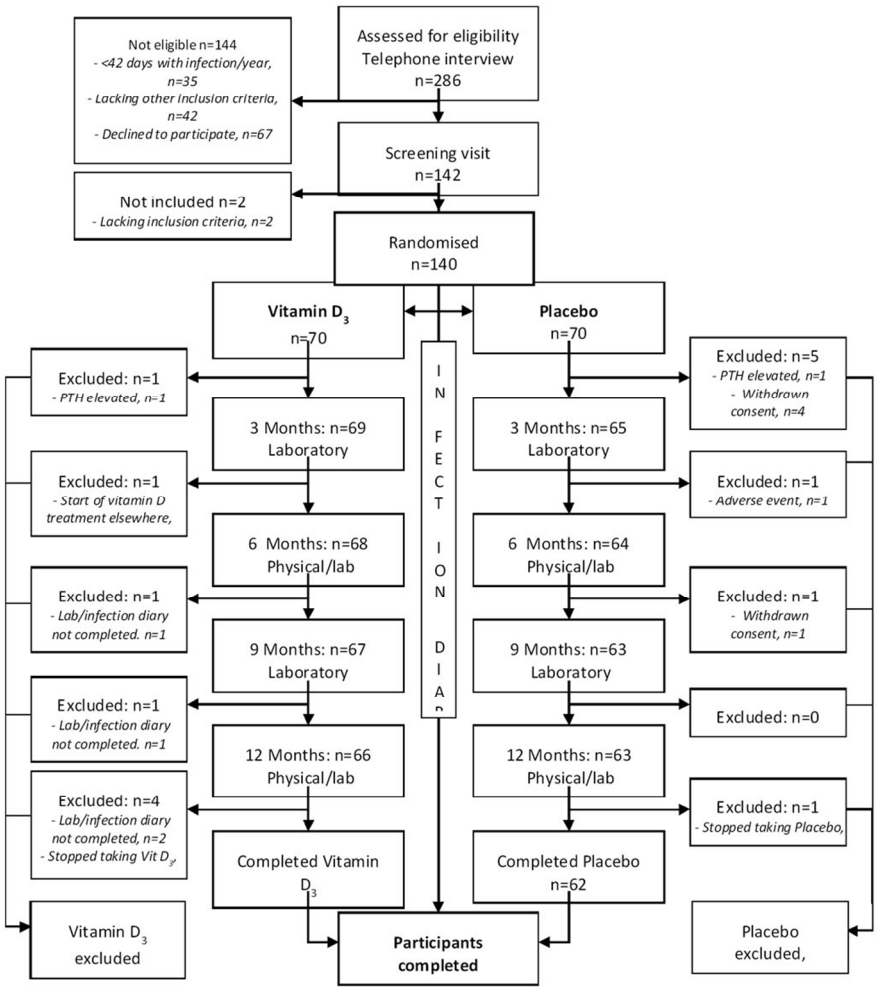

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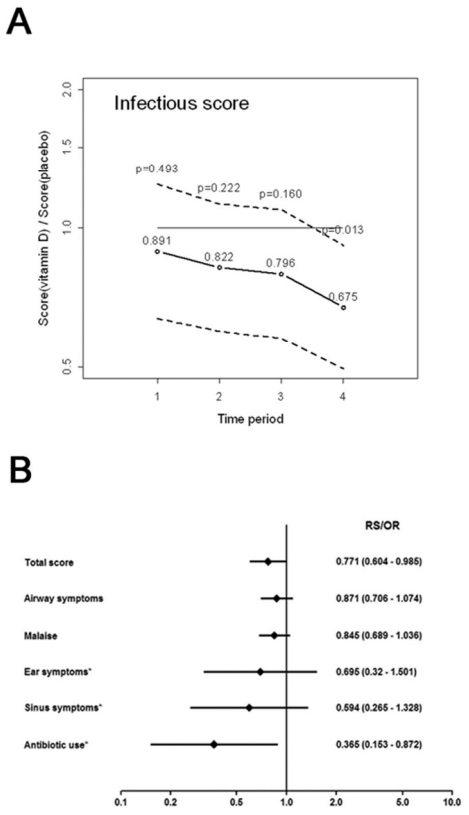

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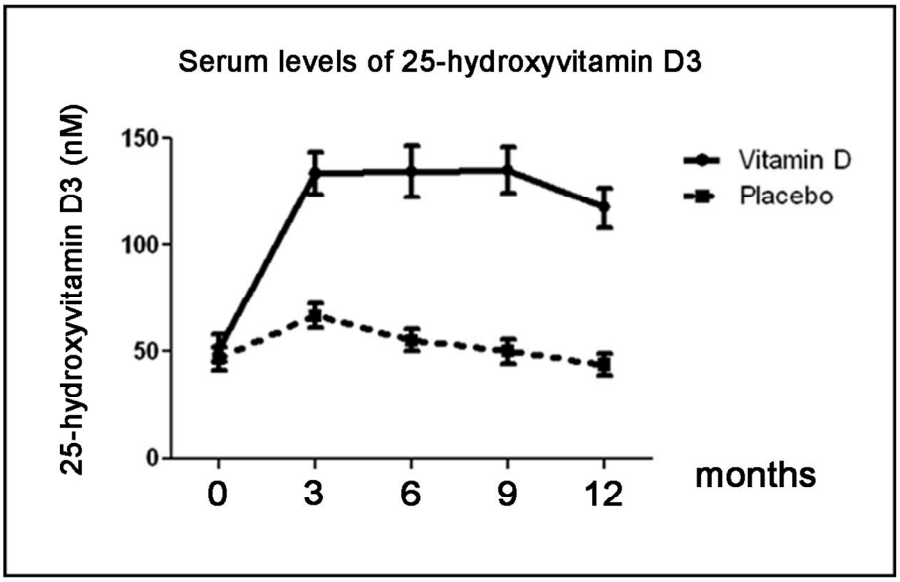

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Bergman et al, Vitamin D_3 supplementation reduces infectious symptoms and antibiotic consumption among patients with antibody deficiency and frequent respiratory tract infections - a randomised controlled trial.

Supplementary tables

Table S1. Primary endpoint. Unadjusted relative score per day calculated per 3 months periods as indicated. Values are expressed as mean +/- SD.

	Month 1-12		Month 1-3		Month 4-6		Month 7-9		Month 10-12	
	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo
Infectious score	0.56(0.58)	0.69(0.54)	0.58(0.66)	0.67(0.70)	0.51(0.61)	0.59(0.57)	0.59(0.67)	0.72(0.65)	0.53(0.56)	0.77(0.61)
Airway symptoms	0.26(0.24)	0.32(0.28)	0.27(0.27)	0.29(0.28)	0.25(0.29)	0.25(0.24)	0.27(0.27)	0.30(0.27)	0.27(0.27)	0.33(0.27)
Malaise	0.16(0.20)	0.18(0.17)	0.16(0.24)	0.18(0.22)	0.14(0.21)	0.15(0.18)	0.17(0.23)	0.19(0.20)	0.15(0.20)	0.22(0.22)
Ear symptoms	0.04(0.09)	0.07(0.15)	0.05(0.12)	0.07(0.17)	0.03(0.09)	0.05(0.14)	0.05(0.10)	0.08(0.18)	0.05(0.10)	0.08(0.18)
Sinus symptoms	0.05(0.12)	0.06(0.10)	0.05(0.12)	0.06(0.13)	0.04(0.10)	0.04(0.10)	0.06(0.15)	0.07(0.13)	0.05(0.14)	0.07(0.12)
Antibiotic use	0.04(0.06)	0.09(0.14)	0.05(0.09)	0.08(0.16)	0.04(0.08)	0.09(0.17)	0.04(0.08)	0.09(0.15)	0.04(0.07)	0.11(0.18)

Table S2: Genotyping. Influence of genetic factors on the outcome of vitamin D_3 treatment in patients with frequent respiratory tract infections. Mean infectious score (0-12 months) are presented per genotype and study group, along with the number of included patients. P-values refer to an interaction between genotype and study group.

Gene / SNP	Allel-group	Vitamin D_3 Mean inf score (n)	Placebo Mean inf score (n)	$p_{interaction}$
VDR: TaqI	tt tT /TT (reference)	145 (11) 202 (49)	225 (12) 253 (47)	0.757
VDR: FoqI	ff fF /FF (reference)	295 (5) 195 (56)	331 (6) 238 (53)	0.575
CYP27B1: Rs10877012	CC AC/CC (reference)	181 (27) 220 (34)	211 (21) 268 (38)	0.194
GC: RS2282679	AA AC/CC (reference)	205 (39) 162 (21)	214 (28) 283 (29)	0.247
CYP2R1: Rs2060793	AA AG/GG(reference)	142 (8) 212 (53)	315 (14) 227 (45)	0.046
CYP24A1: Rs6013897	AA AT /TT(reference)	92 (2) 207 (59)	221 (2) 249 (57)	0.473

Table S3. Detailed description of adverse events.

		VitD$_3$=1 Placebo=2		
Group	**Adverse event**	**1**	**2**	**Total**
CNS	Headache	5		5
	TIA (Transient Ischemic attack)	1		1
	Vertigo	4		4
CNS Total		10		10
Numbness - pain- shakings	Numbness, pain		8	8
	Paresthesias		2	2
	Tremor	1		1
Numbness-pain-shakings Total		1	10	11
Gastrointestinal symptoms	Diarrhoea		2	2
	Diverticulitis		4	4
	Dyspepsia	2	2	4
	Gastroenteritis	2	2	4
	Helicobacter pylori infection		2	2
Gastrointestinal symptoms Total		4	12	16
Heart/ vessels	Congestive heart disease		2	2
	Hypertension		2	2
	Thrombosis		2	2
Heart/ vessels Total			6	6
Infections	Herpes Zoster	1		1
	Pneumonia	1		1
	Sinusitis		2	2
	Urinary tract infection		4	4
	Pyelonephritis		2	2
Infections Total		2	8	10
Body pain – joint pain	Bursitis		2	2
	Body pain	2	2	4
	Joint pain fingers/ hands	1	2	3
	Joint pain hip		4	4
	Pain in feet	1		1
	Back pain	5		5
	Elbow swelling	1		1
Body pain- joint pain Total		10	10	20
Lungs	Asthma exacerbation	1		1
	Pneumothorax		2	2
	Heavy breathing		2	2
Lungs Total		1	4	5
Ears	Hearing problems	1		1
Ears Total		1		1
Other	Shivering	2		2
	Menstruation too often		2	2
	Nose bleeding	1		1
	Toothache	1		1
	Artheritis temporalis		2	2
Other Total		4	4	8
Rash – itch - blisters	Tongue blisters	1		1
	Hand rash	1		1
	Foot rash	1		1
	Facial rash when drinking alcohol		2	2
	Facial rash	1		1
	Chest rash, itching	1		1
Rash – itch – blisters Total		5	2	7
Total		38	56	94

Supplementary figures

Figure S1. The diary that was used for patients to register their daily symptoms.

Symptoms from "airways", "ears" and "sinuses" were calculated as maximum 1 point per anatomical site per day. "Malaise" and "antibiotic consumption" gave maximum 1 point per day. The occurrence of X-ray verified pneumonia resulted in 3 extra points per day. Thus, 8 points was the maximum value that could be obtained per day. These data constituted the primary endpoint of the study. The diaries were filled out by the patient and sent monthly per mail to the study site.

Figure S2. Primary endpoint. Temporal analysis of infectious score components.

The adjusted one-year relative scores presented separately for each 3 month period. (A) airways, (B) malaise, (C) ear symptoms, (D) sinus symptoms and (E) antibiotic consumption. Effects are presented as relative scores (airway and malaise) or odds ratios (ear, sinus and antibiotics). Dashed lines indicate 95% confidence intervals.

Figure S3. Antimicrobial peptides in nasal fluid. Levels of LL-37 (A) and HNP1-3 (B) were measured in nasal fluid extracts at day 0, 6 and 12 months in a randomly selected group of patients (LL-37, n=12; HNP1-3, n=15). There were no statistically significant differences within or between the groups with regards to peptide levels (Mann-Whitney U test). Bacterial growth in these samples were also recorded (C) and expressed as either 'no growth/normal flora' or 'growth of a primary pathogen'. The growth pattern of the vitamin D_3 and Placebo groups were compared at each time-point using Fisher's exact test.

Figure S4. Blood chemistry. Plasma levels of calcium (mmol/L), phosphate (mmol/L), albumine (g/L) and creatinine (µmol/L) were measured at the time points 0, 3, 6, 9 and 12 months after inclusion. Values are expressed as mean values.

Figure S1.

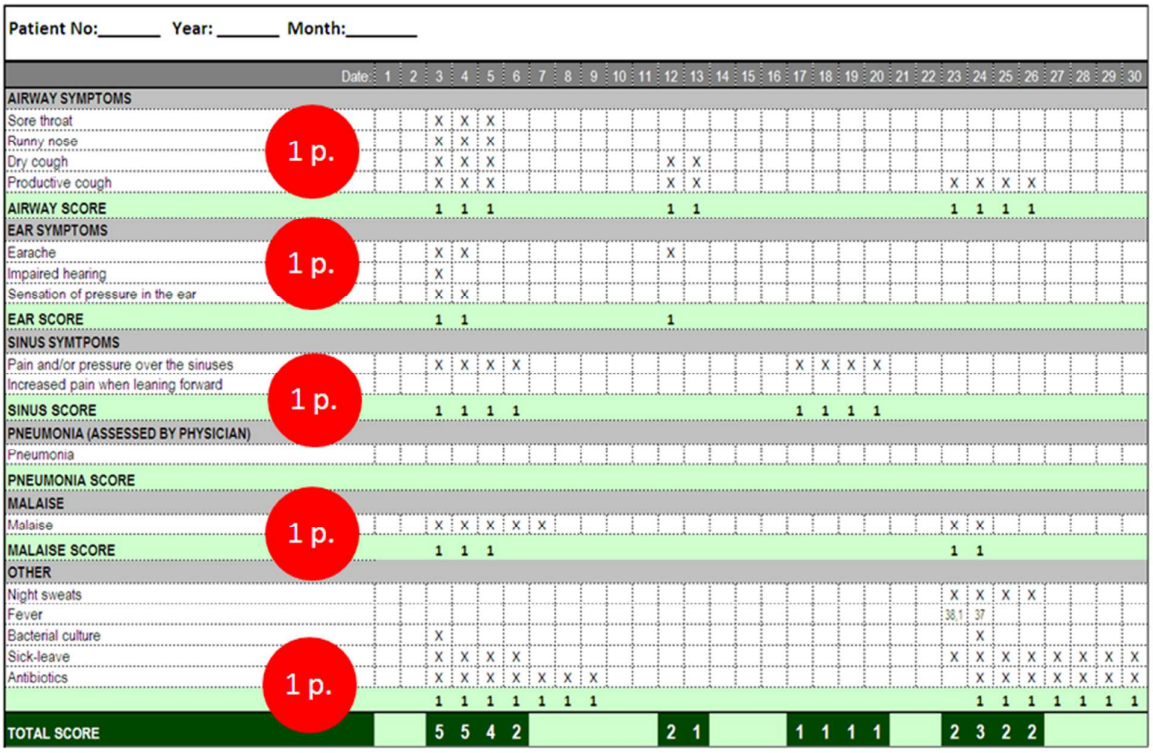


Figure S2

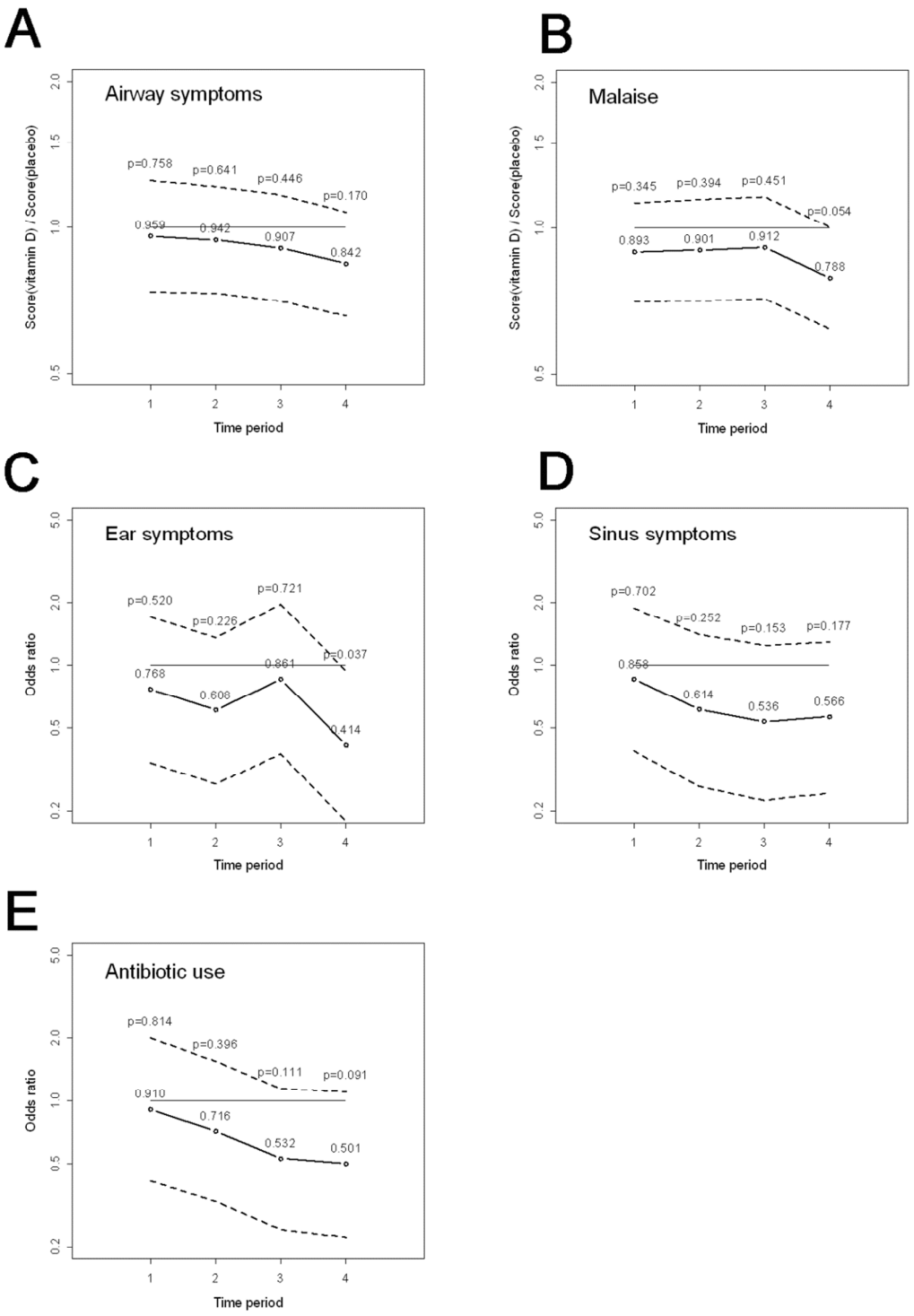

A
Airway symptoms
Score(vitamin D) / Score(placebo)
p=0.758
p=0.641
p=0.446
p=0.170
0.959
0.942
0.907
0.842
Time period
B
Malaise
Score(vitamin D) / Score(placebo)
p=0.345
p=0.394
p=0.451
p=0.054
0.893
0.901
0.912
0.788
Time period
C
Ear symptoms
Odds ratio
p=0.520
p=0.226
p=0.721
p=0.037
0.768
0.608
0.861
0.414
Time period
D
Sinus symptoms
Odds ratio
p=0.702
p=0.252
p=0.153
p=0.177
0.858
0.614
0.536
0.566
Time period
E
Antibiotic use
Odds ratio
p=0.814
p=0.396
p=0.111
p=0.091
0.910
0.716
0.532
0.501
Time period

Figure S3

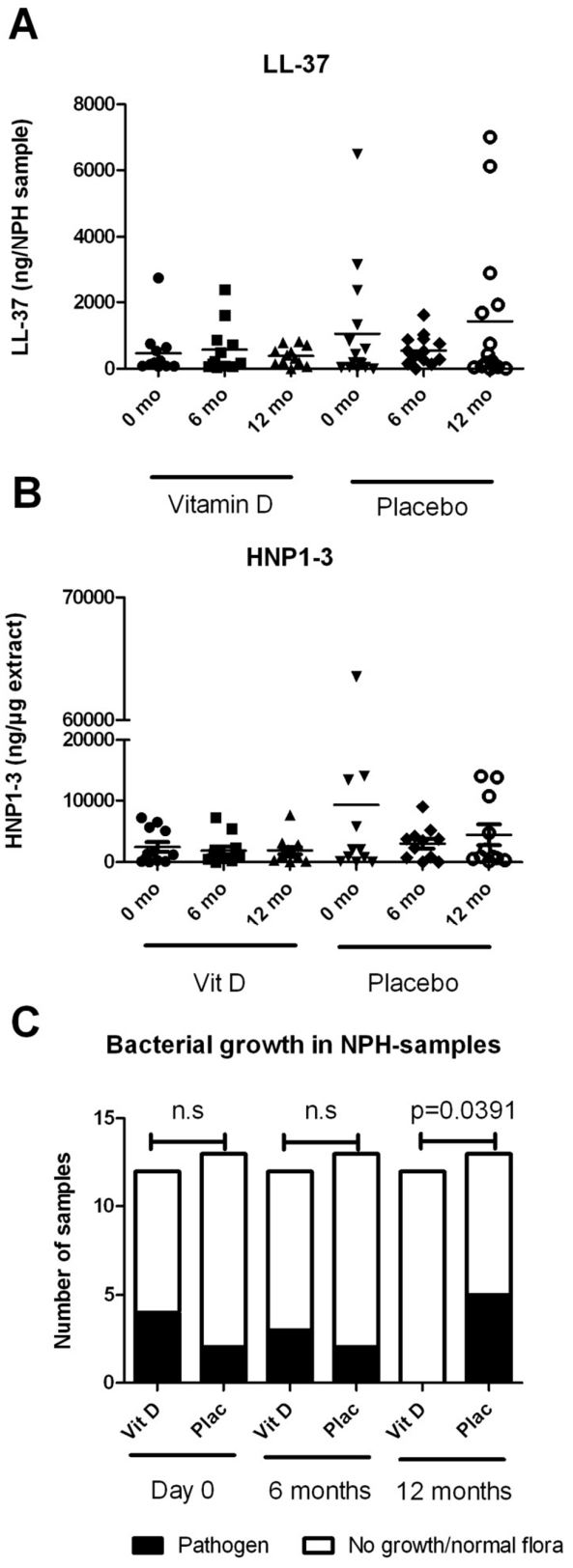
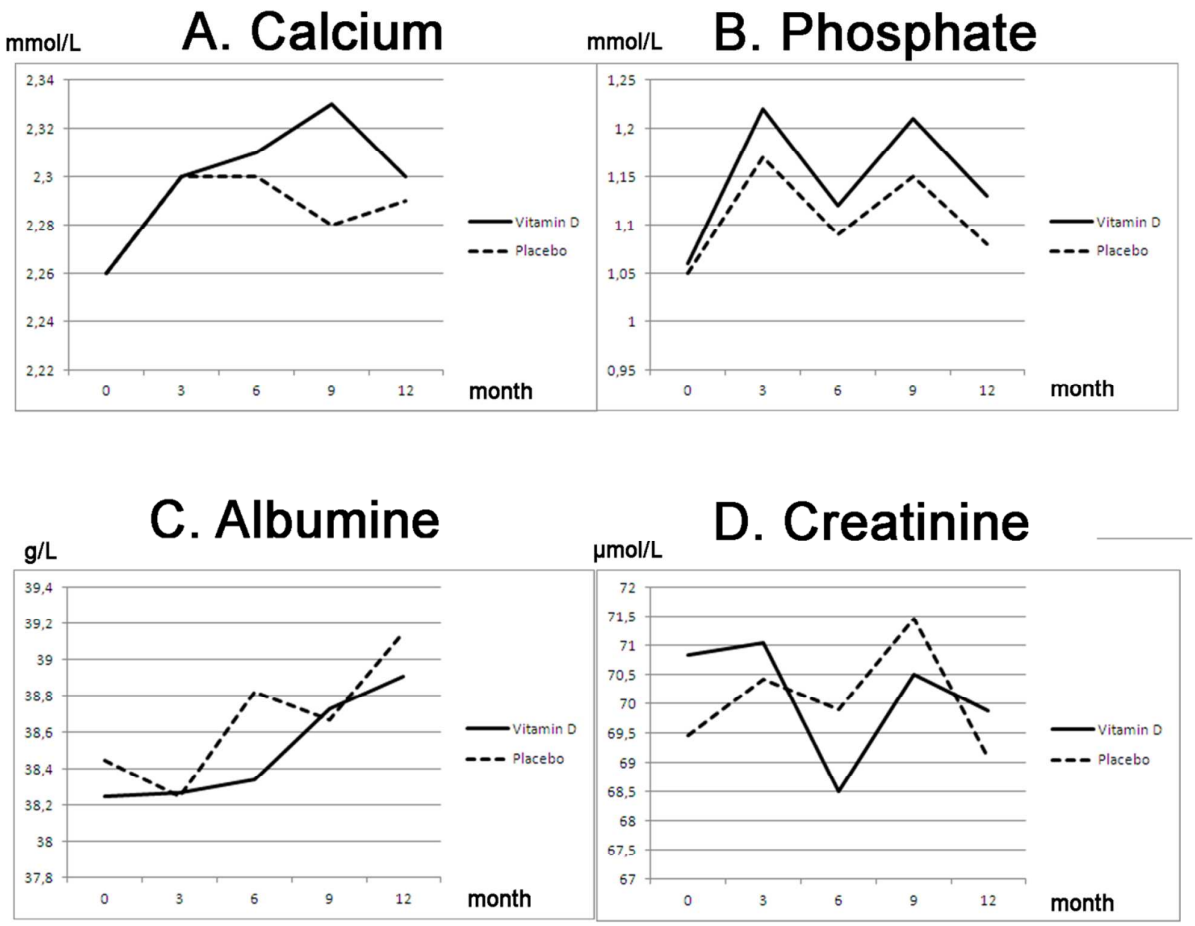


Figure S4



Bergman et al, Vitamin D_3 supplementation reduces infectious symptoms and antibiotic consumption among patients with antibody deficiencies and frequent respiratory tract infections - a randomised controlled trial.

Supplementary Methods

Randomisation and Blinding

A computer-generated list of random numbers was used for patient allocation. Randomization sequence was created using Randomization.com (http://www.randomization.com) and was stratified with a 1:1 allocation using a fixed block size of 10. Within each block two participants were randomly assigned to provide samples of nasal fluid, one for each treatment group.

The vitamin D_3 and placebo were in liquid form and identical in appearance. They were prepacked in bottles and consecutively numbered for each participant according to the randomization schedule. In connection with the inclusion each participant was sequentially assigned a number by the responsible physician and received the corresponding prepacked bottles.

Participants, investigators and staff were kept blinded to the allocation throughout the trial. It was not necessary to un-blind information on any participant during the trial.

Sampling of nasal fluid, NPH swabs

Since vitamin D_3 can induce antimicrobial peptides both in macrophages and in epithelial cells[1], we measured levels of LL-37 and α-defensins (HNP1-3) in nasal fluid (Figure 4A and

B). For logistical reasons we limited patients for nasal fluid collection and only 36/140 patients (20%) were randomised to this procedure. Nasopharyngeal swabs were taken from one nostril and sent to the Clinical Microbiology Laboratory at Karolinska University Hospital, Huddinge for bacterial culture. The bacterial content was evaluated as "no growth of bacteria", "normal flora" (typical findings include α-haemolytic streptococci, *Corynebacteria* spp, *Neisseria* spp. and other nonpathogenic strains) or "pathogenic growth" (defined here as *H. influenzae, S. aureus, S. pneumoniae, M. catharralis* and *Enterobacteriacae* spp). Subsequently, nasal fluid was collected through a thin plastic tube that was carefully placed in the back of the nose using the other nostril as entry port (10-12 cm from the nostril meatus). 5-10 ml of saline was administered into the nose prior to sampling in order to make the epithelial lining moist and to dissolve mucus depositions. A gentle vacuum was applied and 3-5 ml nasal fluid was collected and stored at -20°C, as described in Cederlund et al, PLoS One, 2011[2].

Extraction of peptides and proteins from nasal fluid

Nasal fluid (3-5 ml) was extracted in an equal volume (1:1) of 60% acetonitrile (AcN) in 1% trifluoroacetic acid (TFA) over night at 4°C. The extract was centrifuged at 3500g and the supernatant was lyophilized. The lyophilized extract was resuspended in 0.1% TFA and enriched for polypeptides using solid phase extraction as described in[2]. The lyophilized polypeptide extract was reconstituted in 0.1% TFA to a concentration of 5 µg/µl as determined spectrophotometrically using a Nanodrop-system (Thermo Scientific, Wilmington, U.S.).

Analysis of antimicrobial peptides in nasal fluid

The concentrated and lyophilized extract (25 µg) was dissolved in lithium dodecyl sulphate (LDS) sample buffer, 50 mM Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 70°C for 10 min. The samples were then separated using LDS-PAGE and blotted onto PVDF membranes, as described in[3]. Antibodies used were a LL-37 monoclonal[4] and a HNP1-3 goat polyclonal (sc-22916, Santa Cruz, Santa Cruz, Calif., USA). Proteins and peptides were visualized on chemiluminescence film with ECL plus Western blot detection system (GE Healthcare, Buckinghamshire, United Kingdom). LL-37 and HNP1-3 concentration in nasal fluid were determined by densitometry using the software ImageJ (http://rsbweb.nih.gov/ij/). The intensity of each band was normalized to an external standard on each membrane and the total amount of LL-37 and HNP1-3 was determined by multiplying the densitometric result (ng peptide/µg extract) with the total amount of polypeptide-extract (µg). Thus, the values represent the total amount of LL-37 and HNP1-3 from each nasal fluid sample.

Analysis of 25-OH vitamin D_3 in serum

Levels of 25-hydroxyvitamin D_3 in serum were determined by using DiaSorin immunochemical method (DiaSorin S.p.A, Saluggia, Italy) at the Department of Clinical Chemistry, Karolinska University Hospital.

Genotyping

Specific single nucleotide polymorphisms (SNPs) in key genes for vitamin D metabolism might influence the outcome of vitamin D_3 supplementation. Therefore, all patients were

genotyped for 6 SNPs in the VDR (TaqI and FokI), CYP27B1, CYP24A1, CYP2R1 and GC genes. Six SNPs in five genes involved in vitamin D metabolism and / or effect were analysed in all participants. The aim of these analyses was to investigate whether individuals with a specific genotype would benefit more from vitamin D_3 supplementation. Genomic DNA was isolated from 200 µl peripheral blood leucocytes using the DNA Blood Mini kit (Qiagen, Hilden Geramany). Allelic discrimination reactions were performed using TaqMan® genotyping assays (Applied Biosystems, Foster City CA USA): C_12060045_20 for VDR (FokI); C_2404008_10 for VDR (TaqI); C_29958084 for CYP24A1; C_2958431_10 for CYP2R1; C_26407519_10 for GC. For the CYP27B1 genotyping, primers and probes described previously were used[5]. The final volume for each reaction was 15 µl consisting of 30 ng DNA and 2xTaqman Universal PCR Master mix (Applied Biosystems). The PCR profile consisted of 95° C for 10 minutes followed by 40 cycles of 92° C for 15 sec and 60° C for 1 minute. The fluorescence signal was measured with an ABI 7500 Sequence detector (Applied Biosystems).

Statistical methods: Sample size calculation

The sample size was based on the assumption that the intervention would reduce the number of days with symptoms from 42 days (210 points) to 28 days (140 points), i.e. a reduction of the infectious burden by 30%. Given this assumption, a sample size of 60 patients per study group was predicted to provide the study 90% power at a significance level of p=0.02 (Student's t-test). To compensate for predicted exclusion of participants, the groups were increased to include 70 patients per treatment arm.

Statistical methods: Primary analysis

The distribution of the infectious score was found to be very skewed, thereby violating the normal assumption of the pre-specified t-test analysis. Hence, the Mann Whitney U-test, a non-parametric equivalent of the t-test, was instead uses in all unadjusted analyses.

Further, the randomisation had resulted in age distributions that were not entirely balanced between the two groups. Since there might be concerns that such imbalance could influence the results of the study, the original analysis plan was extended with a multivariable analysis adjusting for potential confounders. In this linear regression model based on log-transformed values of the primary outcome (the total infectious score) and its individual components, adjustment was made for age, gender, smoking, type of immune deficiency and significant co-morbidities (respiratory or non-respiratory). Due to the transformation procedure, the adjusted effect of vitamin D_3 is expressed as a ratio between the score in the vitamin D_3 and the placebo group. In this multiplicative model, an effect size of 1 indicates identical outcome in the two study groups and statistically non-significant results are recognized by confidence intervals encompassing the value 1.

To explore potential divergent effects on different organ systems, both adjusted and unadjusted analyses were repeated separately for each individual item of the infectious score. In addition, the temporal aspects of the vitamin D_3 effect were investigated by dividing the study period into four 90-day periods (starting on the first day of treatment) and repeating the analyses separately for each time period. “Ear” and “sinus” symptoms as well as “antibiotic use” occurred at low frequencies and for these entities normal distributions could not be achieved despite data transformation. Thus, the adjusted analyses of these individual items were based on multivariable logistic regression, after coding the symptom (or antibiotic

therapy) as present or absent during the course of the study. However, this only applies to analysis of the individual items, and not to the primary analysis of the total infectious score, where all item scores were added as originally described.

Most post-randomization exclusions were due to patients failing to fill out the symptoms diary. Hence, no intention to treat (ITT) analysis based on actual outcome data could be performed. However, the potential impact of dropouts was addressed in an ITT analysis based on total infectious scores imputed as follows: In excluded patients, all available outcome data was used and during time periods with missing data (from time of dropout until one year post-randomization) patients' scores were substituted with the average score in placebo-treated patients completing the study. This imputation was used in all excluded patients, regardless of treatment allocation to achieve a conservative estimate of the treatment effect (by assuming zero-effect whenever data was missing).

Statistical methods: Secondary analyses

The number of bacterial cultures taken in each patient and the number of samples with a positive finding were compared between the two study groups by means of the Mann-Whitney U test. To reduce the influence of patients subjected to very frequent sampling, the odds of having one or more culture taken during the course of the study was also compared by means of Fisher's exact test. Similarly, the frequencies of cultures positive for specific pathogens were compared both as number of positive cultures per patient (Mann-Whitney U test) and as fraction of patients presenting with at least one positive culture (Fisher's exact test). The fraction of nasopharyngeal samples exhibiting bacterial growth was compared

between the two groups separately for samples taken at baseline, after six month and after 12 months (Fisher's exact test).

The influence of genetic polymorphisms on the effect of vitamin D_3 treatment was analysed in linear regression models with log-transformed infectious score as dependent variable. Independent variables were study group, genotype and a genotype-study group interaction term. Genotypes were coded as binary variables, based on previous findings reported in the literature[5-10].

In all analyses, P values <0.05 (two-sided) were considered statistically significant. All statistical analyses were performed using R 2.11.1 (R Development Core Team (2010). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org) and GraphPadPrism, version 5.0, GraphPad Software, La Holla, Calif, USA

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Study title:

Study of Vitamin D3 Substitution to Patients With Primary Immunodeficiency (VITAPID)

Date: October 6, 2009

Study site: Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden

Product: Vigantol oil

Substance: Vitamin D3 (cholecalciferol)

Producer: Merck, Germany

EudraCT-number: 2009-011758-16

Sponsor: Professor Jan Andersson, MD, PhD

Co-investigators: Dr Peter Bergman, MD, PhD; Dr Anna-Carin Norlin, MD

Study facts

Protocol identity and aim

EudraCT-number: 2009-011758-16

Protocol title: A placebo-controlled double-blind study of Vitamin D3 supplementation to patients with increased susceptibility to infections.

Aim: To investigate if substitution with vitamin D3 can prevent or ameliorate infectious burden among infection prone patients.

Study drug:

Product:	Vigantol Oil
Pharmaceutic preparation:	Oral mixture (oil)
Administration:	Per os

Methodology

Study design:	Randomized double-blind placebo-controlled
Dose:	Vigantol, 4000 IU/day
Primary endpoint:	Infectious score
Safety parameters:	Plasma levels of calcium, creatinine, albumin and phosphate; serum levels of 25-OH vitamin D_3.
Study population:	Patients with increased susceptibility to respiratory tract infections.
Number:	140

Timeplan:

First patient to be included: Q1, 2010

Last patient to be included: Q4, 2011

Last patient to finish the study: Q4, 2012

Administrative information

Sponsor and Investigators

Professor Jan Andersson, MD, PhD: Sponsor and Principal Investigator

Dr Peter Bergman, MD, PhD: Co-investigator

Dr Anna-Carin Norlin, MD: Co-investigator

Research nurses and study coordinator

Susanne Hansen, Study coordinator, head of Immunodeficiency Unit

Kristina Johansson, Research Nurse

Maria Lindén, Research Nurse

Quality control

Two independent monitors from Karolinska Trial Alliance will monitor the study according to ICH-GCP.

Overview and significance

The innate immune system is depending on antimicrobial peptides, which are potent killers of microbes, such as bacteria, viruses and fungi. These molecules defend epithelial surfaces and are rapidly released after contact with microbes. Vitamin D is a potent inducer of AMPs in epithelial and immune cells. Vitamin D is synthesized in the skin under the influence of UVB-light or can be obtained via the diet. However, in Sweden the UV-radiation has too low intensity during the wintertime and the diet is not enough to maintain adequate levels. Therefore many individuals in Sweden have low levels of vitamin D3, especially during the darker period of the year (October-April). Epidemiological data show a strong association between low vitamin D levels and an increased risk of infection. There is also mechanistic evidence that vitamin D increases the levels of antimicrobial peptides in macrophages and in epithelial cells. However, there are few randomized controlled trials testing the hypothesis that supplementation with vitamin D3 can reduce or ameliorate infections. Therefore, we have designed the study described in this protocol where vitamin D3 will be given to patients with an increased risk of infection. The results may have a great impact on treatment of patients with frequent infections, since vitamin D3 may be used in conjunction with standard care (antibiotics). This may be particularly important in light of the emerging bacterial resistance. Thus, novel strategies to prevent and treat infectious diseases have to be developed and supplementation with Vitamin D3 may constitute one future treatment option.

Aims

To investigate if substitution with vitamin D3 can:

1. Reduce the infectious burden among patients with increased number of infections
2. Increase levels of antimicrobial peptides in nasal fluid
3. Increase serum concentrations of 25-OH vitamin D3

Study design

Participants will be given vitamin D3 or placebo for one year. 140 patients will be recruited and 70 patients will be randomized to placebo or vitamin D3 in a 1:1 randomization. Evaluation of symptoms and antibiotic consumption will be registered by the patient in a diary form that will be sent by mail to the study site on a monthly basis. Patients will be recruited at the tertiary center for primary immune deficiencies. Currently there are 319 patients with IgG-deficiency, 180 patients with selective IgA-deficiency, 90 patients with CVID and 210 patients with an increased susceptibility to infection without a manifest immunological diagnosis. The study patients will be recruited from this group in a nonbiased fashion, ie regardless of diagnosis or IgG-substitution therapy.

Study drug and mechanism

The study drug is cholecalciferol (vitamin D3), which is hydroxylated in the liver to 25-OH vitamin D3 (the storage form in the body). The second hydroxylation step is carried out by 1-alpha hydroxylase. This enzyme is expressed in the kidney but also in immune- and epithelial cells. The kidney is

responsible for the systemic production of 1,25 (OH)2 vitamin D3, which is crucial for skeletal health (endocrine system). The local activation of vitamin D3 in immune- and epithelial cells is described as a paracrine system and is central to the immune effects of vitamin D. The paracrine system is strictly regulated and does not contribute substantially to the systemic levels of 1,25 (OH)2 vitamin D3. This is important since the active and systemically available vitamin D3 is responsible for hypercalcaemia that has been reported as an adverse event for vitamin D3 supplementaton. However, hypercalcaemia is a very rare event and we will strictly follow plasma levels of calcium and 25-OH vitamin D3 during the study period.

Study drug

Vigantol Oil is not a registered drug in Sweden. However, Merck Pharma GmbH has permission to manufacture and sell Vigantol in Germany since many years (permission nr 6154275.00, ATC code A11CC05, mSPC available upon request). The study drug is manufactured according to GMP (GMP certificate from Merck available upon request). The placebo oil is also manufactured by Merck and has identical galenic properties to Vigantol oil. The drugs (vigantol and placebo) will be delivered to Vecura AB at Karolinska University Hospital, Huddinge. Vecura AB is a company specialized in clinical trials and has a GMP-certificate for clinical trials and handling of study drugs. VECURA AB will aliquot the study drug and placebo to the final bottles, carry out randomization and labelling.

Endpoints

Primary endpoint

Infectious score based on patient recorded information registered in a diary-form.

Secondary endpoints

25-OH vitamin D3 in serum

Microbiological findings and numbers of cultures taken

Levels of antimicrobial peptides in nasal fluid

Antibiotic consumption collected from patient records

Design

Evaluations and procedures

Prescreening: Eligible patients fulfilling inclusion criteria will be selected from records and contacted via regular mail. They will be sent a letter of invitation together with information on the study. All these patients will be contacted via telephone one week later and asked for participation.

Visit 1, screening, time=0: Co-investigator (Licenced physician, MD) will meet all patients for screening. Additional information on the study will be given and informed consent will be collected. If the patient is judged to fulfil all criteria for inclusion and all exclusion criteria can be negated, the patient is included in the study. The study drug for 6 months will be given out to the patients as well as diaries and envelopes. The patient will be carefully informed about the procedures with the diaries. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Visit 2, time=6 months +/- 14 days: Co-investigator (Licenced physician, MD) will meet all patients after 6 months. A control for adverse events and compliance will be carried out. Additional bottles of study drug/placebo will be given out for the remaining 6 months of the study. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Visit 3, time= 12 months +/- 14 days: Co-investigator (Licenced physician, MD) will meet all patients after 12 months. A control for adverse events and compliance will be carried out. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Participants

Inclusion Criteria:

- Age 18-75
- Increased number of respiratory tract infections
- At least 42 days of infections during 2008 or 2009
- S-25 OH vitamin D3 < 250 nM
- Not planning a pregnancy during the coming year
- Accepting the use of contraceptives during 1 year

Exclusion Criteria:

- Continuous antibiotic treatment
- Hypercalcaemia
- Sarcoidosis
- Kidney disease
- Tuberculosis
- Pregnancy
- Previous history of kidney stones
- Heart medication (glycosides)

Treatment

Vigantol oil (cholecalciferol). 1 drop contains 500 IU. The patients should take 8 drops per day during the study.

Packing, labelling and handling of study drug: Merck will distribute Vigantol oil and placebo oil to VECURA AB, Karolinska University Hospital, Huddinge, which will handle, pack and label the study drug.

Distribution of the study drug to the participants: At the first visit, the participants will be given study drug for the first 6 months of the study. After 6 months, they will be given the remaining bottles. Oral and written instructions will be given about 8 drops per day .

Blinding and breaking of the code: The design is double-blind. Thus, neither the doctor/nurse nor the patient will have any information on the nature of the study drug. Two monitors will carry out controls of the study. The randomisation list will be stored in such a way that the personnel involved in the study do not have access to it. The Hospital Pharmacy will be given a copy of the list in case of emergency with access via telephone 365 days per year/24 hours per day.

Concomitant medication: All other medication is allowed during the study, including antibiotics. However, recent changes in drug treatments will be documented in the diary and asked for by the study doctors at visits.

Compliance: The compliance to the study drug and diary registration is asked for at visits to the study site.

Control of the study drug: Patients are asked to bring back their empty bottles to the study sites. All bottles will be registered by the study nurses.

Evaluation of efficacy and safety

Evaluation of primary endpoint (clinical endpoint, patient recorded)

The primary endpoint is the infectious score, which is based on the diary form filled out by the patient. The total score is composed of symptoms from airways, sinus and ears together with malaise and antibiotic consumption. The idea is to monitor several aspects of an infectious episode and thus monitor the total infectious burden, rather than a specific symptom.

Evaluation of secondary endpoints (microbiological and biochemical endpoints, collected by the study personnel)

1. 25-OH vitamin D3 in serum
2. Microbiological findings and numbers of cultures taken. This information will be collected from patients' clinical records with a focus on samples taken from the respiratory tract.
3. Levels of antimicrobial peptides in nasal fluid. Every fifth patient (according to a special randomization list) will be asked to leave nasal fluid for analysis of antimicrobial peptides.
4. Antibiotic consumption collected from patient records. Information on how many prescriptions of antibiotics will be collected from patients' records.

Evaluation of clinical safety for participants

Patients will leave blood for analyses of plasma levels of creatinin, calcium, phosphate and albumin as well as serum levels of 25-OH vitamin D3 at times 0, 3, 6, 9 and 12 months. The information regarding all time-points except at inclusion will be sent to an unblinded senior physician who will serve as an external clinical safety monitor. He will contact the study physicians in case of clinically relevant abnormalities in the blood chemistry.

Samples and clinical chemistry

Serum and plasma from the first sampling will be sent to Dept of Clinical Chemistry, Karolinska University Hospital, Huddinge for routine analyses. These answers will be recorded in patients' records. For all other time points samples will be sent to Study Center Karolinska which will coordinate all samples for clinical chemistry and send answers to the unblinded clinical safety monitor. These answers will not appear in patients' records in order to keep the blinded design intact.

Adverse events (AE) and Severe adverse events (SAE)

All adverse events and severe adverse events will be recorded in special forms. They will further be classified for severity (mild, moderate and severe) and for connection with the study drug (probable, possible and unlikely). All SAE will be reported to the sponsor within 24 hours after it has been known by the investigator.

Statistics

Handling of data: All data will be registered in a database especially constructed for the study.

Analysis of excluded patients: Excluded patients will be recorded and followed for adverse events. After the study, special analyses will be performed to understand why these patients did not complete the study.

Statistical analysis and power calculation: The statistical calculation is based on the assumption that the infectious score is reduced with 30 % from 42 days (42x5=240 points) to 28 days (28x5=160 points) with full infectious score. If we include 60 patients per group a significance level of p=0.02 will be reached with a power of 90%. To compensate for expected exclusions, we will increase the number of patients per group to 70. Thus, the total number of patients in the study will be 140.

Quality control

Source data: The information that will be collected from each participant will be: study title, screening number, patient number, written informed consent, main and concomitant diagnoses, study treatment, medication, data on blood chemistry and other investigations carried out.

Monitoring: All study personnel have knowledge on clinical trials and ICH-GCP. The sponsor will sign a contract with Karolinska Trial Alliance for monitoring. The investigator will allocate time for monitoring and supply all available and relevant information to the monitors.

Ethics

The sponsor has applied for ethical approval from the regional Ethical Board. The study will be carried out according to ICH-GCP and the Helsinki-declaration.

Informed consent: The patient will have information sent home via regular mail. One week later the study nurse will call the patient and discuss the study. The first visit will involve the meeting with a physician and time is extended for questions before the written informed consent is signed. One copy will stay at the study site and one copy will go with the patient.

Handling of data

Case Report Forms (CRF) will be used. These will kept at the study site until the end of the study. After completion of the study, all material will be archived at least 10 years.

Insurance

All participants are insured via patient insurance and the Swedisg drug insurance.

Publication of the results

The study group wishes to publish the data in a refereed international scientific journal and to communicate the results at conferences and other venues.

The original protocol is written in Swedish and is available on request.

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	p. 1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	p. 2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	p. 5-6
	2b	Specific objectives or hypotheses	p. 6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	p. 7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	Not Applicable (NA)
Participants	4a	Eligibility criteria for participants	p. 7
	4b	Settings and locations where the data were collected	p. 7
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	p. 8
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	p. 8
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	Suppl meth p4
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	Suppl meth.
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Suppl meth.
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	Suppl meth.

Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	p. 9
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	Suppl meth.
	11b	If relevant, description of the similarity of interventions	NA
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	p. 8-9
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	Suppl meth.
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	p. 10, Fig 1
	13b	For each group, losses and exclusions after randomisation, together with reasons	p. 10, Fig 1
Recruitment	14a	Dates defining the periods of recruitment and follow-up	p. 5
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Yes
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Yes
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Yes
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Yes
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Table 5 +suppl fig/tabl
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Yes
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	Yes
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Yes
Other information			
Registration	23	Registration number and name of trial registry	p. 7
Protocol	24	Where the full trial protocol can be accessed, if available	p. 7
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	p. 20

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

BMJ open

Vitamin D3 supplementation in patients with frequent respiratory tract infections - a randomised, double-blind intervention study

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Vitamin D_3 supplementation in patients with frequent respiratory tract infections

- a randomised, double-blind intervention study

Peter Bergman*[1,3,6#], Anna-Carin Norlin*[2,3], Susanne Hansen[3], Rokeya Sultana Rekha[4], Birgitta Agerberth[4], Linda Björkhem-Bergman[5], Lena Ekström[5], Jonatan D Lindh[5], Jan Andersson[6]

1. Department of Laboratory Medicine, Clinical Microbiology, Karolinska Institutet
2. Department of Laboratory Medicine, Clinical Immunology, Karolinska Institutet
3. Karolinska University Hospital, Clinical Immunology, Immunodeficiency Unit
4. Department of Medical Biochemistry and Biophysics, Karolinska Institutet
5. Department of Laboratory Medicine, Clinical Pharmacology, Karolinska Institutet
6. Department of Medicine, Center for Infectious Medicine (CIM), Karolinska Institutet

*Equal contribution

Positions:

Peter Bergman, MD, PhD, Resident Physician, Clinical Microbiology

Anna-Carin Norlin, MD, Specialist in Clinical Immunology

Susanne Hansen, RN, Head of Immunodeficiency Unit

Rokeya Sultana Rekha, MSc, PhD-student

Birgitta Agerberth, PhD, Professor, Medical Microbial Pathogenesis

Linda Bjorkhem-Bergman, MD, PhD, Resident Physician, Clinical Pharmacology

Lena Ekström, PhD, Associate Professor, Clinical Pharmacology

Jonatan D Lindh, MD, PhD, Specialist in Clinical Pharmacology

Jan Andersson, MD, PhD, Professor, Infectious Diseases

Running title: Vitamin D_3 supplementation in patients with frequent respiratory tract infections

#Corresponding author:

Peter Bergman, MD, PhD

Department of Laboratory Medicine, Clinical Microbiology F68

Karolinska Institutet and Karolinska University Hospital, Huddinge

SE-141 86 Stockholm, Sweden

peter.bergman@ki.se

Phone: +46858587942

Fax: +46858581305

Competing interest statement

All authors have completed the Unified Competing Interest form (available on request from the corresponding author) and declare no support from any organisation for the submitted work, no financial relationships with any organisations that might have an interest in the submitted work in the previous three years and no other relationships or activities that could appear to have influenced the submitted work.

Article Summary

Article focus

• Recent evidence suggests that vitamin D_3 has potent extra-skeletal effects, such as suppression of inflammation and strengthening of mucosal immunity by induction of antimicrobial peptides.

• Data from observational studies suggest that low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections.

• Results from a limited number of randomised controlled trials on the protective role of vitamin D_3 against respiratory tract infections are inconclusive and thus additional studies are warranted.

Key Messages

• Therefore we designed and carried out a randomised controlled trial where a large dose (4000 IU) of vitamin D_3 was given to patients with an increased susceptibility to infections for one year.

• The main conclusion is that vitamin D_3 supplementation reduces symptoms and antibiotic consumption among patients with an increased frequency of respiratory tract infections. Thus, vitamin D_3 supplementation may be an alternative strategy to reduce antibiotic use among patients with recurrent respiratory tract infections.

Strengths and limitations

• Strengths: A high daily dose of vitamin D3 was used, the study time was a full year covering all seasons and patients with an increased frequency of respiratory tract infections were studied.

• Limitations: A single study center, small sample size (n=140) and a selected group of patients.

Abstract

Background: Low serum levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections (RTIs). Clinical trials with vitamin D_3 against various infections have been carried out but data are so far not conclusive. Thus, there is a need for additional randomised controlled trials of effects of vitamin D_3 on infections.

Objective: To investigate if supplementation with vitamin D_3 could reduce infectious symptoms and antibiotic consumption among patients with antibody deficiency or frequent RTIs.

Design: A double-blind randomised controlled trial.

Setting: Karolinska University Hospital, Huddinge

Participants: 140 patients with antibody deficiency (sIgA-, IgG subclass deficiency, CVID) and patients with increased susceptibility to RTIs (>4 bacterial RTIs/year) but without immunological diagnosis.

Intervention: vitamin D3 (4000 IU) or placebo was given daily for one year

Primary and secondary outcome measures: The primary endpoint was an infectious score based on five parameters: symptoms from respiratory tract, ears and sinuses, malaise and antibiotic consumption. Secondary endpoints were serum levels of 25-hydroxyvitamin D_3, microbiological findings and levels of antimicrobial peptides (LL-37, HNP1-3) in nasal fluid.

Results: The overall infectious score was significantly reduced for patients allocated to the vitamin D-group (202 points) compared with the placebo group (249 points) (adjusted relative score 0.771, 95% CI 0.604-0.985, p=0.040).

Limitations: A single study center, small sample size and a selected group of patients.

Conclusions: Supplementation with vitamin D_3 may reduce disease burden in patients with frequent respiratory tract infections.

Primary funding source: Swedish Foundation for Strategic Research (SSF)

The study was registered at www.clinicaltrials.gov (NCT01131858)

273/275 words

Introduction

Vitamin D was discovered when it was noted that rachitic children were improved by exposure to sunlight [1]. It was later shown by Holick *et al* that vitamin D_3 is synthesized in the skin under the influence of UVB-light [2]. Vitamin D_3 is further hydroxylated in the liver to 25-hydroxyvitamin D_3, which is considered to reflect the vitamin D status of an individual patient [3]. The final activation to the active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)D_3$) requires 1-α-hydroxylase activity. This enzyme (also designated CYP27B1) is expressed in the kidney but also in many other cell-types, including epithelial- and immune-cells [4]. The active vitamin D_3 ($1,25(OH)D_3$) binds to the vitamin D receptor (VDR), which belongs to the nuclear receptor family. Active vitamin D_3 is only present in minute amounts in the circulation and local activation in target cells is crucial for vitamin D-mediated effects on the immune system[5].

Low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of tuberculosis [6-8] and respiratory tract infections [9]. The mechanism is not fully elucidated but vitamin D_3 has been shown to induce antimicrobial peptides in immunecells [10]. In addition, active vitamin D_3 ($1,25(OH)D_3$) has broad anti-inflammatory effects on the adaptive immune system by shifting the T-helper cell pool from a Th1/Th17-response to a Th2/Treg-dominated response [11 12]. Vitamin D_3 has also been shown to suppress the Th2-response in allergic bronchopulmonary aspergillosis [13]. Thus, vitamin D_3 modulates both the adaptive and innate immune system [14]. The bulk of data on vitamin D_3 and infections stems from *in vitro* experiments and retrospective observational studies. Results from randomised controlled trials where the effects of vitamin D_3 on infections have been investigated (reviewed in Yamshchikov et al. [15]) are not conclusive and larger clinical trials are therefore warranted.

We designed a study to test the hypothesis that 4000 IU of vitamin D_3 given daily to patients with antibody deficiency and frequent respiratory tract infections for one year could prevent

or ameliorate infections. In addition, we investigated whether genetic polymorphisms in genes involved in the effect and/or metabolism of vitamin D_3 have an influence on the outcome of vitamin D_3 supplementation.

Methods

Study design

A prospective, randomised, double-blind placebo-controlled study of vitamin D_3 supplementation in patients with an increased susceptibility to respiratory tract infections. The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants. The study was registered at www.clinicaltrials.gov prior to inclusion of the first patient (NCT01131858). The EudraCT number is 2009-011758-16. The full protocol is available from the corresponding author upon request.

Participants

Patients at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden, were included between March 2010 and June 2010 by the study nurses (SH, ML, KJ). Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infections; i.e. > 42 days with symptoms from the respiratory tract during a 12 months period prior to study inclusion. Patients registered at the Immunodeficiency Unit are closely followed with a diary of symptoms and antibiotic consumption. Thus, the patients were trained and used to apply such an instrument to assess their infectious status. Data from patients' standard diary was used as an instrument in order to assess patients for eligibility, both via telephone and by the responsible physician (PB, ACN) prior to inclusion. Patients with selective IgA-deficiency (**D80.2**), IgG-subclass deficiency (**D80.3**) and common variable immune disorder (CVID, **D83.0**) as well as patients without a defined immunological diagnosis (**D89.9**) were included. Exclusion criteria were prophylactic treatment with antibiotics, history of

hypercalcemia or stones in the urinary tract, sarcoidosis, ongoing supplementation with vitamin D_3 exceeding 400 IU/day, HIV-infection and pregnancy.

Interventions

Patients were randomised to 12 months' treatment with Vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. One drop contained 500 IU vitamin D_3 or placebo oil (Miglyol oil®) and the participants were asked to take 8 drops daily. The participants had to mark their daily symptoms of infection in a diary, which was sent via regular mail to the study site every month. The following data was recorded: symptoms from the respiratory tract, ears and sinuses, treatment with antibiotics, numbers of bacterial cultures, times and reasons of visits to hospitals, frequency of travelling abroad and adherence to study drug.

Outcomes

The primary outcome was a composite infectious score, based on a daily patient-reported questionnaire, and included five parameters: symptoms from the respiratory tract, ears and sinuses, malaise and use of antibiotics (Figure S1), each parameter gave 1 point/day. The occurrence of X-ray verified pneumonia gave 3 additional points per day for a period of 7 days. Thus each pneumonia resulted in 3x7 points = 21 extra points. Patients were specifically instructed to record only symptoms related to ongoing respiratory tract infections. Symptoms related to infections at other sites (urinary tract, wounds etc) as well as non-infectious symptoms were reported as adverse events. Secondary outcomes were serum levels of 25-hydroxyvitamin D_3 (at baseline and after 3, 6, 9 and 12 months), numbers of bacterial cultures, microbiological findings and levels of antimicrobial peptides (LL-37 and HNP1-3) in nasal fluid (at baseline and after 6 and 12 months). In addition, 6 *post hoc* genotype

analyses were performed in all participants. Analysis of single nucleotide polymorphisms (SNPs) were carried out for VDR (Taq1 and Foq1), CYP27B1, CYP24A1, CYP2R1 and Vitamin D binding protein (GC). Safety tests included plasma levels of creatinine, calcium, phosphate and albumin, measured at baseline and after 3, 6, 9 and 12 months. At inclusion, urine-HCG (human chorionic gonadotropin) in women was measured and p-parathyroid hormone in both genders. The results of the safety tests were reviewed by an independent and un-blinded consultant physician. Two blinded physicians (PB and ACN) were responsible for inclusion and all medical visits to the study site (Immunodeficiency Unit, Karolinska University Hospital, Huddinge).

Randomisation and statistical analysis

Participants were randomised to 12 months' treatment with vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. Block randomization with a block size of ten was used to ascertain equal group sizes. Staff at Karolinska Trial Alliance (KTA) was responsible for randomization procedures. In the statistical analysis, continuous variables were compared using Mann-Whitney U test or linear regression and dichotomous variables by Fisher's exact test or logistic regression. Regressions of log-transformed infectious scores were performed both unadjusted (simple regression) and with adjustment for potential confounders (multiple regression).

Statistical methods: Primary analysis

The distribution of the infectious score was found to be skewed, thereby violating the normal assumption of the pre-specified t-test analysis. Hence, scores were log-transformed prior to analysis.

Further, the randomisation had resulted in age distributions that were not entirely balanced between the two groups. Since there might be concerns that such imbalance could influence the results of the study, the original analysis plan was extended with a multivariable analysis adjusting for potential confounders. In this linear regression model based on log-transformed values of the primary outcome (the total infectious score) and its individual components, adjustment was made for age, gender, smoking, type of immune deficiency and significant co-morbidities (respiratory or non-respiratory). Due to the transformation procedure, the adjusted effect of vitamin D_3 is expressed as a ratio between the score in the vitamin D_3 and the placebo group. In this multiplicative model, an effect size of 1 indicates identical outcome in the two study groups and statistically non-significant results are recognized by confidence intervals encompassing the value 1.

To explore potential divergent effects on different organ systems, both adjusted and unadjusted analyses were repeated separately for each individual item of the infectious score. In addition, the temporal aspects of the vitamin D_3 effect were investigated by dividing the study period into four 90-day periods (starting on the first day of treatment) and repeating the analyses separately for each time period. “Ear” and “sinus” symptoms as well as “antibiotic use” occurred at low frequencies and for these entities normal distributions could not be achieved despite data transformation. Thus, the adjusted analyses of these individual items were based on multivariable logistic regression, after coding the symptom (or antibiotic

therapy) as present or absent during the course of the study. However, this only applies to analysis of the individual items, and not to the primary analysis of the total infectious score, where all item scores were added as originally described.

Most post-randomization exclusions were due to patients failing to fill out the symptoms diary. Hence, no intention to treat (ITT) analysis based on actual outcome data could be performed. However, the potential impact of dropouts was addressed in an ITT analysis based on multiple imputation of missing outcome data. In the imputation process, pooled estimates were derived from 100 datasets created by means of multivariate imputation by chained equations and predictive mean matching for the same covariates as in the adjusted per-protocol analysis

Detailed descriptions of randomisation and blinding, sampling of nasal fluid, measurement of antimicrobial peptides, measurement of 25-hydroxyvitamin D_3, genotyping and statistical analyses of secondary outcomes including sample size calculations are presented in the Supplementary Methods Section.

Results

Baseline data

A total of 286 patients were first assessed for eligibility but 144 were not included because they did not fulfill all inclusion criteria; <42 days with infection/year (n=35), lacked other inclusion criteria (n=42), or declined to participate (n=67). The remaining 142 patients were further screened and 140 patients were included in the study. Of these, 70 were randomised to vitamin D_3 supplementation and 70 to placebo (Figure 1). The groups did not differ with regards to gender, IgG replacement therapy, smoking, baseline 25-hydroxyvitamin D_3 levels, type of immune defect or co-morbidities. Patients with subclass deficiency, selective IgA deficiency (sIgA), common variable immune deficiency (CVID) and patients without a defined immunological diagnosis (ND) but with >4 bacterial respiratory tract infections/year were included. IgG replacement therapy was most common in the CVID-group (100%) and in the subclass deficiency group (63%), but also frequent in the other groups (ND, 54% and sIgA, 38%, table S1). Patients allocated to the placebo group were slightly younger than patients in the treatment group (p=0.025, data not shown). During the course of the study, 16 patients left the study prematurely (8 patients from each study group) and consequently 124 patients were included in the main per-protocol analysis. Reasons for dropout included elevated PTH (n=2), withdrawn consent (n=5), adverse event (n=1), prescription of vitamin D outide the study (n=1), failure to complete diary (n=4) or non-compliance to study medication (n=3) (Figure 1).

Primary endpoint: Infectious score

One year of vitamin D_3 treatment was associated with a significantly reduced total infectious score both in the unadjusted and the adjusted analyses (Table 2, Figure 2A, B and Table S2).

The unadjusted relative score in the intervention group was 0.754 (95% c.i. 0.591-0.963, p=0.024, n=124) corresponding to a 25% reduction and after adjustment for potential confounders, the relative score was 0.771 (95% c.i. 0.604-0.985, p=0.040), corresponding to a 23% reduction (Table 2). According to the temporal analysis, the effect of vitamin D_3 supplementation tended to improve with time (Figure 2A). The absolute unadjusted score per patient was 202 points for the vitamin D group and 249 points for the placebo group, i.e. a significant reduction of 47 points per patient (p=0.023, Mann Whitney U-test, table S3).

When the individual items of the infectious score were analysed separately, all point estimates indicated a reduction in the treatment group (Table 2, figure S2), although only antibiotic consumption reached statistical significance (Figure 2B and S2, panel E). The adjusted OR for antibiotic use was 0.365 (95% c.i. 0.153-0.872, p=0.023, n=124), i.e. a 63.5% reduction of the odds of antibiotic use in the intervention group (Table 2). The absolute values were 33 days on antibiotics for the placebo group and 16 days for the vitamin D_3 group, i.e. a reduction of 17 days in the vitamin D_3 group (table S3). The temporal trends for specific symptoms and antibiotic consumption were similar to the total score and reached statistical significance for 'ear'-symptoms (n=124, p=0.041) and for 'malaise' (n=124, p=0.053) in the final quarter of the study (Figure 2S, panels B and C).

Analyzing the primary outcome according to intention-to-treat (n=170) produced results virtually identical to those of the per-protocol analysis. In the unadjusted ITT analysis, vitamin D_3 reduced the total infectious score by 25% (relative score 0.752, 95% c.i. 0.588-0.962, p=0.024) and after adjustment for potential confounders the reduction was 23% (relative score 0.767, 95% c.i. 0.599-0.982, p=0.036).

Serum levels of 25-OH vitamin D_3

Serum 25-hydroxyvitamin D_3 levels did not differ between the groups at baseline (Table 1). However, already after 3 months the intervention group had a significantly higher level of 25-hydroxyvitamin D_3 (133.4 nmol/L versus 66.6 nmol/L, p<0.001, Figure 3). This increase remained throughout the study (Figure 3).

Bacterial cultures and microbiology

During the course of the study, 173 microbiological samples were obtained in the vitamin D_3 group (n=62, 2.79/patient) and 301 in the placebo group (n=62, 4.85/patient) (p=0.010, Table 3). The number of samples with at least one positive finding was higher in the placebo group, with close to statistical significance (p=0.052), while the fraction of positive samples was similar for both groups (Table 3). Significantly more patients had a microbiological sample taken from the respiratory tract (≥ 1 sample) during the study period in the placebo group; OR 2.63 (95% CI 1.17-5.92), (Table 3).

In total, the vitamin D_3 group generated 76 positive microbiological findings (bacteria or fungi), compared to 159 in the placebo group (p=0.023). There was no difference between the groups for the traditional respiratory pathogens (*H. influenza, M. catharralis and S. pneumonia*), but there were significantly fewer findings of *S. aureus* (p=0.019) and fungi (p=0.028, *Candida* spp. and *Aspergillus* spp.) in the treatment group (Table 4). Likewise, significantly fewer vitamin D_3-treated patients had a bacterial culture positive for *S. aureus* (p=0.019) or fungal species (p=0.058), although the latter difference did not reach statistical significance (Table 4).

Vitamin D_3-treated patients with sub-class deficiency left significantly fewer bacterial or fungal cultures than placebo-treated patients with this diagnosis; 7 cultures in the vitamin D group (n=22) versus 47 cultures in the placebo group (n=24) (Table S4). Also the number of

patients that had ≥1 bacterial culture taken was significantly fewer in the placebo group (12/22 versus 22/24, p=0.0065, table S4). There was no significant effect of other immunological diagnoses on bacterial cultures or microbiology (Table S4).

Since concomitant lung disease may be an important factor for Vitamin D_3-mediated effects on respiratory immunity, we performed a detailed analysis of bacterial cultures and microbiology of patients with asthma, bronchiectasis (BE) and chronic obstructive pulmonary disease (COPD). The numbers of patients with these diagnoses were quite small, which preclude any firm conclusions regarding any effect. However, there was a non-significant trend that Vitamin D_3-treated patients with asthma produced fewer bacterial cultures (average 2.9 cultures/patient versus 7.0 cultures/patients, p=0.080, Figure S3) and fewer positive cultures than placebo-treated asthmatics (average 0.6 positive cultures/patients versus 2.7/patient in the placebo group, p=0.052, Figure S3). In addition, Vitamin D_3-treated asthma patients showed significantly fewer cultures positive for fungi (candida and aspergillus) compared to placebo-treated asthmatics (p=0.0476, table S5). For BE or COPD-patients there was no clear trend or significant effect in bacterial cultures or microbiology.

Levels of antimicrobial peptides (AMPs) in nasal fluid

There was no statistically significant difference between the vitamin D_3 or placebo groups when nasal fluids were analysed for the presence of AMPs. Initially, levels of both LL-37 and HNP1-3 tended to be higher in the placebo group (Figure S3, panels A and B). However, after 12 months the microbiological pattern was reversed and no primary pathogens could be detected in nasal swabs from vitamin D_3-treated patients (n=25, p=0.039) (Figure S4, panel C). The placebo-treated patients exhibited the same mix between normal flora and primary pathogens at all three sampling points (0, 6 and 12 months) (Figure S4, panel C).

SNP variants and treatment effect

Most genetic variants did not affect the primary endpoint. However, patients carrying the 'AA' genotype in the CYP2R1-gene, encoding the 25-hydroxylase enzyme, had a larger benefit of vitamin D_3-supplementation (-55%) compared to AG or GG carriers (-6%) (n=124, p=0.046 for interaction, Table S6).

Adverse events

In total, the vitamin D_3 group reported 38 adverse events (AEs) versus 56 AEs in the placebo group. The most common symptoms in the treatment group were headache (n=5) and lumbago (n=5), whereas placebo-treated patients reported paresthesias (n=8), diverticulitis (n=4) and urinary tract infection (n=4) as most frequent AEs (Table 5, Table S7). There was a general trend towards the number of adverse events being higher in the placebo group. Significantly more patients in the placebo group reported cardiovascular problems, such as heart failure, hypertonia and thrombosis (p=0.028). For gastrointestinal and other (non-respiratory) infections there was also a trend favoring the vitamin D_3 group (p=0.058 and p=0.09, respectively). No clinically relevant changes in serum levels of calcium, phosphate, creatinine or albumin could be observed (Figure S5). There was one severe adverse event (SAE) in each group (rabdomyosarcoma in the vitamin D_3 group and lung bleeding in the placebo group), both judged as being unrelated to the study drug.

Discussion

The main conclusion from this long-term randomised controlled trial (RCT) is that vitamin D_3 supplementation reduces the total burden of respiratory tract infections. The primary endpoint was composed of five different parameters that patients recorded daily throughout the study year. All point estimates favoured the vitamin D_3 group and a statistically significant effect was seen on both the total score and on the probability of receiving antibiotics. The effect on the infectious score was evident both in analysis per-protocol and according to intention-to-treat, and withstood adjustment for potential confounders. In addition, the number of bacterial cultures and microbiological findings was significantly reduced in the intervention group. These findings are potentially important and support that Vitamin D_3 supplementation may prevent respiratory tract infections and reduce antibiotic consumption, particularly in patients with hypogammaglobulinemia or with an increased frequency of respiratory tract infections.

However, our study has several limitations: Firstly, the choice of primary endpoint may be questioned since it relies solely on patient-reported information. To compensate for inherent problems with patient-reported data, the evaluation instrument was designed to cover many aspects of an infectious episode, including various symptoms as well as antibiotic consumption. Together the reported data formed an "infectious score", which constituted the primary endpoint of the study. Similar composite scores have successfully been applied to different diseases, such as tuberculosis (TB-score [16]), pneumonia (CURB-65 [17]) and bacterial meningitis (BMS-score [18]). Notably, vitamin D supplementation had a major effect on the odds of taking antibiotics during the study period (a reduction by 63.5%). In addition, the absolute number of days on antibiotics was reduced by 50% (from 33 days in the placebo group to 16 days in the intervention group), which was statistically significant both in the adjusted and unadjusted analyses (table 2). However, despite the relatively modest reduction for the other components of the primary endpoint the overall infectious score was

significantly reduced – mainly as a result of the large effect on the antibiotic parameter - both in the unadjusted and in the adjusted analyses (table 2, figure 2). Another potential problem was that the patient population was very heterogeneous with regards to immune deficiency and concomitant diseases. We adjusted for these factors in the multivariable analyses of the primary endpoint, but the sample sizes in each subgroup were too small to draw any conclusions of effects in specific disease groups. However, a detailed *post-hoc* analysis of the relation between immunological diagnosis, concomitant lung-disease and the secondary endpoints "taken bacterial cultures", "positive bacterial cultures" and "microbiological findings" was performed. There was a clear trend that Vitamin D_3-treated patients with subclass deficiency and/or asthma produced fewer bacterial cultures, fewer positive cultures and fewer fungal cultures (tables S4 and S5, Figure S3). Although this analysis may lack precision due to the small number of patients included, it could have clinical implications regarding target groups for Vitamin D_3 supplementation.

Nevertheless, our double-blind RCT has several strengths. For example, we chose a high daily dose of vitamin D_3 based on published calculations on metabolism and effects on immunity [14 19]. Other RCTs using lower doses of vitamin D_3, 400-2000 IU/day, have mainly been negative with regards to prevention of infections [20 21]. However, one study using 1200 IU/day showed a significant reduction of influenza among school children in Japan [22]. Notably, also studies using higher doses of vitamin D_3 have been negative. Martineau *et al* used 400,000 IU vitamin D_3 during 42 days (9523 IU/day) with the aim of shortening time to sputum conversion in tuberculosis. No significant effect on the primary endpoint could be observed in that study, except in a subgroup with the *tt* genotype in the vitamin D receptor gene [23]. A recent study investigated whether 100,000 IU vitamin D_3/month (3333 IU/day) could reduce the incidence of chronic obstructive pulmonary disease (COPD) exacerbations. There was no significant effect on the primary endpoint, although a *post hoc* analysis revealed that patients

with a low vitamin D_3 level at baseline had a significant effect of Vitamin D_3 supplementation[24].

Importantly, our study is the first to utilize high daily doses for an extended period of one full year. Thus, we covered all four seasons, which was important in Sweden with a known seasonal variation in 25-hydroxyvitamin D_3 levels [25]. Two previous RCTs were performed during the winter season – when vitamin D levels are low – but only during 4 [22] and 6 months [20], respectively. Previous RCTs have been conducted during shorter periods; 42 days [23], 6 weeks [26] and 12 weeks [21], respectively. Interestingly, we observed a clear time dependent effect suggesting that a long term supplementation approach (> 6 months) may be necessary to affect immunity. To expand on the results of a previous study in healthy individuals where no difference between the intervention and placebo groups was observed [21], we chose a study population with frequent RTIs and at least 42 days with infection during the year prior to inclusion. Notably, patients in the study represent a selected group of individuals with frequent RTI, although the immune disorders that they represent (IgA-, IgG-subclass and patients with no defined immune disorder) are generally mild in character and dominated by mucosal RTIs. We also included a small number of CVID-patients, which can be considered to be a more severe immune disorder, but all these patients are treated with IgG replacement therapy and thus well controlled. Hence, the results from this study cannot directly be applied to the general healthy population. Nevertheless, the results provide solid support for additional interventional studies of vitamin D_3, especially in groups consuming large amounts of antibiotics.

The mechanism of the observed effects remains largely unknown. Vitamin D_3 modulates the immune response at many levels, such as induction of AMPs, skewing of T-cells from Th1/Th17 to Tregs as well as general anti-inflammatory effects [14]. Here, we investigated the role of AMPs in nasal fluid. However, we could not detect any significant changes of LL-37

or HNP1-3 during the study period, but noted that placebo-treated patients tended to have higher levels of AMPs after one year of treatment. This was paralleled by a shift of the microflora in the nasal compartment that could explain the unexpected finding of higher AMP-levels in the placebo group. Recently, it was shown that 1,25 $(OH)_2$-vitamin D_3 induces both HNP1-3 and LL-37 in nasal fluid of healthy volunteers (Hiemstra et al, abstract, European Respiratory Society, 2011), supporting that LL-37 may indeed be induced *in vivo*. However, our study design did not allow such conclusions but rather support that vitamin D_3 affect mucosal immunity, leading to a shift of the microflora. Recently, we showed that the bacterial composition in nasal swabs is an important determinant of AMP-levels in nasal fluid [27].

Given that vitamin D_3 induces LL-37 in epithelial cells and that LL-37 kills bacteria *in vitro,* we expected a reduction of the classical bacterial pathogens *H. influenza*, *M. catharralis* and *S. pneumonia* in the intervention group. However, the frequency of these bacteria was not reduced but a reduction of *S. aureus* and fungal species that often colonise the airways was observed. This could be explained by specific effects by vitamin D_3 on immunity against *S. aureus*. In fact, vitamin D induces human beta defensin-2 (HBD-2) with bactericidal activity against *S. aureus* [28]. A recent study showed that low vitamin D levels were associated with an increased risk of being colonised by this bacterium [29]. Further, vitamin D affects immunity against *C. albicans*, which indicates direct effects of vitamin D on human immunity [30]. Alternatively, it is possible that vitamin D_3 may have prevented symptomatic viral infections, which prompted patients to leave a bacterial sample from the airways. Interestingly, there is both mechanistic and clinical evidence that vitamin D_3 can prevent viral infections [31-33], although we did not address this in the current study.

Notably, we observed a prominent increase in the serum concentration of 25-hydroxyvitamin D_3, which indicated good compliance and tolerability of the study drug. In fact, there was a

trend towards adverse events being reported more often in the placebo group, suggesting that vitamin D_3 possibly could be efficient against other diseases, but this observation requires further studies. No clinically relevant changes of blood chemistry (calcium, phosphate, albumin or creatinine) were observed. Despite few adverse events and high tolerability, 16 exclusions occurred during the study year. The main reason was problems to adhere to the protocol and 6/16 patients dropped out of the study after a few weeks. The rest failed to send in the diaries, did not leave blood for monitoring of safety parameters or did not take the study drug. One patient was excluded based on symptoms that could be attributed to vitamin D_3 (facial paraesthesia). However, this patient was later confirmed to have been allocated to placebo.

In summary, we found that supplementation with vitamin D_3 reduced the total infectious score with 47 points per patient during the study year. A clinically meaningful translation of this effect could be e.g. 47 days with cough (47 points), 23 days with ear and sinus symptoms (23×2=46 points) or 9 days with cough, sinus and ear symptoms together with malaise and antibiotics (9×5=45 points). In addition, our data indicate that vitamin D_3 supplementation reduces the odds of taking antibiotics by approximately 60% in patients with frequent respiratory tract infections. Thus, supplementation with vitamin D_3 could provide a novel strategy to reduce antibiotic use among high consumers and indirectly prevent the emerging epidemic of bacterial resistance.

Acknowledgment

The study was registered at www.clinicaltrials.gov (NCT01131858), prior to the start of the study. The entire process of study design and protocol, data monitoring and analyses was performed by academic authors; there was no industry involvement in the study except that vitamin D_3 (Vigantol®) and placebo oil (Miglyol®) were provided by Merck KGaA (Darmstadt, Germany). Merck did not have any influence on study design, analysis of data, writing or decision to publish. We extend our gratitude to Ilona Skilving, Karolinska Trial Alliance for invaluable help with the protocol. Further, we thank registered nurses Maria Lindén and Kristina Johansson for skillful work with patients. Thanks also to Jenny Lindén and Alicia Hansson for registration of data and to professor Mats Remberger for discussions on statistical methods. Professor Lars Lindqvist, Department of Infectious Diseases, Karolinska University Hospital is gratefully acknowledged for serving as the monitor of the study. PB, LBB and JDL are holding PostDoc-positions financed by Karolinska Institutet and Stockholm County Council (KI/SLL).

Data sharing statement:

There is no additional data available.

Statements

Author contributions:

Peter Bergman, designed the study, collected, analysed and interpreted data, wrote the paper.

Anna-Carin Norlin, designed the study, collected and interpreted data, wrote the paper.

Susanne Hansen, designed and coordinated the study, collected and interpreted data.

Rokeya Sultana Rekha, carried out experimental work, analysed data

Birgitta Agerberth, analysed and interpreted data, wrote the paper.

Linda Björkhem-Bergman, analysed and interpreted data, wrote the paper.

Lena Ekström, analysed and interpreted data

Jonatan Lindh, analysed and interpreted data, wrote the paper.

Jan Andersson, designed the study, interpreted data, wrote the paper.

Role of the funding source

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Merck GmbH provided the study drug (Vigantol) but did not have any influence on study design, analysis of data, writing or decision to publish.

Ethics statement: The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants.

Conflicts of interest: There are no conflicts of interest.

Legends to figures

Figure 1. Study outline.

Figure 2. Primary endpoint. The adjusted total relative infectious score (A) is expressed 'per quarter' (3 month periods). The adjusted one-year scores (total score, airway, malaise, ear, sinus and antibiotics) are depicted in a Forrest-plot (B) together with 95% confidence intervals. Effects are presented as relative scores (total score, airway, malaise) or odds ratios (ear, sinus, antibiotics, indicated with asterisks).

Figure 3. Secondary endpoint. Vitamin D-levels. Serum was collected at day 0, 3, 6, 9 and 12 months and levels of 25-hydroxyvitamin D_3 were measured. Values are expressed as mean +/- 95% confidence interval.

Tables

Table 1. Baseline data. Mann Whitney U-test was used for comparisons of age and 25-OH vitamin D_3. Fisher's exact test was used for all other comparisons. 1) "other disease" includes hypertension, body pain, hypothyroidism and gastritis as most common diagnoses. CVID, common variable immuno deficiency; ND, increased susceptibility to infections without a defined immunological disorder; BE, bronchiectasis; COPD, chronic obstructive pulmonary disease.

	Vitamin D_3	Placebo
Number	70	70
Age (mean)	55,4	50,8
Female	52/70	50/70
Male	18/70	20/70
IgG-replacem.	39/70	42/70
Smoking	4/70	6/70
25-OH levels (mean)	51,5 nmol/L	46,9 nmol/L
Immunological diagnosis		
sIgA-deficiency	9/70	9/70
IgG subclass	27/70	30/70
CVID	6/70	4/70
ND	28/70	27/70
Concomitant disease		
No other disease	16/70	18/70
Lung: Asthma	27/70	25/70
Lung: BE	5/70	7/70
Lung: COPD	5/70	4/70
Other disease[1]	17/70	16/70

Table 2. Primary Endpoint. Treatment effect calculated as the ratio between infectious scores in the vitamin D_3 and the placebo groups. Due to low frequencies, endpoints marked with asterisks were coded as binary outcomes (i.e. present or absent in each patient) and compared by means of logistic regression. In these cases, the effect refers to odds ratios of experiencing the outcome at least once during the course of the study. (The data are based on n=124 patients).

	Univariable regression model (unadjusted values)			Multiple regression model (adjusted values)		
Endpoint	**Effect**	**95% CI**	**p-value**	**Effect**	**95% CI**	**p-value**
Total score	0.754	0.591-0.963	**0.024**	0.771	0.604-0.985	**0.040**
Airway	0.857	0.697-1.053	0.141	0.871	0.706-1.074	0.200
Ear*	0.721	0.352-1.465	0.367	0.695	0.320-1.501	0.357
Sinus*	0.583	0.280-1.198	0.144	0.594	0.265-1.328	0.204
Malaise	0.845	0.692-1.032	0.098	0.845	0.689-1.036	0.108
Antibiotics*	0.355	0.154-0.784	**0.012**	0.365	0.153-0.872	**0.023**

Table 3. Bacterial cultures. [1]Mann-Whitney U-test, [2]Fisher's exact test.

	Vitamin D_3	Placebo	Significance
Number of samples per patient (mean, n=62/62)	2.79	4.85	p=0.010[1]
Number of positive samples per patient (mean, n=62/62)	1.01	2.02	p=0.052[1]
Fraction positive cultures (%)	63/173 (36%)	125/301 (41%)	P=0.28[2]
Patients with ≥ 1 sample taken	38/62 (61%)	50/62 (81%)	p=0.029[2]

Table 4. Microbiological findings. Mann-Whitney U-test was used to analyze the total number of findings, whereas Fisher's exact test was used for analysis of the number of patients (fraction) with a specific finding.

	Number of findings (total)			Number of patients		
Microorganism	Vitamin D_3	Placebo	MW-U	Vitamin D_3	Placebo	Fisher
H. influenzae	28	27	P=0.46	10/62	13/62	P=0.64
M. catharralis	8	17	P=0.39	7/62	10/62	P=0.60
S. pneumoniae	7	6	P=0.74	4/62	5/62	P=1.00
S. aureus	6	33	P=0.010	4/62	14/62	p=0.019
Enterobacteriacae	8	8	P=0.39	4/62	7/62	P=0.53
P. aeruginosa	8	15	P=0.68	3/62	4/62	P=1.00
Fungal infection	11	53	P=0.028	4/62	12/62	p=0.058
Total	76	159	P=0.023			

Table 5. Adverse events. Number of reports. Fisher's exact test was used for between group comparison. (The data are based on AE-reports from n=62 patients/arm).

Organ	Vitamin D_3 n (%)	Placebo n (%)	P-value
CNS	11 (29)	10 (18)	1.00
Gastrointestinal	4 (11)	12 (21)	**0.058**
Cardiovascular	0 (0)	6 (11)	**0.028**
Infections (other than RTI)	2 (5)	8 (14)	**0.09**
Musculoskeletal	10 (26)	10 (18)	1.00
Respiratory (non-infectious)	2 (5)	4 (7)	0.68
Skin	5 (13)	2 (4)	0.44
Other	4 (10)	4 (7)	1.00
Total	**38**	**56**	

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Vitamin D_3 supplementation in patients with frequent respiratory tract infections

- a randomised, double-blind intervention study

Peter Bergman*[1,3,6#], Anna-Carin Norlin*[2,3], Susanne Hansen[3], Rokeya Sultana Rekha[4], Birgitta Agerberth[4], Linda Björkhem-Bergman[5], Lena Ekström[5], Jonatan D Lindh[5], Jan Andersson[6]

1. Department of Laboratory Medicine, Clinical Microbiology, Karolinska Institutet
2. Department of Laboratory Medicine, Clinical Immunology, Karolinska Institutet
3. Karolinska University Hospital, Clinical Immunology, Immunodeficiency Unit
4. Department of Medical Biochemistry and Biophysics, Karolinska Institutet
5. Department of Laboratory Medicine, Clinical Pharmacology, Karolinska Institutet
6. Department of Medicine, Center for Infectious Medicine (CIM), Karolinska Institutet

*Equal contribution

Positions:

Peter Bergman, MD, PhD, Resident Physician, Clinical Microbiology

Anna-Carin Norlin, MD, Specialist in Clinical Immunology

Susanne Hansen, RN, Head of Immunodeficiency Unit

Rokeya Sultana Rekha, MSc, PhD-student

Birgitta Agerberth, PhD, Professor, Medical Microbial Pathogenesis

Linda Bjorkhem-Bergman, MD, PhD, Resident Physician, Clinical Pharmacology

Lena Ekström, PhD, Associate Professor, Clinical Pharmacology

Jonatan D Lindh, MD, PhD, Specialist in Clinical Pharmacology

Jan Andersson, MD, PhD, Professor, Infectious Diseases

Running title: Vitamin D_3 supplementation in patients with frequent respiratory tract infections

#Corresponding author:

Peter Bergman, MD, PhD

Department of Laboratory Medicine, Clinical Microbiology F68

Karolinska Institutet and Karolinska University Hospital, Huddinge

SE-141 86 Stockholm, Sweden

peter.bergman@ki.se

Phone: +46858587942

Fax: +46858581305

Competing interest statement

All authors have completed the Unified Competing Interest form (available on request from the corresponding author) and declare no support from any organisation for the submitted work, no financial relationships with any organisations that might have an interest in the submitted work in the previous three years and no other relationships or activities that could appear to have influenced the submitted work.

Article Summary

Article focus

• Recent evidence suggests that vitamin D_3 has potent extra-skeletal effects, such as suppression of inflammation and strengthening of mucosal immunity by induction of antimicrobial peptides.

• Data from observational studies suggest that low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections.

• Results from a limited number of randomised controlled trials on the protective role of vitamin D_3 against respiratory tract infections are inconclusive and thus additional studies are warranted.

Key Messages

• Therefore we designed and carried out a randomised controlled trial where a large dose (4000 IU) of vitamin D_3 was given to patients with an increased susceptibility to infections for one year.

• The main conclusion is that vitamin D_3 supplementation reduces symptoms and antibiotic consumption among patients with an increased frequency of respiratory tract infections. Thus, vitamin D_3 supplementation may be an alternative strategy to reduce antibiotic use among patients with recurrent respiratory tract infections.

Strengths and limitations

• Strengths: A high daily dose of vitamin D3 was used, the study time was a full year covering all seasons and patients with an increased frequency of respiratory tract infections were studied.

• Limitations: A single study center, small sample size (n=140) and a selected group of patients.

Abstract

Background: Low serum levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections (RTIs). Clinical trials with vitamin D_3 against various infections have been carried out but data are so far not conclusive. Thus, there is a need for additional randomised controlled trials of effects of vitamin D_3 on infections.

Objective: To investigate if supplementation with vitamin D_3 could reduce infectious symptoms and antibiotic consumption among patients with antibody deficiency or frequent RTIs.

Design: A double-blind randomised controlled trial.

Setting: Karolinska University Hospital, Huddinge

Participants: 140 patients with antibody deficiency (sIgA-, IgG subclass deficiency, CVID) and patients with increased susceptibility to RTIs (>4 bacterial RTIs/year) but without immunological diagnosis.

Intervention: vitamin D3 (4000 IU) or placebo was given daily for one year

Primary and secondary outcome measures: The primary endpoint was an infectious score based on five parameters: symptoms from respiratory tract, ears and sinuses, malaise and antibiotic consumption. Secondary endpoints were serum levels of 25-hydroxyvitamin D_3, microbiological findings and levels of antimicrobial peptides (LL-37, HNP1-3) in nasal fluid.

Results: The overall infectious score was significantly reduced for patients allocated to the vitamin D-group (202 points) compared with the placebo group (249 points) (adjusted relative score 0.771, 95% CI 0.604-0.985, p=0.040).

Limitations: A single study center, small sample size and a selected group of patients.

Conclusions: Supplementation with vitamin D_3 may reduce disease burden in patients with frequent respiratory tract infections.

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The study was registered at www.clinicaltrials.gov (NCT01131858)

273/275 words

Introduction

Vitamin D was discovered when it was noted that rachitic children were improved by exposure to sunlight [1]. It was later shown by Holick *et al* that vitamin D_3 is synthesized in the skin under the influence of UVB-light [2]. Vitamin D_3 is further hydroxylated in the liver to 25-hydroxyvitamin D_3, which is considered to reflect the vitamin D status of an individual patient [3]. The final activation to the active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)D_3$) requires 1-α-hydroxylase activity. This enzyme (also designated CYP27B1) is expressed in the kidney but also in many other cell-types, including epithelial- and immune-cells [4]. The active vitamin D_3 ($1,25(OH)D_3$) binds to the vitamin D receptor (VDR), which belongs to the nuclear receptor family. Active vitamin D_3 is only present in minute amounts in the circulation and local activation in target cells is crucial for vitamin D-mediated effects on the immune system[5].

Low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of tuberculosis [6-8] and respiratory tract infections [9]. The mechanism is not fully elucidated but vitamin D_3 has been shown to induce antimicrobial peptides in immunecells [10]. In addition, active vitamin D_3 ($1,25(OH)D_3$) has broad anti-inflammatory effects on the adaptive immune system by shifting the T-helper cell pool from a Th1/Th17-response to a Th2/Treg-dominated response [11 12]. Vitamin D_3 has also been shown to suppress the Th2-response in allergic bronchopulmonary aspergillosis [13]. Thus, vitamin D_3 modulates both the adaptive and innate immune system [14]. The bulk of data on vitamin D_3 and infections stems from *in vitro* experiments and retrospective observational studies. Results from randomised controlled trials where the effects of vitamin D_3 on infections have been investigated (reviewed in Yamshchikov et al. [15]) are not conclusive and larger clinical trials are therefore warranted.

We designed a study to test the hypothesis that 4000 IU of vitamin D_3 given daily to patients with antibody deficiency and frequent respiratory tract infections for one year could prevent

or ameliorate infections. In addition, we investigated whether genetic polymorphisms in genes involved in the effect and/or metabolism of vitamin D_3 have an influence on the outcome of vitamin D_3 supplementation.

Methods

Study design

A prospective, randomised, double-blind placebo-controlled study of vitamin D_3 supplementation in patients with an increased susceptibility to respiratory tract infections. The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants. The study was registered at www.clinicaltrials.gov prior to inclusion of the first patient (NCT01131858). The EudraCT number is 2009-011758-16. The full protocol is available from the corresponding author upon request.

Participants

Patients at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden, were included between March 2010 and June 2010 by the study nurses (SH, ML, KJ). Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infections; i.e. > 42 days with symptoms from the respiratory tract during a 12 months period prior to study inclusion. Patients registered at the Immunodeficiency Unit are closely followed with a diary of symptoms and antibiotic consumption. Thus, the patients were trained and used to apply such an instrument to assess their infectious status. Data from patients' standard diary was used as an instrument in order to assess patients for eligibility, both via telephone and by the responsible physician (PB, ACN) prior to inclusion. Patients with selective IgA-deficiency (**D80.2**), IgG-subclass deficiency (**D80.3**) and common variable immune disorder (CVID, **D83.0**) as well as patients without a defined immunological diagnosis (**D89.9**) were included. Exclusion criteria were prophylactic treatment with antibiotics, history of

hypercalcemia or stones in the urinary tract, sarcoidosis, ongoing supplementation with vitamin D_3 exceeding 400 IU/day, HIV-infection and pregnancy.

Interventions

Patients were randomised to 12 months' treatment with Vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. One drop contained 500 IU vitamin D_3 or placebo oil (Miglyol oil®) and the participants were asked to take 8 drops daily. The participants had to mark their daily symptoms of infection in a diary, which was sent via regular mail to the study site every month. The following data was recorded: symptoms from the respiratory tract, ears and sinuses, treatment with antibiotics, numbers of bacterial cultures, times and reasons of visits to hospitals, frequency of travelling abroad and adherence to study drug.

Outcomes

The primary outcome was a composite infectious score, based on a daily patient-reported questionnaire, and included five parameters: symptoms from the respiratory tract, ears and sinuses, malaise and use of antibiotics (Figure S1), each parameter gave 1 point/day. The occurrence of X-ray verified pneumonia gave 3 additional points per day for a period of 7 days. Thus each pneumonia resulted in 3x7 points = 21 extra points. Patients were specifically instructed to record only symptoms related to ongoing respiratory tract infections. Symptoms related to infections at other sites (urinary tract, wounds etc) as well as non-infectious symptoms were reported as adverse events. Secondary outcomes were serum levels of 25-hydroxyvitamin D_3 (at baseline and after 3, 6, 9 and 12 months), numbers of bacterial cultures, microbiological findings and levels of antimicrobial peptides (LL-37 and HNP1-3) in nasal fluid (at baseline and after 6 and 12 months). In addition, 6 *post hoc* genotype

analyses were performed in all participants. Analysis of single nucleotide polymorphisms (SNPs) were carried out for VDR (Taq1 and Foq1), CYP27B1, CYP24A1, CYP2R1 and Vitamin D binding protein (GC). Safety tests included plasma levels of creatinine, calcium, phosphate and albumin, measured at baseline and after 3, 6, 9 and 12 months. At inclusion, urine-HCG (human chorionic gonadotropin) in women was measured and p-parathyroid hormone in both genders. The results of the safety tests were reviewed by an independent and un-blinded consultant physician. Two blinded physicians (PB and ACN) were responsible for inclusion and all medical visits to the study site (Immunodeficiency Unit, Karolinska University Hospital, Huddinge).

Randomisation and statistical analysis

Participants were randomised to 12 months' treatment with vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. Block randomization with a block size of ten was used to ascertain equal group sizes. Staff at Karolinska Trial Alliance (KTA) was responsible for randomization procedures. In the statistical analysis, continuous variables were compared using Mann-Whitney U test or linear regression and dichotomous variables by Fisher's exact test or logistic regression. Regressions of log-transformed infectious scores were performed both unadjusted (simple regression) and with adjustment for potential confounders (multiple regression).

Statistical methods: Primary analysis

The distribution of the infectious score was found to be skewed, thereby violating the normal assumption of the pre-specified t-test analysis. Hence, scores were log-transformed prior to analysis.

Further, the randomisation had resulted in age distributions that were not entirely balanced between the two groups. Since there might be concerns that such imbalance could influence the results of the study, the original analysis plan was extended with a multivariable analysis adjusting for potential confounders. In this linear regression model based on log-transformed values of the primary outcome (the total infectious score) and its individual components, adjustment was made for age, gender, smoking, type of immune deficiency and significant co-morbidities (respiratory or non-respiratory). Due to the transformation procedure, the adjusted effect of vitamin D_3 is expressed as a ratio between the score in the vitamin D_3 and the placebo group. In this multiplicative model, an effect size of 1 indicates identical outcome in the two study groups and statistically non-significant results are recognized by confidence intervals encompassing the value 1.

To explore potential divergent effects on different organ systems, both adjusted and unadjusted analyses were repeated separately for each individual item of the infectious score. In addition, the temporal aspects of the vitamin D_3 effect were investigated by dividing the study period into four 90-day periods (starting on the first day of treatment) and repeating the analyses separately for each time period. “Ear” and “sinus” symptoms as well as “antibiotic use” occurred at low frequencies and for these entities normal distributions could not be achieved despite data transformation. Thus, the adjusted analyses of these individual items were based on multivariable logistic regression, after coding the symptom (or antibiotic

therapy) as present or absent during the course of the study. However, this only applies to analysis of the individual items, and not to the primary analysis of the total infectious score, where all item scores were added as originally described.

Most post-randomization exclusions were due to patients failing to fill out the symptoms diary. Hence, no intention to treat (ITT) analysis based on actual outcome data could be performed. However, the potential impact of dropouts was addressed in an ITT analysis based on multiple imputation of missing outcome data. In the imputation process, pooled estimates were derived from 100 datasets created by means of multivariate imputation by chained equations and predictive mean matching for the same covariates as in the adjusted per-protocol analysis

Detailed descriptions of randomisation and blinding, sampling of nasal fluid, measurement of antimicrobial peptides, measurement of 25-hydroxyvitamin D_3, genotyping and statistical analyses of secondary outcomes including sample size calculations are presented in the Supplementary Methods Section.

Results

Baseline data

A total of 286 patients were first assessed for eligibility but 144 were not included because they did not fulfill all inclusion criteria; <42 days with infection/year (n=35), lacked other inclusion criteria (n=42), or declined to participate (n=67). The remaining 142 patients were further screened and 140 patients were included in the study. Of these, 70 were randomised to vitamin D_3 supplementation and 70 to placebo (Figure 1). The groups did not differ with regards to gender, IgG replacement therapy, smoking, baseline 25-hydroxyvitamin D_3 levels, type of immune defect or co-morbidities. Patients with subclass deficiency, selective IgA deficiency (sIgA), common variable immune deficiency (CVID) and patients without a defined immunological diagnosis (ND) but with >4 bacterial respiratory tract infections/year were included. IgG replacement therapy was most common in the CVID-group (100%) and in the subclass deficiency group (63%), but also frequent in the other groups (ND, 54% and sIgA, 38%, table S1). Patients allocated to the placebo group were slightly younger than patients in the treatment group (p=0.025, data not shown). During the course of the study, 16 patients left the study prematurely (8 patients from each study group) and consequently 124 patients were included in the main per-protocol analysis. Reasons for dropout included elevated PTH (n=2), withdrawn consent (n=5), adverse event (n=1), prescription of vitamin D outide the study (n=1), failure to complete diary (n=4) or non-compliance to study medication (n=3) (Figure 1).

Primary endpoint: Infectious score

One year of vitamin D_3 treatment was associated with a significantly reduced total infectious score both in the unadjusted and the adjusted analyses (Table 2, Figure 2A, B and Table S2).

The unadjusted relative score in the intervention group was 0.754 (95% c.i. 0.591-0.963, p=0.024, n=124) corresponding to a 25% reduction and after adjustment for potential confounders, the relative score was 0.771 (95% c.i. 0.604-0.985, p=0.040), corresponding to a 23% reduction (Table 2). According to the temporal analysis, the effect of vitamin D_3 supplementation tended to improve with time (Figure 2A). The absolute unadjusted score per patient was 202 points for the vitamin D group and 249 points for the placebo group, i.e. a significant reduction of 47 points per patient (p=0.023, Mann Whitney U-test, table S3).

When the individual items of the infectious score were analysed separately, all point estimates indicated a reduction in the treatment group (Table 2, figure S2), although only antibiotic consumption reached statistical significance (Figure 2B and S2, panel E). The adjusted OR for antibiotic use was 0.365 (95% c.i. 0.153-0.872, p=0.023, n=124), i.e. a 63.5% reduction of the odds of antibiotic use in the intervention group (Table 2). The absolute values were 33 days on antibiotics for the placebo group and 16 days for the vitamin D_3 group, i.e. a reduction of 17 days in the vitamin D_3 group (table S3). The temporal trends for specific symptoms and antibiotic consumption were similar to the total score and reached statistical significance for 'ear'-symptoms (n=124, p=0.041) and for 'malaise' (n=124, p=0.053) in the final quarter of the study (Figure 2S, panels B and C).

Analyzing the primary outcome according to intention-to-treat (n=170) produced results virtually identical to those of the per-protocol analysis. In the unadjusted ITT analysis, vitamin D_3 reduced the total infectious score by 25% (relative score 0.752, 95% c.i. 0.588-0.962, p=0.024) and after adjustment for potential confounders the reduction was 23% (relative score 0.767, 95% c.i. 0.599-0.982, p=0.036).

Serum levels of 25-OH vitamin D_3

Serum 25-hydroxyvitamin D_3 levels did not differ between the groups at baseline (Table 1). However, already after 3 months the intervention group had a significantly higher level of 25-hydroxyvitamin D_3 (133.4 nmol/L versus 66.6 nmol/L, p<0.001, Figure 3). This increase remained throughout the study (Figure 3).

Bacterial cultures and microbiology

During the course of the study, 173 microbiological samples were obtained in the vitamin D_3 group (n=62, 2.79/patient) and 301 in the placebo group (n=62, 4.85/patient) (p=0.010, Table 3). The number of samples with at least one positive finding was higher in the placebo group, with close to statistical significance (p=0.052), while the fraction of positive samples was similar for both groups (Table 3). Significantly more patients had a microbiological sample taken from the respiratory tract (≥ 1 sample) during the study period in the placebo group; OR 2.63 (95% CI 1.17-5.92), (Table 3).

In total, the vitamin D_3 group generated 76 positive microbiological findings (bacteria or fungi), compared to 159 in the placebo group (p=0.023). There was no difference between the groups for the traditional respiratory pathogens (*H. influenza, M. catharralis and S. pneumonia*), but there were significantly fewer findings of *S. aureus* (p=0.019) and fungi (p=0.028, *Candida* spp. and *Aspergillus* spp.) in the treatment group (Table 4). Likewise, significantly fewer vitamin D_3-treated patients had a bacterial culture positive for *S. aureus* (p=0.019) or fungal species (p=0.058), although the latter difference did not reach statistical significance (Table 4).

Vitamin D_3-treated patients with sub-class deficiency left significantly fewer bacterial or fungal cultures than placebo-treated patients with this diagnosis; 7 cultures in the vitamin D group (n=22) versus 47 cultures in the placebo group (n=24) (Table S4). Also the number of

patients that had ≥1 bacterial culture taken was significantly fewer in the placebo group (12/22 versus 22/24, p=0.0065, table S4). There was no significant effect of other immunological diagnoses on bacterial cultures or microbiology (Table S4).

Since concomitant lung disease may be an important factor for Vitamin D_3-mediated effects on respiratory immunity, we performed a detailed analysis of bacterial cultures and microbiology of patients with asthma, bronchiectasis (BE) and chronic obstructive pulmonary disease (COPD). The numbers of patients with these diagnoses were quite small, which preclude any firm conclusions regarding any effect. However, there was a non-significant trend that Vitamin D_3-treated patients with asthma produced fewer bacterial cultures (average 2.9 cultures/patient versus 7.0 cultures/patients, p=0.080, Figure S3) and fewer positive cultures than placebo-treated asthmatics (average 0.6 positive cultures/patients versus 2.7/patient in the placebo group, p=0.052, Figure S3). In addition, Vitamin D_3-treated asthma patients showed significantly fewer cultures positive for fungi (candida and aspergillus) compared to placebo-treated asthmatics (p=0.0476, table S5). For BE or COPD-patients there was no clear trend or significant effect in bacterial cultures or microbiology.

Levels of antimicrobial peptides (AMPs) in nasal fluid

There was no statistically significant difference between the vitamin D_3 or placebo groups when nasal fluids were analysed for the presence of AMPs. Initially, levels of both LL-37 and HNP1-3 tended to be higher in the placebo group (Figure S3, panels A and B). However, after 12 months the microbiological pattern was reversed and no primary pathogens could be detected in nasal swabs from vitamin D_3-treated patients (n=25, p=0.039) (Figure S4, panel C). The placebo-treated patients exhibited the same mix between normal flora and primary pathogens at all three sampling points (0, 6 and 12 months) (Figure S4, panel C).

SNP variants and treatment effect

Most genetic variants did not affect the primary endpoint. However, patients carrying the 'AA' genotype in the CYP2R1-gene, encoding the 25-hydroxylase enzyme, had a larger benefit of vitamin D_3-supplementation (-55%) compared to AG or GG carriers (-6%) (n=124, p=0.046 for interaction, Table S6).

Adverse events

In total, the vitamin D_3 group reported 38 adverse events (AEs) versus 56 AEs in the placebo group. The most common symptoms in the treatment group were headache (n=5) and lumbago (n=5), whereas placebo-treated patients reported paresthesias (n=8), diverticulitis (n=4) and urinary tract infection (n=4) as most frequent AEs (Table 5, Table S7). There was a general trend towards the number of adverse events being higher in the placebo group. Significantly more patients in the placebo group reported cardiovascular problems, such as heart failure, hypertonia and thrombosis (p=0.028). For gastrointestinal and other (non-respiratory) infections there was also a trend favoring the vitamin D_3 group (p=0.058 and p=0.09, respectively). No clinically relevant changes in serum levels of calcium, phosphate, creatinine or albumin could be observed (Figure S5). There was one severe adverse event (SAE) in each group (rabdomyosarcoma in the vitamin D_3 group and lung bleeding in the placebo group), both judged as being unrelated to the study drug.

Discussion

The main conclusion from this long-term randomised controlled trial (RCT) is that vitamin D_3 supplementation reduces the total burden of respiratory tract infections. The primary endpoint was composed of five different parameters that patients recorded daily throughout the study year. All point estimates favoured the vitamin D_3 group and a statistically significant effect was seen on both the total score and on the probability of receiving antibiotics. The effect on the infectious score was evident both in analysis per-protocol and according to intention-to-treat, and withstood adjustment for potential confounders. In addition, the number of bacterial cultures and microbiological findings was significantly reduced in the intervention group. These findings are potentially important and support that Vitamin D_3 supplementation may prevent respiratory tract infections and reduce antibiotic consumption, particularly in patients with hypogammaglobulinemia or with an increased frequency of respiratory tract infections.

However, our study has several limitations: Firstly, the choice of primary endpoint may be questioned since it relies solely on patient-reported information. To compensate for inherent problems with patient-reported data, the evaluation instrument was designed to cover many aspects of an infectious episode, including various symptoms as well as antibiotic consumption. Together the reported data formed an "infectious score", which constituted the primary endpoint of the study. Similar composite scores have successfully been applied to different diseases, such as tuberculosis (TB-score [16]), pneumonia (CURB-65 [17]) and bacterial meningitis (BMS-score [18]). Notably, vitamin D supplementation had a major effect on the odds of taking antibiotics during the study period (a reduction by 63.5%). In addition, the absolute number of days on antibiotics was reduced by 50% (from 33 days in the placebo group to 16 days in the intervention group), which was statistically significant both in the adjusted and unadjusted analyses (table 2). However, despite the relatively modest reduction for the other components of the primary endpoint the overall infectious score was

significantly reduced – mainly as a result of the large effect on the antibiotic parameter - both in the unadjusted and in the adjusted analyses (table 2, figure 2). Another potential problem was that the patient population was very heterogeneous with regards to immune deficiency and concomitant diseases. We adjusted for these factors in the multivariable analyses of the primary endpoint, but the sample sizes in each subgroup were too small to draw any conclusions of effects in specific disease groups. However, a detailed *post-hoc* analysis of the relation between immunological diagnosis, concomitant lung-disease and the secondary endpoints "taken bacterial cultures", "positive bacterial cultures" and "microbiological findings" was performed. There was a clear trend that Vitamin D_3-treated patients with subclass deficiency and/or asthma produced fewer bacterial cultures, fewer positive cultures and fewer fungal cultures (tables S4 and S5, Figure S3). Although this analysis may lack precision due to the small number of patients included, it could have clinical implications regarding target groups for Vitamin D_3 supplementation.

Nevertheless, our double-blind RCT has several strengths. For example, we chose a high daily dose of vitamin D_3 based on published calculations on metabolism and effects on immunity [14 19]. Other RCTs using lower doses of vitamin D_3, 400-2000 IU/day, have mainly been negative with regards to prevention of infections [20 21]. However, one study using 1200 IU/day showed a significant reduction of influenza among school children in Japan [22]. Notably, also studies using higher doses of vitamin D_3 have been negative. Martineau *et al* used 400,000 IU vitamin D_3 during 42 days (9523 IU/day) with the aim of shortening time to sputum conversion in tuberculosis. No significant effect on the primary endpoint could be observed in that study, except in a subgroup with the *tt* genotype in the vitamin D receptor gene [23]. A recent study investigated whether 100,000 IU vitamin D_3/month (3333 IU/day) could reduce the incidence of chronic obstructive pulmonary disease (COPD) exacerbations. There was no significant effect on the primary endpoint, although a *post hoc* analysis revealed that patients

with a low vitamin D_3 level at baseline had a significant effect of Vitamin D_3 supplementation[24].

Importantly, our study is the first to utilize high daily doses for an extended period of one full year. Thus, we covered all four seasons, which was important in Sweden with a known seasonal variation in 25-hydroxyvitamin D_3 levels [25]. Two previous RCTs were performed during the winter season – when vitamin D levels are low – but only during 4 [22] and 6 months [20], respectively. Previous RCTs have been conducted during shorter periods; 42 days [23], 6 weeks [26] and 12 weeks [21], respectively. Interestingly, we observed a clear time dependent effect suggesting that a long term supplementation approach (> 6 months) may be necessary to affect immunity. To expand on the results of a previous study in healthy individuals where no difference between the intervention and placebo groups was observed [21], we chose a study population with frequent RTIs and at least 42 days with infection during the year prior to inclusion. Notably, patients in the study represent a selected group of individuals with frequent RTI, although the immune disorders that they represent (IgA-, IgG-subclass and patients with no defined immune disorder) are generally mild in character and dominated by mucosal RTIs. We also included a small number of CVID-patients, which can be considered to be a more severe immune disorder, but all these patients are treated with IgG replacement therapy and thus well controlled. Hence, the results from this study cannot directly be applied to the general healthy population. Nevertheless, the results provide solid support for additional interventional studies of vitamin D_3, especially in groups consuming large amounts of antibiotics.

The mechanism of the observed effects remains largely unknown. Vitamin D_3 modulates the immune response at many levels, such as induction of AMPs, skewing of T-cells from Th1/Th17 to Tregs as well as general anti-inflammatory effects [14]. Here, we investigated the role of AMPs in nasal fluid. However, we could not detect any significant changes of LL-37

or HNP1-3 during the study period, but noted that placebo-treated patients tended to have higher levels of AMPs after one year of treatment. This was paralleled by a shift of the microflora in the nasal compartment that could explain the unexpected finding of higher AMP-levels in the placebo group. Recently, it was shown that 1,25 $(OH)_2$-vitamin D_3 induces both HNP1-3 and LL-37 in nasal fluid of healthy volunteers (Hiemstra et al, abstract, European Respiratory Society, 2011), supporting that LL-37 may indeed be induced *in vivo*. However, our study design did not allow such conclusions but rather support that vitamin D_3 affect mucosal immunity, leading to a shift of the microflora. Recently, we showed that the bacterial composition in nasal swabs is an important determinant of AMP-levels in nasal fluid [27].

Given that vitamin D_3 induces LL-37 in epithelial cells and that LL-37 kills bacteria *in vitro,* we expected a reduction of the classical bacterial pathogens *H. influenza*, *M. catharralis* and *S. pneumonia* in the intervention group. However, the frequency of these bacteria was not reduced but a reduction of *S. aureus* and fungal species that often colonise the airways was observed. This could be explained by specific effects by vitamin D_3 on immunity against *S. aureus*. In fact, vitamin D induces human beta defensin-2 (HBD-2) with bactericidal activity against *S. aureus* [28]. A recent study showed that low vitamin D levels were associated with an increased risk of being colonised by this bacterium [29]. Further, vitamin D affects immunity against *C. albicans*, which indicates direct effects of vitamin D on human immunity [30]. Alternatively, it is possible that vitamin D_3 may have prevented symptomatic viral infections, which prompted patients to leave a bacterial sample from the airways. Interestingly, there is both mechanistic and clinical evidence that vitamin D_3 can prevent viral infections [31-33], although we did not address this in the current study.

Notably, we observed a prominent increase in the serum concentration of 25-hydroxyvitamin D_3, which indicated good compliance and tolerability of the study drug. In fact, there was a

trend towards adverse events being reported more often in the placebo group, suggesting that vitamin D_3 possibly could be efficient against other diseases, but this observation requires further studies. No clinically relevant changes of blood chemistry (calcium, phosphate, albumin or creatinine) were observed. Despite few adverse events and high tolerability, 16 exclusions occurred during the study year. The main reason was problems to adhere to the protocol and 6/16 patients dropped out of the study after a few weeks. The rest failed to send in the diaries, did not leave blood for monitoring of safety parameters or did not take the study drug. One patient was excluded based on symptoms that could be attributed to vitamin D_3 (facial paraesthesia). However, this patient was later confirmed to have been allocated to placebo.

In summary, we found that supplementation with vitamin D_3 reduced the total infectious score with 47 points per patient during the study year. A clinically meaningful translation of this effect could be e.g. 47 days with cough (47 points), 23 days with ear and sinus symptoms (23×2=46 points) or 9 days with cough, sinus and ear symptoms together with malaise and antibiotics (9×5=45 points). In addition, our data indicate that vitamin D_3 supplementation reduces the odds of taking antibiotics by approximately 60% in patients with frequent respiratory tract infections. Thus, supplementation with vitamin D_3 could provide a novel strategy to reduce antibiotic use among high consumers and indirectly prevent the emerging epidemic of bacterial resistance.

Acknowledgment

The study was registered at www.clinicaltrials.gov (NCT01131858), prior to the start of the study. The entire process of study design and protocol, data monitoring and analyses was performed by academic authors; there was no industry involvement in the study except that vitamin D_3 (Vigantol®) and placebo oil (Miglyol®) were provided by Merck KGaA (Darmstadt, Germany). Merck did not have any influence on study design, analysis of data, writing or decision to publish. We extend our gratitude to Ilona Skilving, Karolinska Trial Alliance for invaluable help with the protocol. Further, we thank registered nurses Maria Lindén and Kristina Johansson for skillful work with patients. Thanks also to Jenny Lindén and Alicia Hansson for registration of data and to professor Mats Remberger for discussions on statistical methods. Professor Lars Lindqvist, Department of Infectious Diseases, Karolinska University Hospital is gratefully acknowledged for serving as the monitor of the study. PB, LBB and JDL are holding PostDoc-positions financed by Karolinska Institutet and Stockholm County Council (KI/SLL).

Data sharing statement:

There is no additional data available.

Statements

Author contributions:

Peter Bergman, designed the study, collected, analysed and interpreted data, wrote the paper.

Anna-Carin Norlin, designed the study, collected and interpreted data, wrote the paper.

Susanne Hansen, designed and coordinated the study, collected and interpreted data.

Rokeya Sultana Rekha, carried out experimental work, analysed data

Birgitta Agerberth, analysed and interpreted data, wrote the paper.

Linda Björkhem-Bergman, analysed and interpreted data, wrote the paper.

Lena Ekström, analysed and interpreted data

Jonatan Lindh, analysed and interpreted data, wrote the paper.

Jan Andersson, designed the study, interpreted data, wrote the paper.

Role of the funding source

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Merck GmbH provided the study drug (Vigantol) but did not have any influence on study design, analysis of data, writing or decision to publish.

Ethics statement: The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants.

Conflicts of interest: There are no conflicts of interest.

Legends to figures

Figure 1. Study outline.

Figure 2. Primary endpoint. The adjusted total relative infectious score (A) is expressed 'per quarter' (3 month periods). The adjusted one-year scores (total score, airway, malaise, ear, sinus and antibiotics) are depicted in a Forrest-plot (B) together with 95% confidence intervals. Effects are presented as relative scores (total score, airway, malaise) or odds ratios (ear, sinus, antibiotics, indicated with asterisks).

Figure 3. Secondary endpoint. Vitamin D-levels. Serum was collected at day 0, 3, 6, 9 and 12 months and levels of 25-hydroxyvitamin D_3 were measured. Values are expressed as mean +/- 95% confidence interval.

Tables

Table 1. Baseline data. Mann Whitney U-test was used for comparisons of age and 25-OH vitamin D_3. Fisher's exact test was used for all other comparisons. 1) "other disease" includes hypertension, body pain, hypothyroidism and gastritis as most common diagnoses. CVID, common variable immuno deficiency; ND, increased susceptibility to infections without a defined immunological disorder; BE, bronchiectasis; COPD, chronic obstructive pulmonary disease.

	Vitamin D_3	Placebo
Number	70	70
Age (mean)	55,4	50,8
Female	52/70	50/70
Male	18/70	20/70
IgG-replacem.	39/70	42/70
Smoking	4/70	6/70
25-OH levels (mean)	51,5 nmol/L	46,9 nmol/L
Immunological diagnosis		
sIgA-deficiency	9/70	9/70
IgG subclass	27/70	30/70
CVID	6/70	4/70
ND	28/70	27/70
Concomitant disease		
No other disease	16/70	18/70
Lung: Asthma	27/70	25/70
Lung: BE	5/70	7/70
Lung: COPD	5/70	4/70
Other disease[1]	17/70	16/70

Table 2. Primary Endpoint. Treatment effect calculated as the ratio between infectious scores in the vitamin D_3 and the placebo groups. Due to low frequencies, endpoints marked with asterisks were coded as binary outcomes (i.e. present or absent in each patient) and compared by means of logistic regression. In these cases, the effect refers to odds ratios of experiencing the outcome at least once during the course of the study. (The data are based on n=124 patients).

	Univariable regression model (unadjusted values)			Multiple regression model (adjusted values)		
Endpoint	**Effect**	**95% CI**	**p-value**	**Effect**	**95% CI**	**p-value**
Total score	0.754	0.591-0.963	**0.024**	0.771	0.604-0.985	**0.040**
Airway	0.857	0.697-1.053	0.141	0.871	0.706-1.074	0.200
Ear*	0.721	0.352-1.465	0.367	0.695	0.320-1.501	0.357
Sinus*	0.583	0.280-1.198	0.144	0.594	0.265-1.328	0.204
Malaise	0.845	0.692-1.032	0.098	0.845	0.689-1.036	0.108
Antibiotics*	0.355	0.154-0.784	**0.012**	0.365	0.153-0.872	**0.023**

Table 3. Bacterial cultures. [1]Mann-Whitney U-test, [2]Fisher's exact test.

	Vitamin D_3	Placebo	Significance
Number of samples per patient (mean, n=62/62)	2.79	4.85	p=0.010[1]
Number of positive samples per patient (mean, n=62/62)	1.01	2.02	p=0.052[1]
Fraction positive cultures (%)	63/173 (36%)	125/301 (41%)	P=0.28[2]
Patients with ≥ 1 sample taken	38/62 (61%)	50/62 (81%)	p=0.029[2]

Table 4. Microbiological findings. Mann-Whitney U-test was used to analyze the total number of findings, whereas Fisher's exact test was used for analysis of the number of patients (fraction) with a specific finding.

	Number of findings (total)			Number of patients		
Microorganism	Vitamin D_3	Placebo	MW-U	Vitamin D_3	Placebo	Fisher
H. influenzae	28	27	P=0.46	10/62	13/62	P=0.64
M. catharralis	8	17	P=0.39	7/62	10/62	P=0.60
S. pneumoniae	7	6	P=0.74	4/62	5/62	P=1.00
S. aureus	6	33	P=0.010	4/62	14/62	p=0.019
Enterobacteriacae	8	8	P=0.39	4/62	7/62	P=0.53
P. aeruginosa	8	15	P=0.68	3/62	4/62	P=1.00
Fungal infection	11	53	P=0.028	4/62	12/62	p=0.058
Total	76	159	P=0.023			

Table 5. Adverse events. Number of reports. Fisher's exact test was used for between group comparison. (The data are based on AE-reports from n=62 patients/arm).

Organ	Vitamin D_3 n (%)	Placebo n (%)	P-value
CNS	11 (29)	10 (18)	1.00
Gastrointestinal	4 (11)	12 (21)	**0.058**
Cardiovascular	0 (0)	6 (11)	**0.028**
Infections (other than RTI)	2 (5)	8 (14)	**0.09**
Musculoskeletal	10 (26)	10 (18)	1.00
Respiratory (non-infectious)	2 (5)	4 (7)	0.68
Skin	5 (13)	2 (4)	0.44
Other	4 (10)	4 (7)	1.00
Total	**38**	**56**	

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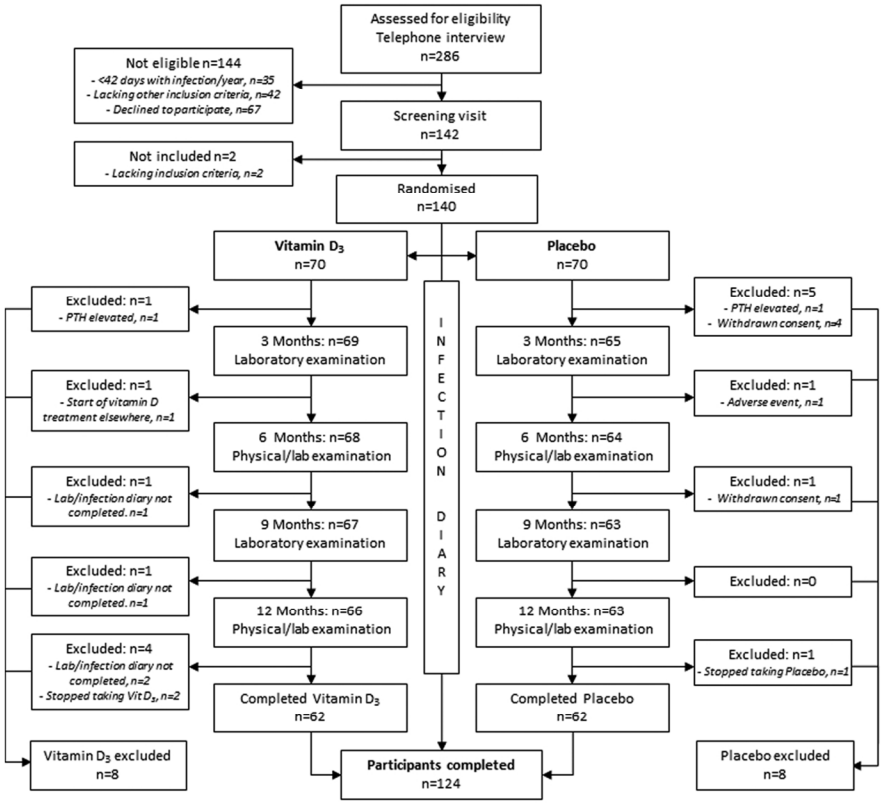

Figure 1
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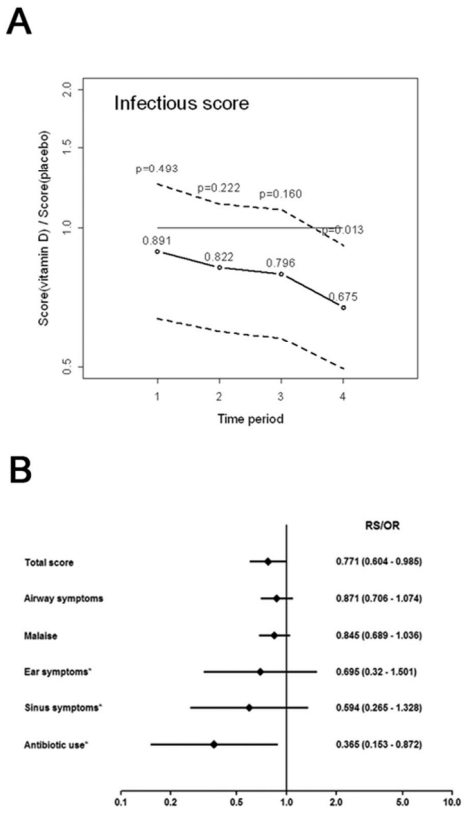

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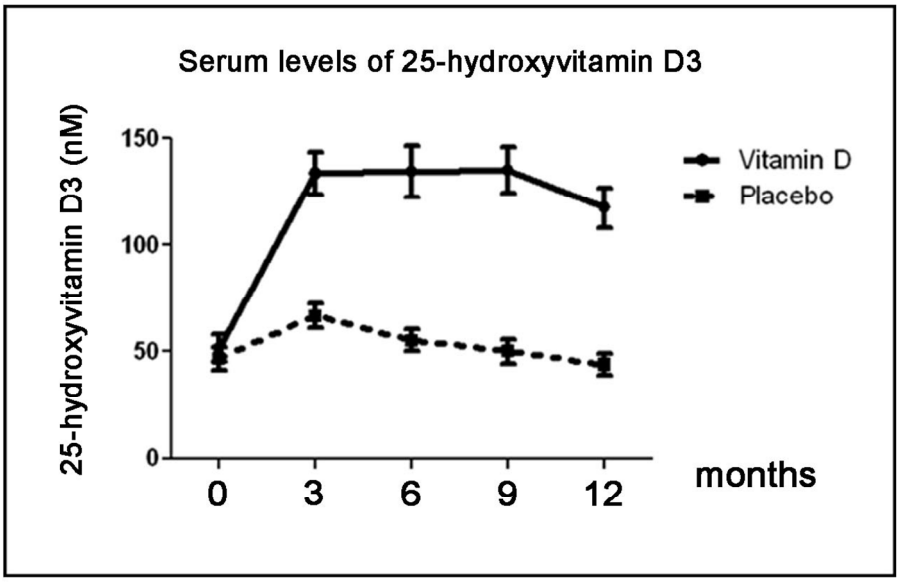

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***Bergman et al,* Vitamin D_3 supplementation in patients with frequent respiratory tract infections - a randomised and double blind intervention study**

Supplementary Methods

Randomisation and Blinding

A computer-generated list of random numbers was used for patient allocation. Randomization sequence was created using Randomization.com (http://www.randomization.com) and was stratified with a 1:1 allocation using a fixed block size of 10. Within each block two participants were randomly assigned to provide samples of nasal fluid, one for each treatment group.

The vitamin D_3 and placebo were in liquid form and identical in appearance. They were prepacked in bottles and consecutively numbered for each participant according to the randomization schedule. In connection with the inclusion each participant was sequentially assigned a number by the responsible physician and received the corresponding prepacked bottles.

Participants, investigators and staff were kept blinded to the allocation throughout the trial. It was not necessary to un-blind information on any participant during the trial.

Sampling of nasal fluid, NPH swabs

Since vitamin D_3 can induce antimicrobial peptides both in macrophages and in epithelial cells[1], we measured levels of LL-37 and α-defensins (HNP1-3) in nasal fluid (Figure 4A and B). For logistical reasons we limited patients for nasal fluid collection and only 36/140 patients (20%) were randomised to this procedure. Nasopharyngeal swabs were taken from

one nostril and sent to the Clinical Microbiology Laboratory at Karolinska University Hospital, Huddinge for bacterial culture. The bacterial content was evaluated as "no growth of bacteria", "normal flora" (typical findings include α-haemolytic streptococci, *Corynebacteria* spp, *Neisseria* spp. and other nonpathogenic strains) or "pathogenic growth" (defined here as *H. influenzae, S. aureus, S. pneumoniae, M. catharralis* and *Enterobacteriacae* spp). Subsequently, nasal fluid was collected through a thin plastic tube that was carefully placed in the back of the nose using the other nostril as entry port (10-12 cm from the nostril meatus). 5-10 ml of saline was administered into the nose prior to sampling in order to make the epithelial lining moist and to dissolve mucus depositions. A gentle vacuum was applied and 3-5 ml nasal fluid was collected and stored at -20°C, as described in Cederlund et al, PLoS One, 2011[2].

Extraction of peptides and proteins from nasal fluid

Nasal fluid (3-5 ml) was extracted in an equal volume (1:1) of 60% acetonitrile (AcN) in 1% trifluoroacetic acid (TFA) over night at 4°C. The extract was centrifuged at 3500g and the supernatant was lyophilized. The lyophilized extract was resuspended in 0.1% TFA and enriched for polypeptides using solid phase extraction as described in[2]. The lyophilized polypeptide extract was reconstituted in 0.1% TFA to a concentration of 5 µg/µl as determined spectrophotometrically using a Nanodrop-system (Thermo Scientific, Wilmington, U.S.).

Analysis of antimicrobial peptides in nasal fluid

The concentrated and lyophilized extract (25 µg) was dissolved in lithium dodecyl sulphate (LDS) sample buffer, 50 mM Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 70°C for 10 min. The samples were then separated using LDS-PAGE and blotted onto PVDF membranes, as described in[3]. Antibodies used were a LL-37 monoclonal[4] and a HNP1-3 goat polyclonal (sc-22916, Santa Cruz, Santa Cruz, Calif., USA). Proteins and peptides were visualized on chemiluminescence film with ECL plus Western blot detection system (GE Healthcare, Buckinghamshire, United Kingdom). LL-37 and HNP1-3 concentration in nasal fluid were determined by densitometry using the software ImageJ (http://rsbweb.nih.gov/ij/). The intensity of each band was normalized to an external standard on each membrane and the total amount of LL-37 and HNP1-3 was determined by multiplying the densitometric result (ng peptide/µg extract) with the total amount of polypeptide-extract (µg). Thus, the values represent the total amount of LL-37 and HNP1-3 from each nasal fluid sample.

Analysis of 25-OH vitamin D_3 in serum

Levels of 25-hydroxyvitamin D_3 in serum were determined by using DiaSorin immunochemical method (DiaSorin S.p.A, Saluggia, Italy) at the Department of Clinical Chemistry, Karolinska University Hospital.

Genotyping

Specific single nucleotide polymorphisms (SNPs) in key genes for vitamin D metabolism might influence the outcome of vitamin D_3 supplementation. Therefore, all patients were

genotyped for 6 SNPs in the VDR (TaqI and FokI), CYP27B1, CYP24A1, CYP2R1 and GC genes. Six SNPs in five genes involved in vitamin D metabolism and / or effect were analysed in all participants. The aim of these analyses was to investigate whether individuals with a specific genotype would benefit more from vitamin D_3 supplementation. Genomic DNA was isolated from 200 µl peripheral blood leucocytes using the DNA Blood Mini kit (Qiagen, Hilden Geramany). Allelic discrimination reactions were performed using TaqMan® genotyping assays (Applied Biosystems, Foster City CA USA): C_12060045_20 for VDR (FokI); C_2404008_10 for VDR (TaqI); C_29958084 for CYP24A1; C_2958431_10 for CYP2R1; C_26407519_10 for GC. For the CYP27B1 genotyping, primers and probes described previously were used[5]. The final volume for each reaction was 15 µl consisting of 30 ng DNA and 2xTaqman Universal PCR Master mix (Applied Biosystems). The PCR profile consisted of 95° C for 10 minutes followed by 40 cycles of 92° C for 15 sec and 60° C for 1 minute. The fluorescence signal was measured with an ABI 7500 Sequence detector (Applied Biosystems).

Statistical methods: Sample size calculation

The sample size was based on the assumption that the intervention would reduce the number of days with symptoms from 6 weeks (42 days x 5 points = 210 points) to 4 weeks (28 days x 5 points = 140 points), i.e. a reduction of the infectious burden by 30%. The estimated standard deviation was 3 weeks (21 days x 5 points = 105 points). Given these assumptions, a sample size of 60 patients per study group was predicted to provide the study 90% power at a significance level of p=0.02 (Student's t-test). To compensate for predicted exclusion of participants, the groups were increased to include 70 patients per treatment arm.

Statistical methods: Secondary analyses

The number of bacterial cultures taken in each patient and the number of samples with a positive finding were compared between the two study groups by means of the Mann-Whitney U test. To reduce the influence of patients subjected to very frequent sampling, the odds of having one or more culture taken during the course of the study was also compared by means of Fisher's exact test. Similarly, the frequencies of cultures positive for specific pathogens were compared both as number of positive cultures per patient (Mann-Whitney U test) and as fraction of patients presenting with at least one positive culture (Fisher's exact test). The fraction of nasopharyngeal samples exhibiting bacterial growth was compared between the two groups separately for samples taken at baseline, after six month and after 12 months (Fisher's exact test).

The influence of genetic polymorphisms on the effect of vitamin D_3 treatment was analysed in linear regression models with log-transformed infectious score as dependent variable. Independent variables were study group, genotype and a genotype-study group interaction term. Genotypes were coded as binary variables, based on previous findings reported in the literature[5-10].

In all analyses, P values <0.05 (two-sided) were considered statistically significant (the significance level of 0.02 in the power calculation was chosen to provide an extra safety margin). All statistical analyses were performed using R 2.11.1 (R Development Core Team (2010). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org) and GraphPadPrism, version 5.0, GraphPad Software, La Holla, Calif, USA

Supplementary References

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***Bergman et al*, Vitamin D_3 supplementation in patients with frequent respiratory tract infections - a randomised, double blind intervention study**

Supplementary tables

Table S1. Data on IgG substitution for participants that were included in the per protocol analysis (n=62/arm).

Treatment with IgG per diagnosis	Yes	No	Total
Subclass deficiency	29	17	46
IgA deficiency	6	10	16
CVID	9	0	9
ND	29	24	53
Total:			124

Table S2. Primary endpoint. Unadjusted relative score per day calculated per 3 months periods as indicated. Values are expressed as mean +/- SD. (n=62/arm).

	Month 1-12		Month 1-3		Month 4-6		Month 7-9		Month 10-12	
	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo
Infectious score	0.56(0.58)	0.69(0.54)	0.58(0.66)	0.67(0.70)	0.51(0.61)	0.59(0.57)	0.59(0.67)	0.72(0.65)	0.53(0.56)	0.77(0.61)
Airway symptoms	0.26(0.24)	0.32(0.28)	0.27(0.27)	0.29(0.28)	0.25(0.29)	0.25(0.24)	0.27(0.27)	0.30(0.27)	0.27(0.27)	0.33(0.27)
Malaise	0.16(0.20)	0.18(0.17)	0.16(0.24)	0.18(0.22)	0.14(0.21)	0.15(0.18)	0.17(0.23)	0.19(0.20)	0.15(0.20)	0.22(0.22)
Ear symptoms	0.04(0.09)	0.07(0.15)	0.05(0.12)	0.07(0.17)	0.03(0.09)	0.05(0.14)	0.05(0.10)	0.08(0.18)	0.05(0.10)	0.08(0.18)
Sinus symptoms	0.05(0.12)	0.06(0.10)	0.05(0.12)	0.06(0.13)	0.04(0.10)	0.04(0.10)	0.06(0.15)	0.07(0.13)	0.05(0.14)	0.07(0.12)
Antibiotic use	0.04(0.06)	0.09(0.14)	0.05(0.09)	0.08(0.16)	0.04(0.08)	0.09(0.17)	0.04(0.08)	0.09(0.15)	0.04(0.07)	0.11(0.18)

Table S3: Primary endpoint. Unadjusted score.

	Mean score / patient / year			MWU (unadjusted)
Endpoint	**Vitamin D**	**Placebo**	**Diff.**	**p-value**
Total score	202	249	-47	0,023
Airway	94	101	-7	0,302
Ear*	16	25	-9	0,225
Sinus*	18	21	-3	0,126
Malaise	56	66	-10	0,041
Antibiotics*	16	33	-17	0,024

Table S4: Secondary endpoint. Number of bacterial cultures in relation to immunological diagnosis. Patients can have several bacteria or fungi in the same culture. Values within parentheses indicate the number of patients that provided the positive cultures. **p=0.0065, Fisher's exact test (12/22 vs 22/24).

								Number of patients	Number of patients
Number of bacterial cultures per diagnosis and treatment		(n)	H. infl.	M. Cath	S. pneum.	S. aur	Fungi	>1 culture taken	>1 culture positive
Subclass deficiency	Vitamin D	22	2(1)	3(3)	2(2)	0	0	12**	5
	Placebo	24	15(6)	3(2)	2(2)	13(5)	14(4)	22**	12
IgA deficiency	Vitamin D	9	4(1)	1(1)	0	0	0	4	3
	Placebo	7	1(1)	2(2)	0	1(1)	0	5	2
CVID	Vitamin D	5	1(1)	3(2)	0	0	0	4	1
	Placebo	4	3(1)	0	2(1)	1(1)	3(1)	4	3
ND	Vitamin D	26	21(7)	1(1)	5(2)	6(4)	11(4)	17	12
	Placebo	27	8(5)	1(1)	2(2)	18(7)	36(7)	19	14
Total		124						0	0

Table S5: Secondary endpoint. Microbiological findings in relation to concomitant lung disease. Values within parentheses indicate the number of patients that provided the positive cultures. H. infl: *Haemophilus influenza*; M. cath: *Moraxella catharralis*; S. pne: *Streptococcus pneumonia*; S. aur: *Staphylococcus aureus*; Eba: *Enterobacteriaceae spp*; P. aer: *Pseudomonas aeruginosa*. **Fishers exact test, p=0.0467 (0/27 vs 4/25).

		H. infl.	M. cath	S. pne	S. aur	Eba	P. aer	Fungi	Sum
Asthma	Vit D, n=27	7(4)	4(2)	2(2)	2(2)	1(1)	1(1)	0**	17
	Plac, n=25	9(5)	6(5)	1(1)	4(4)	2(2)	4(1)	11(4)**	37
BE	Vit D, n=5	5(2)	1(19	0	4(1)	2(1)	0	1(1)	13
	Plac, n=7	1(1)	0	0	8(1)	1(1)	0	3(1)	13
COPD	Vit D, n=5	9(2)	1(1)	0	3(1)	2(1)	0	0	15
	Plac, n=4	0	0	0	8(2)	0	0	0	8

Table S6: Genotyping. Influence of genetic factors on the outcome of vitamin D_3 treatment in patients with frequent respiratory tract infections. Mean infectious score (0-12 months) are presented per genotype and study group, along with the number of included patients. P-values refer to an interaction between genotype and study group.

Gene / SNP	Allel-group	Vitamin D_3 Mean inf score (n)	Placebo Mean inf score (n)	$p_{interaction}$
VDR: TaqI	tt tT /TT (reference)	145 (11) 202 (49)	225 (12) 253 (47)	0.757
VDR: FokI	ff fF /FF (reference)	295 (5) 195 (56)	331 (6) 238 (53)	0.575
CYP27B1: Rs10877012	CC AC/CC (reference)	181 (27) 220 (34)	211 (21) 268 (38)	0.194
GC: RS2282679	AA AC/CC (reference)	205 (39) 162 (21)	214 (28) 283 (29)	0.247
CYP2R1: Rs2060793	AA AG/GG(reference)	142 (8) 212 (53)	315 (14) 227 (45)	0.046
CYP24A1: Rs6013897	AA AT /TT(reference)	92 (2) 207 (59)	221 (2) 249 (57)	0.473

Table S7. Detailed description of adverse events.

Group	Adverse event	Vit D	Placebo	Total
CNS	Headache	5		5
	TIA (Transient Ischemic attack)	1		1
	Vertigo	4		4
CNS Total		10		10
Numbness - pain- shakings	Numbness, pain		8	8
	Paresthesias		2	2
	Tremor	1		1
Numbness-pain-shakings Total		1	10	11
Gastrointestinal symptoms	Diarrhoea		2	2
	Diverticulitis		4	4
	Dyspepsia	2	2	4
	Gastroenteritis	2	2	4
	Helicobacter pylori infection		2	2
Gastrointestinal symptoms Total		4	12	16
Heart/ vessels	Congestive heart disease		2	2
	Hypertension		2	2
	Thrombosis		2	2
Heart/ vessels Total			6	6
Infections	Herpes Zoster	1		1
	Pneumonia	1		1
	Sinusitis		2	2
	Urinary tract infection		4	4
	Pyelonephritis		2	2
Infections Total		2	8	10
Body pain – joint pain	Bursitis		2	2
	Body pain	2	2	4
	Joint pain fingers/ hands	1	2	3
	Joint pain hip		4	4
	Pain in feet	1		1
	Back pain	5		5
	Elbow swelling	1		1
Body pain- joint pain Total		10	10	20
Lungs	Asthma exacerbation	1		1
	Pneumothorax		2	2
	Heavy breathing		2	2
Lungs Total		1	4	5
Ears	Hearing problems	1		1
Ears Total		1		1
Other	Shivering	2		2
	Menstruation too often		2	2
	Nose bleeding	1		1
	Toothache	1		1
	Artheritis temporalis		2	2
Other Total		4	4	8
Rash – itch - blisters	Tongue blisters	1		1
	Hand rash	1		1
	Foot rash	1		1
	Facial rash when drinking alcohol		2	2
	Facial rash	1		1
	Chest rash, itching	1		1
Rash – itch – blisters Total		5	2	7
Total		38	56	94

Supplementary figures

Figure S1. The diary that was used for patients to register their daily symptoms.

Symptoms from "airways", "ears" and "sinuses" were calculated as maximum 1 point per anatomical site per day. "Malaise" and "antibiotic consumption" gave maximum 1 point per day. The occurrence of X-ray verified pneumonia resulted in 3 extra points per day for one week. Thus, 8 points was the maximum value that could be obtained per day. These data constituted the primary endpoint of the study. The diaries were filled out by the patient and sent monthly per mail to the study site.

Figure S2. Primary endpoint. Temporal analysis of infectious score components.

The adjusted one-year relative scores presented separately for each 3 month period. (A) airways, (B) malaise, (C) ear symptoms, (D) sinus symptoms and (E) antibiotic consumption. Effects are presented as relative scores (airway and malaise) or odds ratios (ear, sinus and antibiotics). Dashed lines indicate 95% confidence intervals.

Figure S3. Secondary endpoint. Number of bacterial cultures in relation to concomitant lung disease. The number of bacterial cultures taken (A) and positive bacterial cultures (B). Asthma (vit D, n=22; Placebo, n=22). Bronchiectasis, BE (Vit D, n=5; Placebo, n=7). Chronic Obstructive Pulmonary Disease, COPD (Vit D, n=4; Placebo, n=4).

Figure S4. Antimicrobial peptides in nasal fluid. Levels of LL-37 (A) and HNP1-3 (B) were measured in nasal fluid extracts at day 0, 6 and 12 months in a randomly selected group of patients (LL-37, n=12; HNP1-3, n=15). There were no statistically significant differences within or between the groups with regards to peptide levels (Mann-Whitney U test). Bacterial

growth in these samples were also recorded (C) and expressed as either 'no growth/normal flora' or 'growth of a primary pathogen'. The growth pattern of the vitamin D_3 and Placebo groups were compared at each time-point using Fisher's exact test.

Figure S5. Blood chemistry. Plasma levels of calcium (mmol/L), phosphate (mmol/L), albumine (g/L) and creatinine (µmol/L) were measured at the time points 0, 3, 6, 9 and 12 months after inclusion. Values are expressed as mean values.

Figure S1.

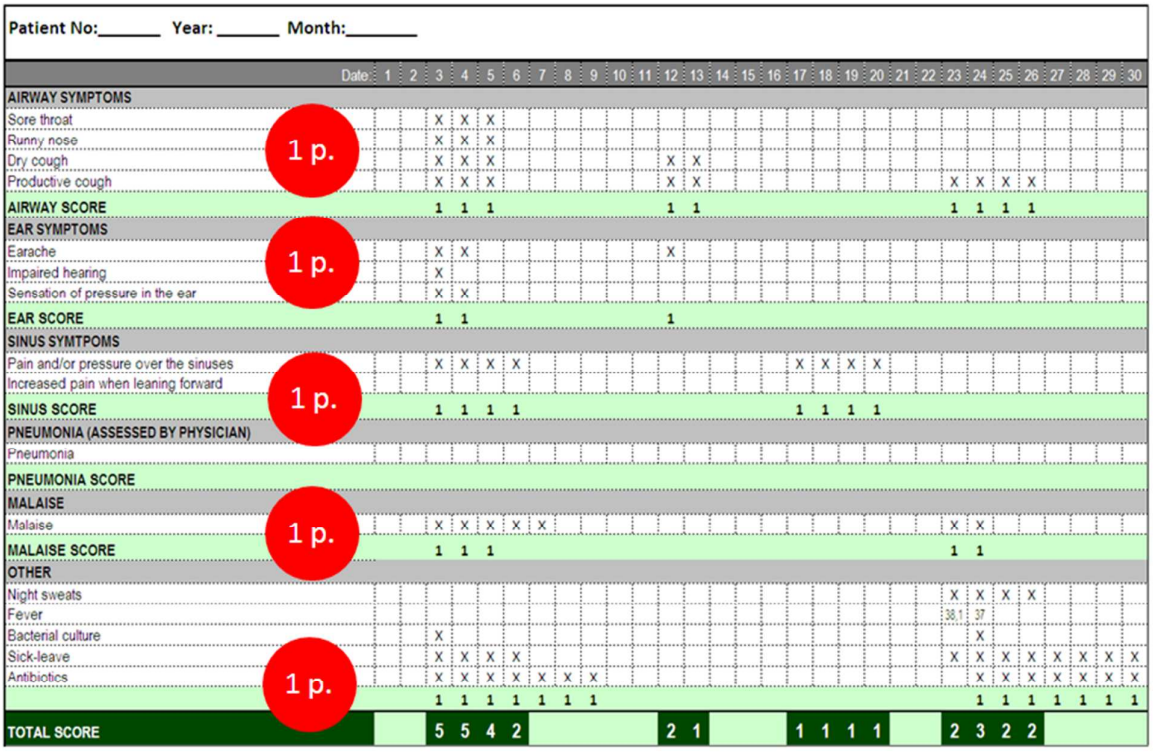


Figure S2

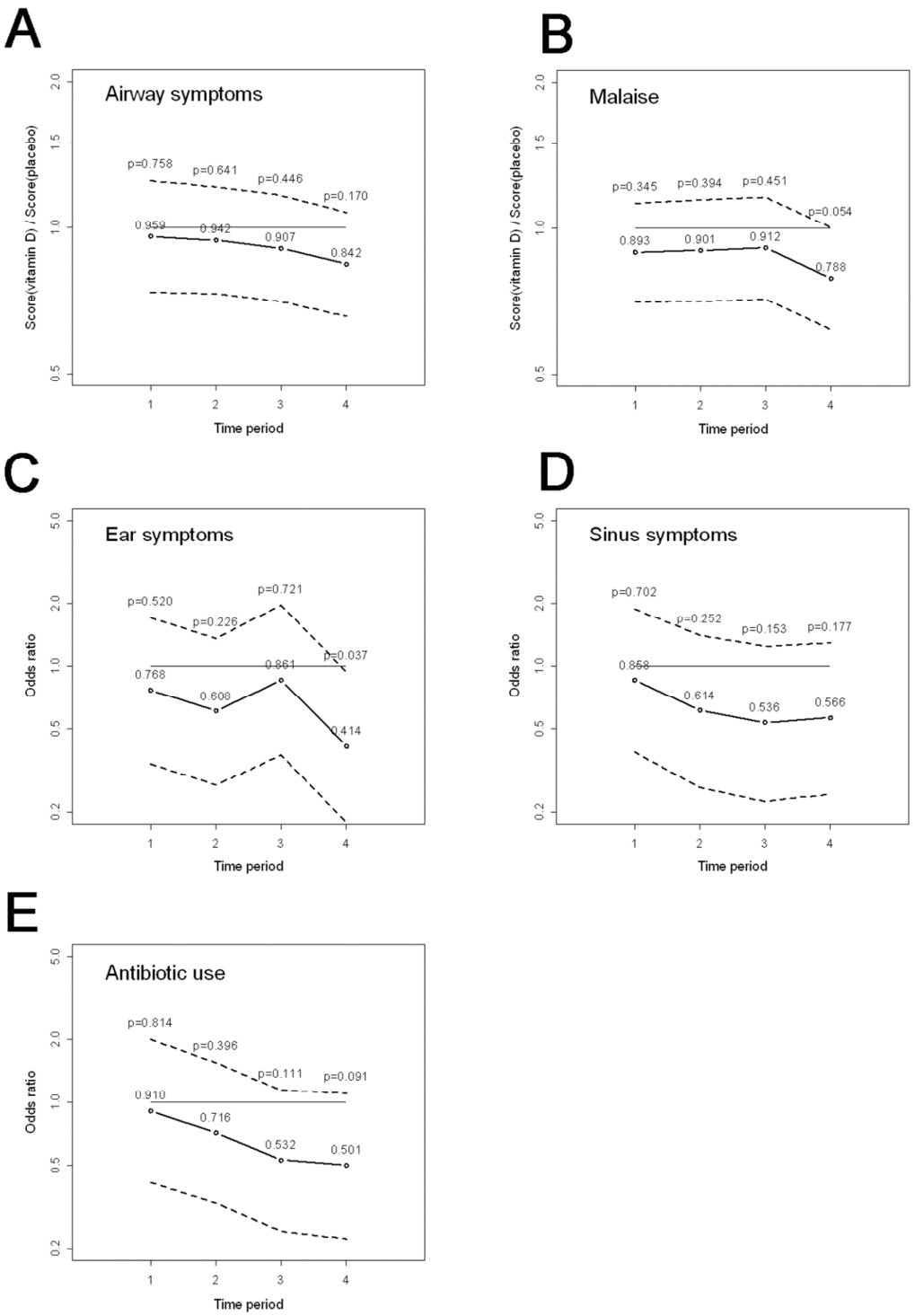

A
Airway symptoms
Score(vitamin D) / Score(placebo)
p=0.758
p=0.641
p=0.446
p=0.170
0.959
0.942
0.907
0.842
Time period
B
Malaise
Score(vitamin D) / Score(placebo)
p=0.345
p=0.394
p=0.451
p=0.054
0.893
0.901
0.912
0.788
Time period
C
Ear symptoms
Odds ratio
p=0.520
p=0.226
p=0.721
p=0.037
0.768
0.608
0.861
0.414
Time period
D
Sinus symptoms
Odds ratio
p=0.702
p=0.252
p=0.153
p=0.177
0.858
0.614
0.536
0.566
Time period
E
Antibiotic use
Odds ratio
p=0.814
p=0.396
p=0.111
p=0.091
0.910
0.716
0.532
0.501
Time period

Figure S3

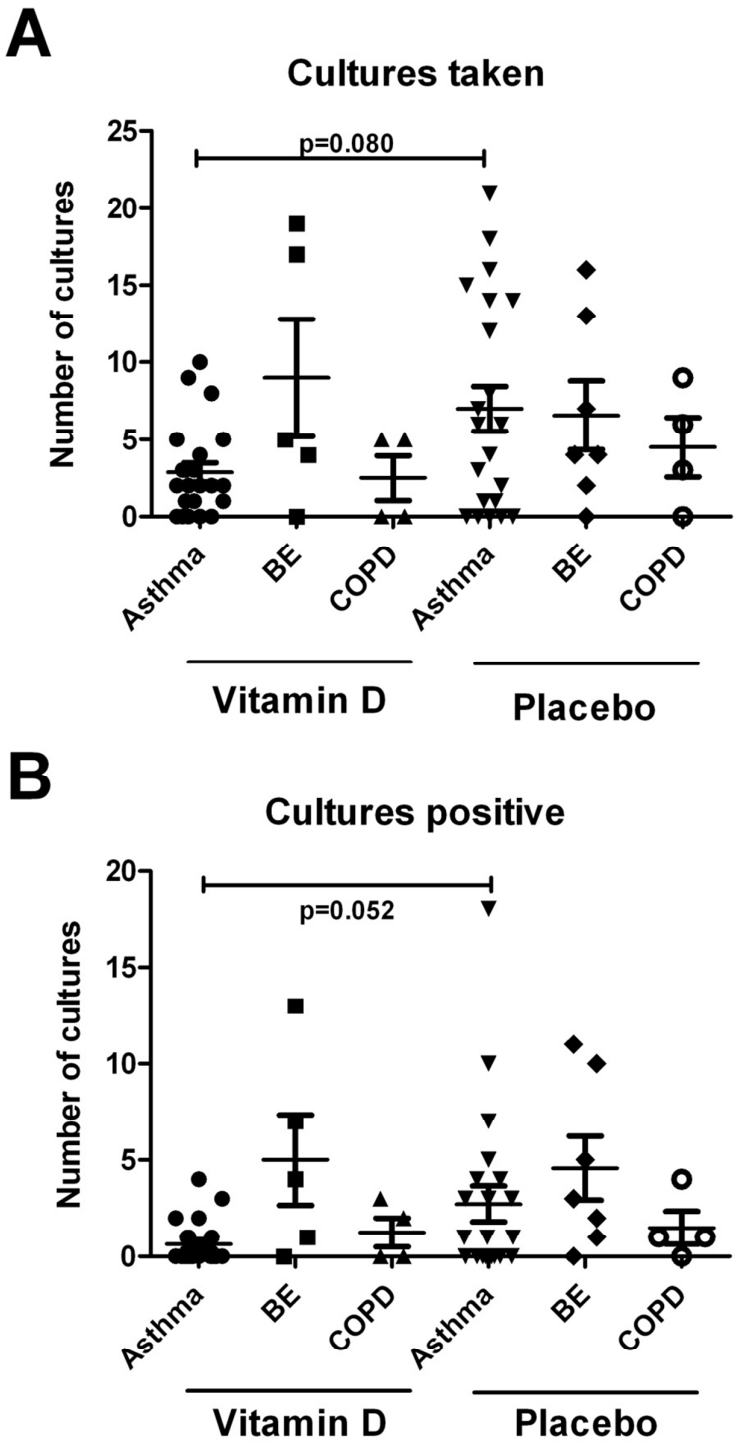


Figure S4

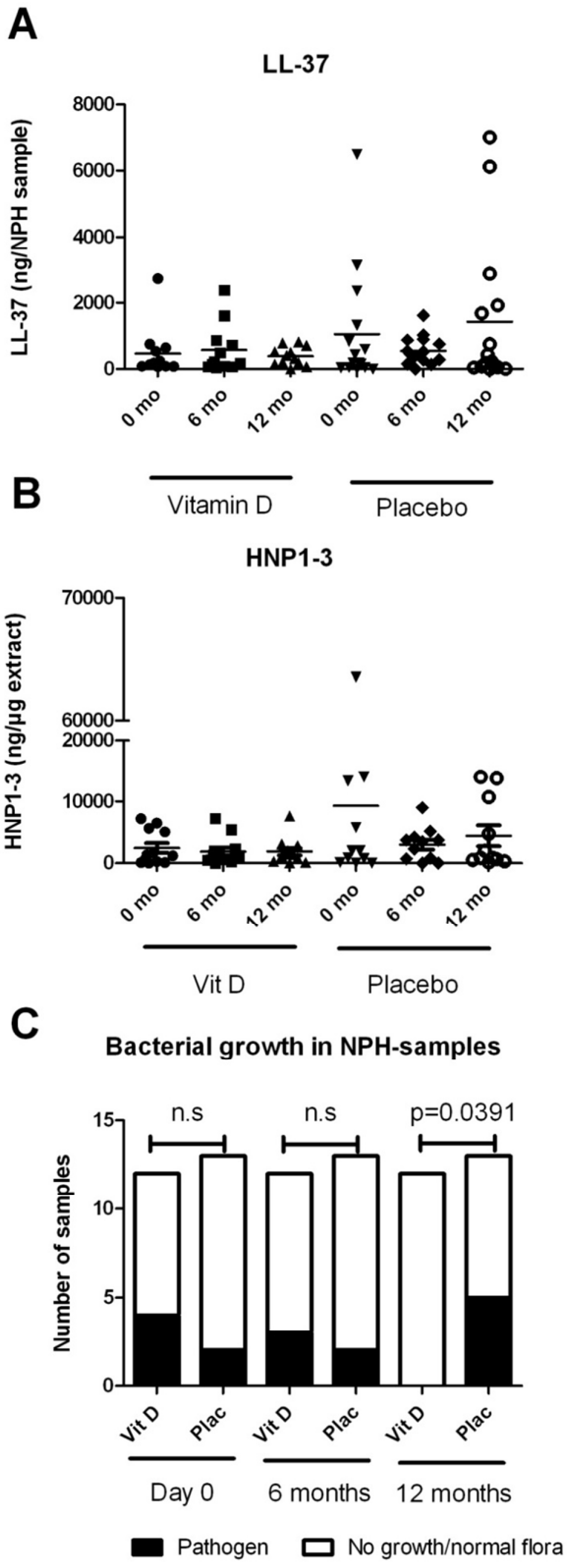
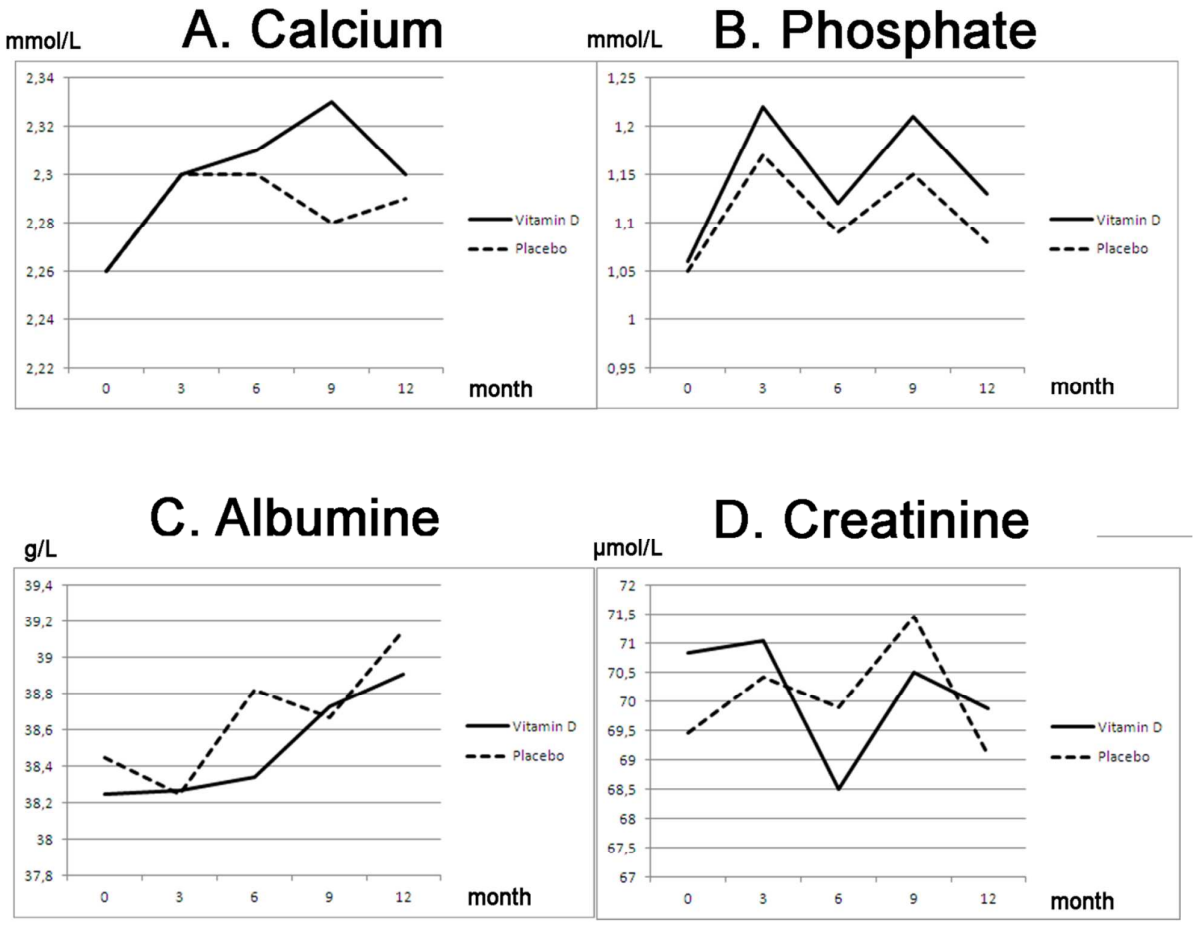


Figure S5



CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	p. 1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	p. 2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	p. 5-6
	2b	Specific objectives or hypotheses	p. 6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	p. 7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	Not Applicable (NA)
Participants	4a	Eligibility criteria for participants	p. 7
	4b	Settings and locations where the data were collected	p. 7
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	p. 8
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	p. 8
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	Suppl meth p4
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	Suppl meth.
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Suppl meth.
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	Suppl meth.

Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	p. 9
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	Suppl meth.
	11b	If relevant, description of the similarity of interventions	NA
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	p. 8-9
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	Suppl meth.
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	p. 10, Fig 1
	13b	For each group, losses and exclusions after randomisation, together with reasons	p. 10, Fig 1
Recruitment	14a	Dates defining the periods of recruitment and follow-up	p. 5
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Yes
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Yes
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Yes
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Yes
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Table 5 +suppl fig/tabl
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Yes
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	Yes
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Yes
Other information			
Registration	23	Registration number and name of trial registry	p. 7
Protocol	24	Where the full trial protocol can be accessed, if available	p. 7
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	p. 20

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Study title:

Study of Vitamin D3 Substitution to Patients With Primary Immunodeficiency (VITAPID)

Date: October 6, 2009

Study site: Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden

Product: Vigantol oil

Substance: Vitamin D3 (cholecalciferol)

Producer: Merck, Germany

EudraCT-number: 2009-011758-16

Sponsor: Professor Jan Andersson, MD, PhD

Co-investigators: Dr Peter Bergman, MD, PhD; Dr Anna-Carin Norlin, MD

Study facts

Protocol identity and aim

EudraCT-number: 2009-011758-16

Protocol title: A placebo-controlled double-blind study of Vitamin D3 supplementation to patients with increased susceptibility to infections.

Aim: To investigate if substitution with vitamin D3 can prevent or ameliorate infectious burden among infection prone patients.

Study drug:

Product:	Vigantol Oil
Pharmaceutic preparation:	Oral mixture (oil)
Administration:	Per os

Methodology

Study design:	Randomized double-blind placebo-controlled
Dose:	Vigantol, 4000 IU/day
Primary endpoint:	Infectious score
Safety parameters:	Plasma levels of calcium, creatinine, albumin and phosphate; serum levels of 25-OH vitamin D_3.
Study population:	Patients with increased susceptibility to respiratory tract infections.
Number:	140

Timeplan:

First patient to be included: Q1, 2010

Last patient to be included: Q4, 2011

Last patient to finish the study: Q4, 2012

Administrative information

Sponsor and Investigators

Professor Jan Andersson, MD, PhD: Sponsor and Principal Investigator

Dr Peter Bergman, MD, PhD: Co-investigator

Dr Anna-Carin Norlin, MD: Co-investigator

Research nurses and study coordinator

Susanne Hansen, Study coordinator, head of Immunodeficiency Unit

Kristina Johansson, Research Nurse

Maria Lindén, Research Nurse

Quality control

Two independent monitors from Karolinska Trial Alliance will monitor the study according to ICH-GCP.

Overview and significance

The innate immune system is depending on antimicrobial peptides, which are potent killers of microbes, such as bacteria, viruses and fungi. These molecules defend epithelial surfaces and are rapidly released after contact with microbes. Vitamin D is a potent inducer of AMPs in epithelial and immune cells. Vitamin D is synthesized in the skin under the influence of UVB-light or can be obtained via the diet. However, in Sweden the UV-radiation has too low intensity during the wintertime and the diet is not enough to maintain adequate levels. Therefore many individuals in Sweden have low levels of vitamin D3, especially during the darker period of the year (October-April). Epidemiological data show a strong association between low vitamin D levels and an increased risk of infection. There is also mechanistic evidence that vitamin D increases the levels of antimicrobial peptides in macrophages and in epithelial cells. However, there are few randomized controlled trials testing the hypothesis that supplementation with vitamin D3 can reduce or ameliorate infections. Therefore, we have designed the study described in this protocol where vitamin D3 will be given to patients with an increased risk of infection. The results may have a great impact on treatment of patients with frequent infections, since vitamin D3 may be used in conjunction with standard care (antibiotics). This may be particularly important in light of the emerging bacterial resistance. Thus, novel strategies to prevent and treat infectious diseases have to be developed and supplementation with Vitamin D3 may constitute one future treatment option.

Aims

To investigate if substitution with vitamin D3 can:

1. Reduce the infectious burden among patients with increased number of infections
2. Increase levels of antimicrobial peptides in nasal fluid
3. Increase serum concentrations of 25-OH vitamin D3

Study design

Participants will be given vitamin D3 or placebo for one year. 140 patients will be recruited and 70 patients will be randomized to placebo or vitamin D3 in a 1:1 randomization. Evaluation of symptoms and antibiotic consumption will be registered by the patient in a diary form that will be sent by mail to the study site on a monthly basis. Patients will be recruited at the tertiary center for primary immune deficiencies. Currently there are 319 patients with IgG-deficiency, 180 patients with selective IgA-deficiency, 90 patients with CVID and 210 patients with an increased susceptibility to infection without a manifest immunological diagnosis. The study patients will be recruited from this group in a nonbiased fashion, ie regardless of diagnosis or IgG-substitution therapy.

Study drug and mechanism

The study drug is cholecalciferol (vitamin D3), which is hydroxylated in the liver to 25-OH vitamin D3 (the storage form in the body). The second hydroxylation step is carried out by 1-alpha hydroxylase. This enzyme is expressed in the kidney but also in immune- and epithelial cells. The kidney is

responsible for the systemic production of 1,25 (OH)2 vitamin D3, which is crucial for skeletal health (endocrine system). The local activation of vitamin D3 in immune- and epithelial cells is described as a paracrine system and is central to the immune effects of vitamin D. The paracrine system is strictly regulated and does not contribute substantially to the systemic levels of 1,25 (OH)2 vitamin D3. This is important since the active and systemically available vitamin D3 is responsible for hypercalcaemia that has been reported as an adverse event for vitamin D3 supplementaton. However, hypercalcaemia is a very rare event and we will strictly follow plasma levels of calcium and 25-OH vitamin D3 during the study period.

Study drug

Vigantol Oil is not a registered drug in Sweden. However, Merck Pharma GmbH has permission to manufacture and sell Vigantol in Germany since many years (permission nr 6154275.00, ATC code A11CC05, mSPC available upon request). The study drug is manufactured according to GMP (GMP certificate from Merck available upon request). The placebo oil is also manufactured by Merck and has identical galenic properties to Vigantol oil. The drugs (vigantol and placebo) will be delivered to Vecura AB at Karolinska University Hospital, Huddinge. Vecura AB is a company specialized in clinical trials and has a GMP-certificate for clinical trials and handling of study drugs. VECURA AB will aliquot the study drug and placebo to the final bottles, carry out randomization and labelling.

Endpoints

Primary endpoint

Infectious score based on patient recorded information registered in a diary-form.

Secondary endpoints

25-OH vitamin D3 in serum

Microbiological findings and numbers of cultures taken

Levels of antimicrobial peptides in nasal fluid

Antibiotic consumption collected from patient records

Design

Evaluations and procedures

Prescreening: Eligible patients fulfilling inclusion criteria will be selected from records and contacted via regular mail. They will be sent a letter of invitation together with information on the study. All these patients will be contacted via telephone one week later and asked for participation.

Visit 1, screening, time=0: Co-investigator (Licenced physician, MD) will meet all patients for screening. Additional information on the study will be given and informed consent will be collected. If the patient is judged to fulfil all criteria for inclusion and all exclusion criteria can be negated, the patient is included in the study. The study drug for 6 months will be given out to the patients as well as diaries and envelopes. The patient will be carefully informed about the procedures with the diaries. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Visit 2, time=6 months +/- 14 days: Co-investigator (Licenced physician, MD) will meet all patients after 6 months. A control for adverse events and compliance will be carried out. Additional bottles of study drug/placebo will be given out for the remaining 6 months of the study. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Visit 3, time= 12 months +/- 14 days: Co-investigator (Licenced physician, MD) will meet all patients after 12 months. A control for adverse events and compliance will be carried out. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Participants

Inclusion Criteria:

- Age 18-75
- Increased number of respiratory tract infections
- At least 42 days of infections during 2008 or 2009
- S-25 OH vitamin D3 < 250 nM
- Not planning a pregnancy during the coming year
- Accepting the use of contraceptives during 1 year

Exclusion Criteria:

- Continuous antibiotic treatment
- Hypercalcaemia
- Sarcoidosis
- Kidney disease
- Tuberculosis
- Pregnancy
- Previous history of kidney stones
- Heart medication (glycosides)

Treatment

Vigantol oil (cholecalciferol). 1 drop contains 500 IU. The patients should take 8 drops per day during the study.

Packing, labelling and handling of study drug: Merck will distribute Vigantol oil and placebo oil to VECURA AB, Karolinska University Hospital, Huddinge, which will handle, pack and label the study drug.

Distribution of the study drug to the participants: At the first visit, the participants will be given study drug for the first 6 months of the study. After 6 months, they will be given the remaining bottles. Oral and written instructions will be given about 8 drops per day .

Blinding and breaking of the code: The design is double-blind. Thus, neither the doctor/nurse nor the patient will have any information on the nature of the study drug. Two monitors will carry out controls of the study. The randomisation list will be stored in such a way that the personnel involved in the study do not have access to it. The Hospital Pharmacy will be given a copy of the list in case of emergency with access via telephone 365 days per year/24 hours per day.

Concomitant medication: All other medication is allowed during the study, including antibiotics. However, recent changes in drug treatments will be documented in the diary and asked for by the study doctors at visits.

Compliance: The compliance to the study drug and diary registration is asked for at visits to the study site.

Control of the study drug: Patients are asked to bring back their empty bottles to the study sites. All bottles will be registered by the study nurses.

Evaluation of efficacy and safety

Evaluation of primary endpoint (clinical endpoint, patient recorded)

The primary endpoint is the infectious score, which is based on the diary form filled out by the patient. The total score is composed of symptoms from airways, sinus and ears together with malaise and antibiotic consumption. The idea is to monitor several aspects of an infectious episode and thus monitor the total infectious burden, rather than a specific symptom.

Evaluation of secondary endpoints (microbiological and biochemical endpoints, collected by the study personnel)

1. 25-OH vitamin D3 in serum
2. Microbiological findings and numbers of cultures taken. This information will be collected from patients' clinical records with a focus on samples taken from the respiratory tract.
3. Levels of antimicrobial peptides in nasal fluid. Every fifth patient (according to a special randomization list) will be asked to leave nasal fluid for analysis of antimicrobial peptides.
4. Antibiotic consumption collected from patient records. Information on how many prescriptions of antibiotics will be collected from patients' records.

Evaluation of clinical safety for participants

Patients will leave blood for analyses of plasma levels of creatinin, calcium, phosphate and albumin as well as serum levels of 25-OH vitamin D3 at times 0, 3, 6, 9 and 12 months. The information regarding all time-points except at inclusion will be sent to an unblinded senior physician who will serve as an external clinical safety monitor. He will contact the study physicians in case of clinically relevant abnormalities in the blood chemistry.

Samples and clinical chemistry

Serum and plasma from the first sampling will be sent to Dept of Clinical Chemistry, Karolinska University Hospital, Huddinge for routine analyses. These answers will be recorded in patients' records. For all other time points samples will be sent to Study Center Karolinska which will coordinate all samples for clinical chemistry and send answers to the unblinded clinical safety monitor. These answers will not appear in patients' records in order to keep the blinded design intact.

Adverse events (AE) and Severe adverse events (SAE)

All adverse events and severe adverse events will be recorded in special forms. They will further be classified for severity (mild, moderate and severe) and for connection with the study drug (probable, possible and unlikely). All SAE will be reported to the sponsor within 24 hours after it has been known by the investigator.

Statistics

Handling of data: All data will be registered in a database especially constructed for the study.

Analysis of excluded patients: Excluded patients will be recorded and followed for adverse events. After the study, special analyses will be performed to understand why these patients did not complete the study.

Statistical analysis and power calculation: The statistical calculation is based on the assumption that the infectious score is reduced with 30 % from 42 days (42x5=240 points) to 28 days (28x5=160 points) with full infectious score. If we include 60 patients per group a significance level of p=0.02 will be reached with a power of 90%. To compensate for expected exclusions, we will increase the number of patients per group to 70. Thus, the total number of patients in the study will be 140.

Quality control

Source data: The information that will be collected from each participant will be: study title, screening number, patient number, written informed consent, main and concomitant diagnoses, study treatment, medication, data on blood chemistry and other investigations carried out.

Monitoring: All study personnel have knowledge on clinical trials and ICH-GCP. The sponsor will sign a contract with Karolinska Trial Alliance for monitoring. The investigator will allocate time for monitoring and supply all available and relevant information to the monitors.

Ethics

The sponsor has applied for ethical approval from the regional Ethical Board. The study will be carried out according to ICH-GCP and the Helsinki-declaration.

Informed consent: The patient will have information sent home via regular mail. One week later the study nurse will call the patient and discuss the study. The first visit will involve the meeting with a physician and time is extended for questions before the written informed consent is signed. One copy will stay at the study site and one copy will go with the patient.

Handling of data

Case Report Forms (CRF) will be used. These will kept at the study site until the end of the study. After completion of the study, all material will be archived at least 10 years.

Insurance

All participants are insured via patient insurance and the Swedisg drug insurance.

Publication of the results

The study group wishes to publish the data in a refereed international scientific journal and to communicate the results at conferences and other venues.

The original protocol is written in Swedish and is available on request.

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Vitamin D3 supplementation in patients with frequent respiratory tract infections - a randomised, double-blind intervention study

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Vitamin D_3 supplementation in patients with frequent respiratory tract infections - a randomized and double blind intervention study

Peter Bergman*[1,3,6#], Anna-Carin Norlin*[2,3], Susanne Hansen[3], Rokeya Sultana Rekha[4], Birgitta Agerberth[4], Linda Björkhem-Bergman[5], Lena Ekström[5], Jonatan D Lindh[5], Jan Andersson[6]

1. Department of Laboratory Medicine, Clinical Microbiology, Karolinska Institutet
2. Department of Laboratory Medicine, Clinical Immunology, Karolinska Institutet
3. Karolinska University Hospital, Clinical Immunology, Immunodeficiency Unit
4. Department of Medical Biochemistry and Biophysics, Karolinska Institutet
5. Department of Laboratory Medicine, Clinical Pharmacology, Karolinska Institutet
6. Department of Medicine, Center for Infectious Medicine (CIM), Karolinska Institutet

*Equal contribution

Positions:

Peter Bergman, MD, PhD, Resident Physician, Clinical Microbiology

Anna-Carin Norlin, MD, Specialist in Clinical Immunology

Susanne Hansen, RN, Head of Immunedeficiency Unit

Rokeya Sultana Rekha, MSc, PhD-student

Birgitta Agerberth, PhD, Professor, Medical Microbial Pathogenesis

Linda Bjorkhem-Bergman, MD, PhD, Resident Physician, Clinical Pharmacology

Lena Ekström, PhD, Associate Professor, Clinical Pharmacology

Jonatan D Lindh, MD, PhD, Specialist in Clinical Pharmacology

Jan Andersson, MD, PhD, Professor, Infectious Diseases

Running title: Vitamin D_3 supplementation in patients with frequent respiratory tract infections

#Corresponding author:

Peter Bergman, MD, PhD

Department of Laboratory Medicine, Clinical Microbiology F68

Karolinska Institutet and Karolinska University Hospital, Huddinge

SE-141 86 Stockholm, Sweden

peter.bergman@ki.se

Phone: +46858587942

Fax: +46858581305

Competing interest statement

All authors have completed the Unified Competing Interest form (available on request from the corresponding author) and declare no support from any organisation for the submitted work, no financial relationships with any organisations that might have an interest in the submitted work in the previous three years and no other relationships or activities that could appear to have influenced the submitted work.

Article Summary

Article focus

• Recent evidence suggests that vitamin D_3 has potent extra-skeletal effects, such as suppression of inflammation and strengthening of mucosal immunity by induction of antimicrobial peptides.

• Data from observational studies suggest that low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections.

• Results from a limited number of randomized controlled trials on the protective role of vitamin D_3 against respiratory tract infections are inconclusive and thus additional studies are warranted.

Key Messages

• Therefore we designed and carried out a randomized controlled trial where a large dose (4000 IU) of vitamin D_3 was given to patients with an increased susceptibility to infections for one year.

• The main conclusion is that vitamin D_3 supplementation reduces symptoms and antibiotic consumption among patients with an increased frequency of respiratory tract infections. Thus, vitamin D_3 supplementation may be an alternative strategy to reduce antibiotic use among patients with recurrent respiratory tract infections.

Strengths and limitations

• Strengths: A high daily dose of vitamin D3 was used, the study time was a full year covering all seasons and patient with an increased frequency of respiratory tract infections were studied.

• Limitations: A single study center, small sample size (n=140) and a selected group of patients.

Abstract

Background: Low serum levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections (RTIs). Clinical trials with vitamin D_3 against various infections have been carried out but data are so far not conclusive. Thus, there is a need for additional randomized controlled trials of effects of vitamin D_3 on infections.

Objective: To investigate if supplementation with vitamin D_3 could reduce infectious symptoms and antibiotic consumption among patients with antibody deficiency or frequent RTIs.

Design: A double-blind randomized controlled trial.

Setting: Karolinska University Hospital, Huddinge

Participants: 140 patients with antibody deficiency (sIgA-, IgG subclass deficiency, CVID) and patients with increased susceptibility to RTIs (>4 bacterial RTIs/year) but without immunological diagnosis.

Intervention: vitamin D3 (4000 IU) or placebo was given daily for one year

Primary and secondary outcome measures: The primary endpoint was an infectious score based on five parameters: symptoms from respiratory tract, ears and sinuses, malaise and antibiotic consumption. Secondary endpoints were serum levels of 25-hydroxyvitamin D_3, microbiological findings and levels of antimicrobial peptides (LL-37, HNP1-3) in nasal fluid.

Results: The overall infectious score was significantly reduced for patients allocated to the vitamin D-group (202 points) compared with the placebo group (249 points) (adjusted relative score 0.771, 95% CI 0.604-0.985, p=0.04).

Limitations: A single study center, small sample size and a selected group of patients.

Conclusions: Supplementation with vitamin D_3 may reduce disease burden in patients with frequent respiratory tract infections.

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The study was registered at www.clinicaltrials.gov (NCT01131858)

273/275 words

Introduction

Vitamin D was discovered when it was noted that rachitic children were improved by exposure to sunlight [1]. It was later shown by Holick *et al* that vitamin D_3 is synthesized in the skin under the influence of UVB-light [2]. Vitamin D_3 is further hydroxylated in the liver to 25-hydroxyvitamin D_3, which is considered to reflect the vitamin D status of an individual patient [3]. The final activation to the active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)D_3$) requires 1-α-hydroxylase activity. This enzyme (also designated CYP27B1) is expressed in the kidney but also in many other cell-types, including epithelial- and immune-cells [4]. The active vitamin D_3 ($1,25(OH)D_3$) binds to the vitamin D receptor (VDR), which belongs to the nuclear receptor family. Active vitamin D_3 is only present in minute amounts in the circulation and local activation in target cells is crucial for vitamin D-mediated effects on the immune system[5].

Low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of tuberculosis [6-8] and respiratory tract infections [9]. The mechanism is not fully elucidated but vitamin D_3 has been shown to induce antimicrobial peptides in immunecells [10]. In addition, active vitamin D_3 ($1,25(OH)D_3$) has broad anti-inflammatory effects on the adaptive immune system by shifting the T-helper cell pool from a Th1/Th17-response to a Th2/Treg-dominated response [11 12]. Vitamin D_3 has also been shown to suppress the Th2-response in allergic bronchopulmonary aspergillosis [13]. Thus, vitamin D_3 modulates both the adaptive and innate immune system [14]. The bulk of data on vitamin D_3 and infections stems from *in vitro* experiments and retrospective observational studies. Results from randomized controlled trials where the effects of vitamin D_3 on infections have been investigated (reviewed in Yamshchikov et al. [15]) are not conclusive and larger clinical trials are therefore warranted.

We designed a study to test the hypothesis that 4000 IU of vitamin D_3 given daily to patients with antibody deficiency and frequent respiratory tract infections for one year could prevent

or ameliorate infections. In addition, we investigated whether genetic polymorphisms in genes involved in the effect and/or metabolism of vitamin D_3 have an influence on the outcome of vitamin D_3 supplementation.

Methods

Study design

A prospective, randomized, double-blind placebo-controlled study of vitamin D_3 supplementation in patients with an increased susceptibility to respiratory tract infections. The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants. The study was registered at www.clinicaltrials.gov prior to inclusion of the first patient (NCT01131858). The EudraCT number is 2009-011758-16. The full protocol is available from the corresponding author upon request.

Sample size calculation

The sample size was based on the assumption that the intervention would reduce the number of days with symptoms from 42 days (210 points) to 28 days (140 points), i.e. a reduction of the infectious burden by 30%. Given this assumption, a sample size of 60 patients per study group was predicted to provide the study 90% power at a significance level of p=0.02 (Student's t-test). To compensate for predicted exclusion of participants, the groups were increased to include 70 patients per treatment arm. Importantly, the significance level of p=0.02 was chosen in the power calculation to ensure that a sufficient number of patients were recruited in order to avoid a type II error in the primary analysis. However, the widely accepted significance level of p=0.05 was used for statistical analyses of the primary and secondary endpoints. Consequently, p-values are written out together with the 95% confidence interval (CI).

Participants

Patients at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden, were included between March 2010 and June 2010 by the study nurses (SH, ML, KJ). Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infections; i.e. > 42 days with symptoms from the respiratory tract during a 12 months period prior to study inclusion. Patients registered at the Immunodeficiency Unit are closely followed with a diary of symptoms and antibiotic consumption. Thus, the patients are trained and used to apply such an instrument to assess their infectious status. Data from patients' standard diary was used as an instrument in order to assess patients for eligibility, both via telephone and by the responsible physician (PB, ACN) prior to inclusion. Patients with selective IgA-deficiency (**D80.2**), IgG-subclass deficiency (**D80.3**) and common variable immune disorder (CVID, **D83.0**) as well as patients without a defined immunological diagnosis (**D89.9**) were included. Exclusion criteria were prophylactic treatment with antibiotics, history of hypercalcemia or stones in the urinary tract, sarcoidosis, ongoing supplementation with vitamin D_3 exceeding 400 IU/day, HIV-infection and pregnancy.

Interventions

Patients were randomized to 12 months' treatment with Vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. One drop contained 500 IU vitamin D_3 or placebo oil (Miglyol oil®) and the participants were asked to take 8 drops daily. The participants had to mark their daily symptoms of infection in a diary, which was sent via regular mail to the study site every month. The following data was recorded: symptoms from the respiratory tract, ears and sinuses, treatment with antibiotics, numbers of bacterial cultures, times and reasons of visits to hospitals, frequency of travelling abroad and adherence to study drug.

Outcomes

The primary outcome was a composite infectious score, based on a daily patient-reported questionnaire, and included five parameters: symptoms from the respiratory tract, ears and sinuses, malaise and use of antibiotics (Figure S1), each parameter gave 1 point/day. The occurrence of X-ray verified pneumonia gave 3 additional points per day for a period of 7 days. Thus each pneumonia resulted in 3x7 points = 21 extra points. Patients were specifically instructed to record only symptoms related to ongoing respiratory tract infections. Symptoms related to infections at other sites (urinary tract, wounds etc) as well as non-infectious symptoms were reported as adverse events. Secondary outcomes were serum levels of 25-hydroxyvitamin D_3 (at baseline and after 3, 6, 9 and 12 months), numbers of bacterial cultures, microbiological findings and levels of antimicrobial peptides (LL-37 and HNP1-3) in nasal fluid (at baseline and after 6 and 12 months). In addition, 6 *post hoc* genotype analyses were performed in all participants. Analysis of single nucleotide polymorphisms (SNPs) were carried out for VDR (Taq1 and Foq1), CYP27B1, CYP24A1, CYP2R1 and Vitamin D binding protein (GC). Safety tests included plasma levels of creatinine, calcium, phosphate and albumin, measured at baseline and after 3, 6, 9 and 12 months. At inclusion, urine-HCG (human chorionic gonadotropin) in women was measured and p-parathyroid hormone in both genders. The results of the safety tests were reviewed by an independent and un-blinded consultant physician. Two blinded physicians (PB and ACN) were responsible for inclusion and all medical visits to the study site (Immunodeficiency Unit, Karolinska University Hospital, Huddinge).

Randomisation and statistical analysis

Participants were randomised to 12 months' treatment with vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. Block randomization with a block size of ten was used to ascertain equal group sizes. Staff at Karolinska Trial Alliance (KTA) was responsible for randomization procedures. In the statistical analysis, continuous variables were compared using Mann-Whitney U test or linear regression and dichotomous variables by Fisher's exact test or logistic regression. Regressions of log-transformed infectious scores were performed both unadjusted (simple regression) and with adjustment for potential confounders (multiple regression).

Statistical methods: Primary analysis

The distribution of the infectious score was found to be very skewed, thereby violating the normal assumption of the pre-specified t-test analysis. Hence, scores were log-transformed prior to analysis.

Further, the randomisation had resulted in age distributions that were not entirely balanced between the two groups. Since there might be concerns that such imbalance could influence the results of the study, the original analysis plan was extended with a multivariable analysis adjusting for potential confounders. In this linear regression model based on log-transformed values of the primary outcome (the total infectious score) and its individual components, adjustment was made for age, gender, smoking, type of immune deficiency and significant co-morbidities (respiratory or non-respiratory). Due to the transformation procedure, the adjusted effect of vitamin D_3 is expressed as a ratio between the score in the vitamin D_3 and the placebo group. In this multiplicative model, an effect size of 1 indicates identical outcome in the two study groups and statistically non-significant results are recognized by confidence intervals encompassing the value 1.

To explore potential divergent effects on different organ systems, both adjusted and unadjusted analyses were repeated separately for each individual item of the infectious score. In addition, the temporal aspects of the vitamin D_3 effect were investigated by dividing the study period into four 90-day periods (starting on the first day of treatment) and repeating the analyses separately for each time period. “Ear” and “sinus” symptoms as well as “antibiotic use” occurred at low frequencies and for these entities normal distributions could not be achieved despite data transformation. Thus, the adjusted analyses of these individual items were based on multivariable logistic regression, after coding the symptom (or antibiotic therapy) as present or absent during the course of the study. However, this only applies to analysis of the individual items, and not to the primary analysis of the total infectious score, where all item scores were added as originally described.

Most post-randomization exclusions were due to patients failing to fill out the symptoms diary. Hence, no intention to treat (ITT) analysis based on actual outcome data could be performed. However, the potential impact of dropouts was addressed in an ITT analysis based on multiple imputation of missing outcome data. In the imputation process, pooled estimates were derived from 100 datasets created by means of multivariate imputation by chained equations and predictive mean matching for the same covariates as in the adjusted per-protocol analysis

Detailed descriptions of randomisation and blinding, sampling of nasal fluid, measurement of antimicrobial peptides, measurement of 25-hydroxyvitamin D_3, genotyping and statistical analyses of secondary outcomes including sample size calculations are presented in the Supplementary Methods Section.

Results

Baseline data

A total of 286 patients were first assessed for eligibility but 144 were not included because they did not fulfill all inclusion criteria; <42 days with infection/year (n=35), lacked other inclusion criteria (n=42), or declined to participate (n=67). The remaining 142 patients were further screened and 140 patients were included in the study. Of these, 70 were randomized to vitamin D_3 supplementation and 70 to placebo (Figure 1). The groups did not differ with regards to gender, IgG replacement therapy, smoking, baseline 25-hydroxyvitamin D_3 levels, type of immune defect or co-morbidities. Patients with subclass deficiency, selective IgA deficiency (sIgA), common variable immune deficiency (CVID) and patients without a defined immunological diagnosis (ND) but with >4 bacterial respiratory tract infections/year were included. IgG replacement therapy was most common in the CVID-group (100%) and in the subclass deficiency group (63%), but also frequent in the other groups (ND, 54% and sIgA, 38%, table S1). Patients allocated to the placebo group were slightly younger than patients in the treatment group (p=0.025, data not shown). During the course of the study, 16 patients left the study prematurely (8 patients from each study group) and consequently 124 patients were included in the main per-protocol analysis. Reasons for dropout included elevated PTH (n=2), withdrawn consent (n=5), adverse event (n=1), prescription of vitamin D outide the study (n=1), failure to complete diary (n=4) or non-compliance to study medication (n=3) (Figure 1).

Primary endpoint: Infectious score

One year of vitamin D_3 treatment was associated with a significantly reduced total infectious score both in the unadjusted (n=124, p=0.024, Table 2) and the adjusted analyses (n=124,

p=0.040) (Table 2, Figure 2A, B and Table S2). The unadjusted relative score in the intervention group was 0.754 (95% c.i. 0.591-0.963, p=0.024, n=124) corresponding to a 25% reduction and after adjustment for potential confounders, the relative score was 0.771 (95% c.i. 0.604-0.985, p=0.04), corresponding to a 23% reduction (Table 2). According to the temporal analysis, the effect of vitamin D_3 supplementation tended to improve with time (Figure 2A). The absolute unadjusted score per patient was 202 points for the vitamin D group and 249 points for the placebo group, which was a significant reduction of 47 points per patient (p=0.023, Mann Whitney U-test, table S3).

When the individual items of the infectious score were analyzed separately, all point estimates indicated a reduction in the treatment group (Table 2, figure S2), although only antibiotic consumption reached statistical significance (Figure 2B and S2, panel E). The adjusted OR for antibiotic use was 0.365 (95% c.i. 0.153-0.872, p=0.023, n=124), i.e. a 63.5% reduction of the odds of antibiotic use in the intervention group (Table 2). The absolute values were 33 days on antibiotics for the placebo group and 16 days for the vitamin D_3 group, i.e. a reduction of 17 days in the vitamin D_3 group (table S3). The temporal trends for specific symptoms and antibiotic consumption were similar to the total score and reached statistical significance for 'ear'-symptoms (n=124, p=0.041) and for 'malaise' (n=124, p=0.053) in the final quarter of the study (Figure 2S, panels B and C).

Analyzing the primary outcome according to intention-to-treat (n=170) produced results virtually identical to those of the per-protocol analysis. In the unadjusted ITT analysis, vitamin D_3 reduced the total infectious score by 25% (relative score 0.752, 95% c.i. 0.588-0.962, p=0.024) and after adjustment for potential confounders the reduction was 23% (relative score 0.767, 95% c.i. 0.599-0.982, p=0.036).

Serum levels of 25-OH vitamin D_3

Serum 25-hydroxyvitamin D_3 levels did not differ between the groups at baseline (Table 1) but already after 3 months the intervention group had a significantly higher level of 25-hydroxyvitamin D_3 (133.4 nmol/L versus 66.6 nmol/L, p<0.001, Figure 3). This increase remained throughout the study (Figure 3).

Bacterial cultures and microbiology

During the course of the study, 173 microbiological samples were obtained in the vitamin D_3 group (n=62, 2.79/patient) and 301 in the placebo group (n=62, 4.85/patient) (p=0.010, Table 3). The number of samples with at least one positive finding was higher in the placebo group, with close to statistical significance (p=0.052), while the fraction of positive samples was similar for both groups (Table 3). Significantly more patients had a microbiological sample taken from the respiratory tract (≥ 1 sample) during the study period in the placebo group; OR 2.63 (95% CI 1.17-5.92), (Table 3).

In total, the vitamin D_3 group generated 76 positive microbiological findings (bacteria or fungi), compared to 159 in the placebo group (p=0.023). There was no difference between the groups for the traditional respiratory pathogens (*H. influenza, M. catharralis and S. pneumonia*), but there were significantly fewer findings of *S. aureus* (p=0.019) and fungi (p=0.028, *Candida* spp. and *Aspergillus* spp.) in the treatment group (Table 4). Likewise, significantly fewer vitamin D_3-treated patients had a bacterial culture positive for *S. aureus* (p=0.019) or fungal species (p=0.058), although the latter difference did not reach statistical significance (Table 4).

Vitamin D treated patients with sub-class deficiency left significantly fewer bacterial or fungal cultures than placebo-treated patients with this diagnosis; 7 cultures in the vitamin D group (n=22) versus 47 cultures in the placebo group (n=24) (Table S4). Also the number of patients that had ≥1 bacterial culture taken was significantly fewer in the placebo group (12/22 versus 22/24, p=0.0065, table S4). There was no significant effect of other immunological diagnoses on bacterial cultures or microbiology (Table S4).

Since concomitant lung disease may be an important factor for vitamin D mediated effects on respiratory immunity, we performed a detailed analysis of bacterial cultures and microbiology of patients with asthma, bronchiectasis (BE) and chronic obstructive pulmonary disease (COPD). The numbers of patients with these diagnoses were quite small, which preclude any firm conclusions regarding any effect. However, there was a trend – however not significant – that vitamin D treated patients with asthma produced fewer bacterial cultures (average 2.9 cultures/patient versus 7.0 cultures/patients, p=0.080, Figure S3) and fewer positive cultures than placebo treated asthmatics (average 0.6 positive cultures/patients versus 2.7/patient in the placebo group, p=0.052, Figure S3). In addition, vitamin D treated asthma patients showed significantly fewer cultures positive for fungi (candida and aspergillus) compared to placebo treated asthmatics (p=0.0476, table S5). For BE or COPD-patients there was no clear trend or significant effect in bacterial cultures or microbiology.

Levels of antimicrobial peptides (AMPs) in nasal fluid

There was no statistically significant difference between the vitamin D_3 or placebo groups when nasal fluids were analyzed for the presence of AMPs. Initially, levels of both LL-37 and HNP1-3 tended to be higher in the placebo group (Figure S3, panels A and B). However, after 12 months the microbiological pattern was reversed and no primary pathogens could be

detected in nasal swabs from vitamin D_3-treated patients (n=25, p=0.039) (Figure S4, panel C). The placebo-treated patients exhibited the same mix between normal flora and primary pathogens at all three sampling points (0, 6 and 12 months) (Figure S4, panel C).

SNP variants and treatment effect

Most genetic variants did not affect the primary endpoint. However, patients carrying the 'AA' genotype in the CYP2R1-gene, encoding the 25-hydroxylase enzyme, had a larger benefit of vitamin D_3-supplementation (-55%) compared to AG or GG carriers (-6%) (n=124, p=0.046 for interaction, Table S6).

Adverse events

In total, the vitamin D_3 group reported 38 adverse events (AEs) versus 56 AEs in the placebo group. The most common symptoms in the treatment group were headache (n=5) and lumbago (n=5), whereas placebo-treated patients reported paresthesias (n=8), diverticulitis (n=4) and urinary tract infection (n=4) as most frequent AEs (Table 5, Table S7). There was a general trend towards the number of adverse events being higher in the placebo group. Significantly more patients in the placebo group reported cardiovascular problems, such as heart failure, hypertonia and thrombosis (p=0.028). For gastrointestinal and other (non-respiratory) infections there was also a trend favoring the vitamin D_3 group (p=0.058 and p=0.09, respectively). No clinically relevant changes in serum levels of calcium, phosphate, creatinine or albumin could be observed (Figure S5). There was one severe adverse event (SAE) in each group (rabdomyosarcoma in the vitamin D_3 group and lung bleeding in the placebo group), both judged as being unrelated to the study drug.

Discussion

The main conclusion from this long-term randomized controlled trial (RCT) is that vitamin D_3 supplementation reduces the total burden of respiratory tract infections. The primary endpoint was composed of five different parameters that patients recorded daily throughout the study year. All point estimates favoured the vitamin D_3 group and a statistically significant effect was seen on both the total score and on the probability of receiving antibiotics. The effect on the infectious score was evident both in analysis per-protocol and according to intention-to-treat, and withstood adjustment for potential confounders. In addition, the number of bacterial cultures and microbiological findings was significantly reduced in the intervention group. These findings are potentially important and support that Vitamin D_3 supplementation may prevent respiratory tract infections and reduce antibiotic consumption, particularly in patients with hypogammaglobulinemia or with an increased frequency of respiratory tract infections.

However, our study has several limitations: Firstly, the choice of primary endpoint may be questioned since it relies solely on patient-reported information. To compensate for inherent problems with patient-reported data, the evaluation instrument was designed to cover many aspects of an infectious episode, including various symptoms as well as antibiotic consumption. Together the reported data formed an "infectious score", which constituted the primary endpoint of the study. Similar composite scores have successfully been applied to different diseases, such as tuberculosis (TB-score [16]), pneumonia (CURB-65 [17]) and bacterial meningitis (BMS-score [18]). Notably, vitamin D supplementation had a major effect on the odds of taking antibiotics during the study period (a reduction by 63.5%). In addition, the absolute number of days on antibiotics was reduced by 50% (from 33 days in the placebo group to 16 days in the intervention group), which was statistically significant both in the adjusted and unadjusted analyses (table 2). However, despite the relatively modest reduction for the other components of the primary endpoint the overall infectious score was

significantly reduced – mainly as a result of the large effect on the antibiotic parameter - both in the unadjusted and in the adjusted analyses (table 2, figure 2). It is important to interpret the statistical significance in the light of our power calculation, which was based on a significance level of p=0.02. This unusual significance level was chosen as a means of accounting for uncertainties in the power calculation assumptions, thereby asserting a sample size large enough to produce results significant at the p=0.05 without an unacceptably high risk of a type II error. However, we have used the widely accepted and conventionally used significance level p=0.05 in the statistical analyses, although this is not in full accordance with the significance level mentioned in the power calculation. Another potential problem was that the patient population was very heterogeneous with regards to immune deficiency and concomitant diseases. We adjusted for these factors in the multivariable analyses of the primary endpoint, but the sample sizes in each subgroup were too small to draw any conclusions of effects in specific disease groups. However, a detailed *post-hoc* analysis of the relation between immunological diagnosis, concomitant lung-disease and the secondary endpoints "taken bacterial cultures", "positive bacterial" cultures and "microbiological findings" was performed. There was a clear trend that Vitamin D treated patients with subclass deficiency and/or asthma produced fewer bacterial cultures, fewer positive cultures and fewer fungal cultures (tables S4 and S5, Figure S3). Although this analysis may lack precision by the small number of patients included, it could have clinical implications regarding target groups for vitamin D supplementation.

Nevertheless, our double-blind RCT has several strengths. For example, we chose a high daily dose of vitamin D_3 based on published calculations on metabolism and effects on immunity [14 19]. Other RCTs using lower doses of vitamin D_3, 400-2000 IU/day, have mainly been negative with regards to prevention of infections [20 21]. However, one study using 1200 IU/day showed a significant reduction of influenza among school children in Japan [22]. Notably, also studies

using higher doses of vitamin D_3 have been negative. Martineau *et al* used 400,000 IU vitamin D_3 during 42 days (9523 IU/day) with the aim of shortening time to sputum conversion in tuberculosis. No significant effect on the primary endpoint could be observed in that study, except in a subgroup with the *tt* genotype in the vitamin D receptor gene [23]. A recent study investigated whether 100,000 IU vitamin D_3/month (3333 IU/day) could reduce the incidence of chronic obstructive pulmonary disease (COPD) exacerbations. There was no significant effect on the primary endpoint, although a *post hoc* analysis revealed that patients with a low vitamin D_3 level at baseline had a significant effect of Vitamin D_3 supplementation[24].

Importantly, our study is the first to utilize high daily doses for an extended period of one full year. Thus, we covered all four seasons, which was important in Sweden with a known seasonal variation in 25-hydroxyvitamin D_3 levels [25]. Two previous RCTs were performed during the winter season – when vitamin D levels are low – but only during 4 [22] and 6 months [20], respectively. Previous RCTs have been conducted during shorter periods; 42 days [23], 6 weeks [26] and 12 weeks [21], respectively. Interestingly, we observed a clear time dependent effect suggesting that a long term supplementation approach (> 6 months) may be necessary to affect immunity. To expand on the results of a previous study in healthy individuals where no difference between the intervention and placebo groups was observed [21], we chose a study population with frequent RTIs and at least 42 days with infection during the year prior to inclusion. Notably, patients in the study represent a selected group of individuals with frequent RTI, although the immune disorders that they represent (IgA-, IgG-subclass and patients with no defined immune disorder) are generally mild in character and dominated by mucosal RTIs. We also included a small number of CVID-patients, which can be considered to be a more severe immune disorder, but all these patients are treated with IgG replacement therapy and thus well controlled. Hence, the results from this study cannot directly be applied

to the general healthy population. Nevertheless, the results provide solid support for additional interventional studies of vitamin D_3, especially in groups consuming large amounts of antibiotics.

The mechanism of the observed effects remains largely unknown. Vitamin D_3 modulates the immune response at many levels, such as induction of AMPs, skewing of T-cells from Th1/Th17 to Tregs as well as general anti-inflammatory effects [14]. Here, we investigated the role of AMPs in nasal fluid. However, we could not detect any significant changes of LL-37 or HNP1-3 during the study period, but noted that placebo-treated patients tended to have higher levels of AMPs after one year of treatment. This was paralleled by a shift of the microflora in the nasal compartment that could explain the unexpected finding of higher AMP-levels in the placebo group. Recently, it was shown that 1,25 $(OH)_2$-vitamin D_3 induces both HNP1-3 and LL-37 in nasal fluid of healthy volunteers (Hiemstra et al, abstract, European Respiratory Society, 2011), supporting that LL-37 may indeed be induced *in vivo*. However, our study design did not allow such conclusions but rather support that vitamin D_3 affect mucosal immunity, leading to a shift of the microflora. Recently, we showed that the bacterial composition in nasal swabs is an important determinant of AMP-levels in nasal fluid [27].

Given that vitamin D_3 induces LL-37 in epithelial cells and that LL-37 kills bacteria *in vitro,* we expected a reduction of the classical bacterial pathogens *H. influenza*, *M. catharralis* and *S. pneumonia* in the intervention group. However, the frequency of these bacteria was not reduced but a reduction of *S. aureus* and fungal species that often colonise the airways was observed. This could be explained by specific effects by vitamin D_3 on immunity against *S. aureus*. In fact, vitamin D induces human beta defensin-2 (HBD-2) with bactericidal activity against *S. aureus* [28]. A recent study showed that low vitamin D levels were associated with an increased risk of being colonised by this bacterium [29]. Further, vitamin D affects immunity

against *C. albicans*, which indicates direct effects of vitamin D on human immunity [30]. Alternatively, it is possible that vitamin D_3 may have prevented symptomatic viral infections, which prompted patients to leave a bacterial sample from the airways. Interestingly, there is both mechanistic and clinical evidence that vitamin D_3 can prevent viral infections [31-33], although we did not address this in the current study.

Notably, we observed a prominent increase in the serum concentration of 25-hydroxyvitamin D_3, which indicated good compliance and tolerability of the study drug. In fact, there was a trend towards adverse events being reported more often in the placebo group, suggesting that vitamin D_3 possibly could be efficient against other diseases, but this observation requires further studies. No clinically relevant changes of blood chemistry (calcium, phosphate, albumin or creatinine) were observed. Despite few adverse events and high tolerability, 16 exclusions occurred during the study year. The main reason was problems to adhere to the protocol and 6/16 patients dropped out of the study after a few weeks. The rest failed to send in the diaries, did not leave blood for monitoring of safety parameters or did not take the study drug. One patient was excluded based on symptoms that could be attributed to vitamin D_3 (facial paraesthesia). However, this patient was later confirmed to have been allocated to placebo.

In summary, we found that supplementation with vitamin D_3 reduced the total infectious score with 47 points per patient (23% reduction in the adjusted analysis) during the study year. The observed reduction was lower than the assumed reduction of 70 points per patient (predefined assumption: 210 points => 140 points; a reduction of 30%) that formed the basis for the power calculation. However, despite the predefined level of a reduction of infectious score by 30% as a clinically meaningful effect, we believe that effects lower than this also could be relevant for the individual patient. We base this line of reasoning on the fact that a reduction of 47 points per patient can be translated into 47 days with cough (47 points), 23 days with ear

and sinus symptoms (23×2=46 points) or 9 days with cough, sinus and ear symptoms together with malaise and antibiotics (9×5=45 points). In addition, our data indicate that vitamin D_3 supplementation reduces the odds of taking antibiotics by approximately 60% in patients with frequent respiratory tract infections. Thus, supplementation with vitamin D_3 could provide a novel strategy to reduce antibiotic use among high consumers and indirectly prevent the emerging epidemic of bacterial resistance.

Acknowledgment

The study was registered at www.clinicaltrials.gov (NCT01131858), prior to the start of the study. The entire process of study design and protocol, data monitoring and analyses was performed by academic authors; there was no industry involvement in the study except that vitamin D_3 (Vigantol®) and placebo oil (Miglyol®) were provided by Merck KGaA (Darmstadt, Germany). Merck did not have any influence on study design, analysis of data, writing or decision to publish. We extend our gratitude to Ilona Skilving, Karolinska Trial Alliance for invaluable help with the protocol. Further, we thank registered nurses Maria Lindén and Kristina Johansson for skillful work with patients. Thanks also to Jenny Lindén and Alicia Hansson for registration of data and to professor Mats Remberger for discussions on statistical methods. Professor Lars Lindqvist, Department of Infectious Diseases, Karolinska University Hospital is gratefully acknowledged for serving as the monitor of the study. PB, LBB and JDL are holding PostDoc-positions financed by Karolinska Institutet and Stockholm County Council (KI/SLL).

Data sharing statement:

There is no additional data available.

Statements

Author contributions:

Peter Bergman, designed the study, collected, analysed and interpreted data, wrote the paper.

Anna-Carin Norlin, designed the study, collected and interpreted data, wrote the paper.

Susanne Hansen, designed and coordinated the study, collected and interpreted data.

Rokeya Sultana Rekha, carried out experimental work, analysed data

Birgitta Agerberth, analysed and interpreted data, wrote the paper.

Linda Björkhem-Bergman, analysed and interpreted data, wrote the paper.

Lena Ekström, analysed and interpreted data

Jonatan Lindh, analysed and interpreted data, wrote the paper.

Jan Andersson, designed the study, interpreted data, wrote the paper.

Role of the funding source

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Merck GmbH provided the study drug (Vigantol) but did not have any influence on study design, analysis of data, writing or decision to publish.

Ethics statement: The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants.

Conflicts of interest: There are no conflicts of interest.

Legends to figures

Figure 1. Study outline.

Figure 2. Primary endpoint. The adjusted total relative infectious score (A) is expressed 'per quarter' (3 month periods). The adjusted one-year scores (total score, airway, malaise, ear, sinus and antibiotics) are depicted in a Forrest-plot (B) together with 95% confidence intervals. Effects are presented as relative scores (total score, airway, malaise) or odds ratios (ear, sinus, antibiotics, indicated with asterisks).

Figure 3. Secondary endpoint. Vitamin D-levels. Serum was collected at day 0, 3, 6, 9 and 12 months and levels of 25-hydroxyvitamin D_3 were measured. Values are expressed as mean +/- 95% confidence interval.

Tables

Table 1. Baseline data. Mann Whitney U-test was used for comparisons of age and 25-OH vitamin D_3. Fisher's exact test was used for all other comparisons. 1) "other disease" includes hypertension, body pain, hypothyroidism and gastritis as most common diagnoses. CVID, common variable immuno deficiency; ND, increased susceptibility to infections without a defined immunological disorder; BE, bronchiectasis; COPD, chronic obstructive pulmonary disease.

	Vitamin D_3	Placebo
Number	70	70
Age (mean)	55,4	50,8
Female	52/70	50/70
Male	18/70	20/70
IgG-replacem.	39/70	42/70
Smoking	4/70	6/70
25-OH levels (mean)	51,5 nmol/L	46,9 nmol/L
Immunological diagnosis		
sIgA-deficiency	9/70	9/70
IgG subclass	27/70	30/70
CVID	6/70	4/70
ND	28/70	27/70
Concomitant disease		
No other disease	16/70	18/70
Lung: Asthma	27/70	25/70
Lung: BE	5/70	7/70
Lung: COPD	5/70	4/70
Other disease[1]	17/70	16/70

Table 2. Primary Endpoint. Treatment effect calculated as the ratio between infectious scores in the vitamin D_3 and the placebo groups. Due to low frequencies, endpoints marked with asterisks were coded as binary outcomes (i.e. present or absent in each patient) and compared by means of logistic regression. In these cases, the effect refers to odds ratios of experiencing the outcome at least once during the course of the study. (The data are based on n=124 patients).

	Univariable regression model (unadjusted values)			Multiple regression model (adjusted values)		
Endpoint	**Effect**	**95% CI**	**p-value**	**Effect**	**95% CI**	**p-value**
Total score	0.754	0.591-0.963	**0.024**	0.771	0.604-0.985	**0.040**
Airway	0.857	0.697-1.053	0.141	0.871	0.706-1.074	0.200
Ear*	0.721	0.352-1.465	0.367	0.695	0.320-1.501	0.357
Sinus*	0.583	0.280-1.198	0.144	0.594	0.265-1.328	0.204
Malaise	0.845	0.692-1.032	0.098	0.845	0.689-1.036	0.108
Antibiotics*	0.355	0.154-0.784	**0.012**	0.365	0.153-0.872	**0.023**

Table 3. Bacterial cultures. [1]Mann-Whitney U-test, [2]Fisher's exact test.

	Vitamin D_3	Placebo	Significance
Number of samples per patient (mean, n=62/62)	2.79	4.85	p=0.010[1]
Number of positive samples per patient (mean, n=62/62)	1.01	2.02	p=0.052[1]
Fraction positive cultures (%)	63/173 (36%)	125/301 (41%)	P=0.28[2]
Patients with ≥ 1 sample taken	38/62 (61%)	50/62 (81%)	p=0.029[2]

Table 4. Microbiological findings. Mann-Whitney U-test was used to analyze the total number of findings, whereas Fisher's exact test was used for analysis of the number of patients (fraction) with a specific finding.

	Number of findings (total)			Number of patients		
Microorganism	Vitamin D_3	Placebo	MW-U	Vitamin D_3	Placebo	Fisher
H. influenzae	28	27	P=0.46	10/62	13/62	P=0.64
M. catharralis	8	17	P=0.39	7/62	10/62	P=0.60
S. pneumoniae	7	6	P=0.74	4/62	5/62	P=1.00
S. aureus	6	33	P=0.010	4/62	14/62	p=0.019
Enterobacteriacae	8	8	P=0.39	4/62	7/62	P=0.53
P. aeruginosa	8	15	P=0.68	3/62	4/62	P=1.00
Fungal infection	11	53	P=0.028	4/62	12/62	p=0.058
Total	76	159	P=0.023			

Table 5. Adverse events. Number of reports. Fisher's exact test was used for between group comparison. (The data are based on AE-reports from n=62 patients/arm).

Organ	Vitamin D_3 n (%)	Placebo n (%)	P-value
CNS	11 (29)	10 (18)	1.00
Gastrointestinal	4 (11)	12 (21)	**0.058**
Cardiovascular	0 (0)	6 (11)	**0.028**
Infections (other than RTI)	2 (5)	8 (14)	**0.09**
Musculoskeletal	10 (26)	10 (18)	1.00
Respiratory (non-infectious)	2 (5)	4 (7)	0.68
Skin	5 (13)	2 (4)	0.44
Other	4 (10)	4 (7)	1.00
Total	**38**	**56**	

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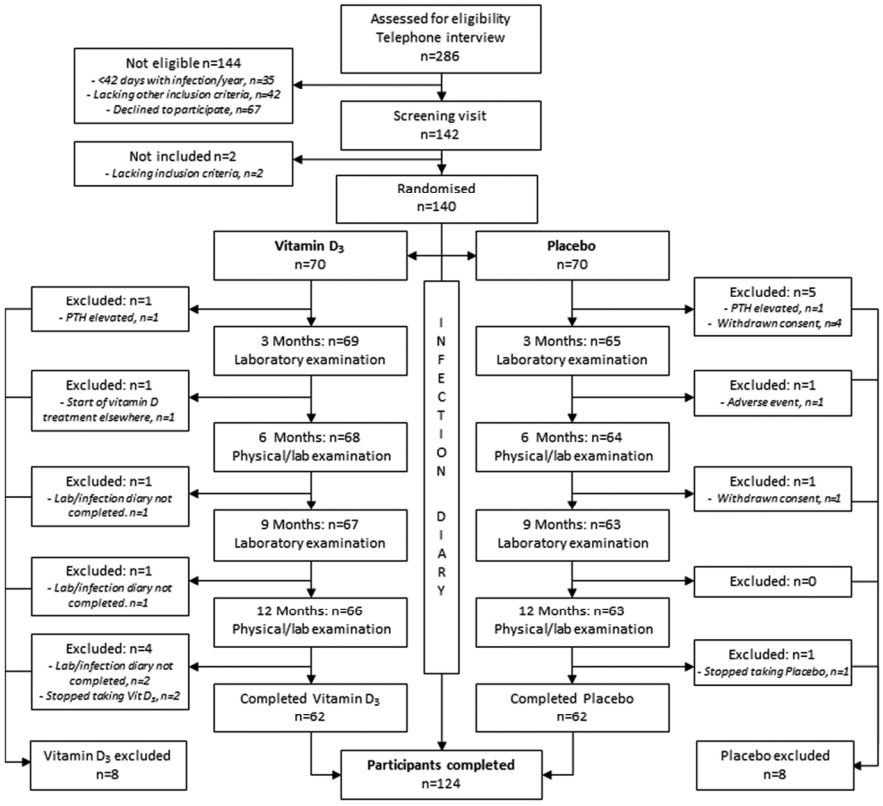

Figure 1
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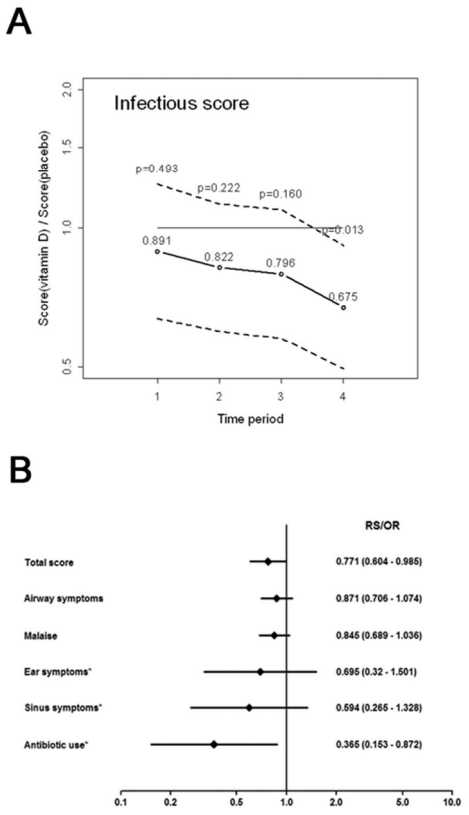

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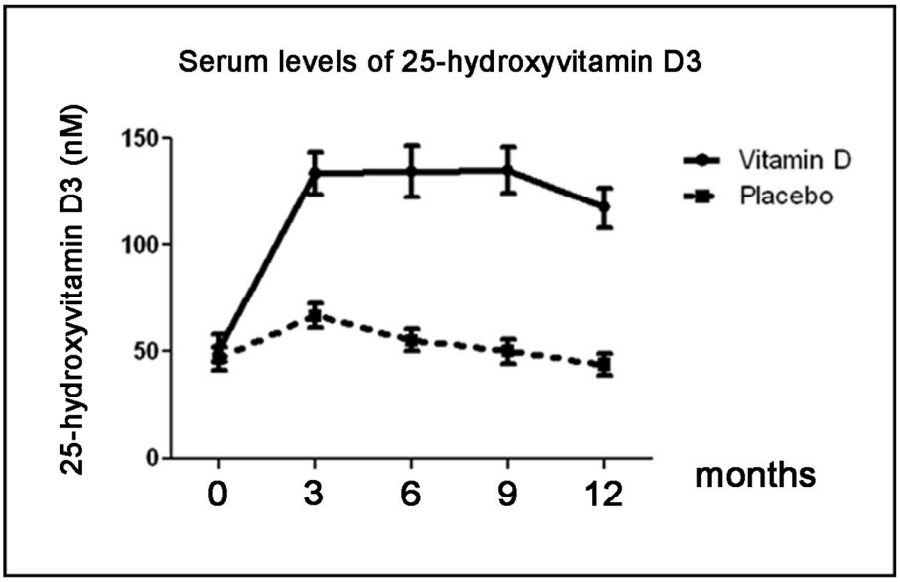

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***Bergman et al,* Vitamin D_3 supplementation in patients with frequent respiratory tract infections - a randomised and double blind intervention study**

Supplementary Methods

Randomisation and Blinding

A computer-generated list of random numbers was used for patient allocation. Randomization sequence was created using Randomization.com (http://www.randomization.com) and was stratified with a 1:1 allocation using a fixed block size of 10. Within each block two participants were randomly assigned to provide samples of nasal fluid, one for each treatment group.

The vitamin D_3 and placebo were in liquid form and identical in appearance. They were prepacked in bottles and consecutively numbered for each participant according to the randomization schedule. In connection with the inclusion each participant was sequentially assigned a number by the responsible physician and received the corresponding prepacked bottles.

Participants, investigators and staff were kept blinded to the allocation throughout the trial. It was not necessary to un-blind information on any participant during the trial.

Sampling of nasal fluid, NPH swabs

Since vitamin D_3 can induce antimicrobial peptides both in macrophages and in epithelial cells[1], we measured levels of LL-37 and α-defensins (HNP1-3) in nasal fluid (Figure 4A and B). For logistical reasons we limited patients for nasal fluid collection and only 36/140 patients (20%) were randomised to this procedure. Nasopharyngeal swabs were taken from

one nostril and sent to the Clinical Microbiology Laboratory at Karolinska University Hospital, Huddinge for bacterial culture. The bacterial content was evaluated as "no growth of bacteria", "normal flora" (typical findings include α-haemolytic streptococci, *Corynebacteria* spp, *Neisseria* spp. and other nonpathogenic strains) or "pathogenic growth" (defined here as *H. influenzae, S. aureus, S. pneumoniae, M. catharralis* and *Enterobacteriacae* spp). Subsequently, nasal fluid was collected through a thin plastic tube that was carefully placed in the back of the nose using the other nostril as entry port (10-12 cm from the nostril meatus). 5-10 ml of saline was administered into the nose prior to sampling in order to make the epithelial lining moist and to dissolve mucus depositions. A gentle vacuum was applied and 3-5 ml nasal fluid was collected and stored at -20°C, as described in Cederlund et al, PLoS One, 2011[2].

Extraction of peptides and proteins from nasal fluid

Nasal fluid (3-5 ml) was extracted in an equal volume (1:1) of 60% acetonitrile (AcN) in 1% trifluoroacetic acid (TFA) over night at 4°C. The extract was centrifuged at 3500g and the supernatant was lyophilized. The lyophilized extract was resuspended in 0.1% TFA and enriched for polypeptides using solid phase extraction as described in[2]. The lyophilized polypeptide extract was reconstituted in 0.1% TFA to a concentration of 5 µg/µl as determined spectrophotometrically using a Nanodrop-system (Thermo Scientific, Wilmington, U.S.).

Analysis of antimicrobial peptides in nasal fluid

The concentrated and lyophilized extract (25 µg) was dissolved in lithium dodecyl sulphate (LDS) sample buffer, 50 mM Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 70°C for 10 min. The samples were then separated using LDS-PAGE and blotted onto PVDF membranes, as described in[3]. Antibodies used were a LL-37 monoclonal[4] and a HNP1-3 goat polyclonal (sc-22916, Santa Cruz, Santa Cruz, Calif., USA). Proteins and peptides were visualized on chemiluminescence film with ECL plus Western blot detection system (GE Healthcare, Buckinghamshire, United Kingdom). LL-37 and HNP1-3 concentration in nasal fluid were determined by densitometry using the software ImageJ (http://rsbweb.nih.gov/ij/). The intensity of each band was normalized to an external standard on each membrane and the total amount of LL-37 and HNP1-3 was determined by multiplying the densitometric result (ng peptide/µg extract) with the total amount of polypeptide-extract (µg). Thus, the values represent the total amount of LL-37 and HNP1-3 from each nasal fluid sample.

Analysis of 25-OH vitamin D_3 in serum

Levels of 25-hydroxyvitamin D_3 in serum were determined by using DiaSorin immunochemical method (DiaSorin S.p.A, Saluggia, Italy) at the Department of Clinical Chemistry, Karolinska University Hospital.

Genotyping

Specific single nucleotide polymorphisms (SNPs) in key genes for vitamin D metabolism might influence the outcome of vitamin D_3 supplementation. Therefore, all patients were

genotyped for 6 SNPs in the VDR (TaqI and FokI), CYP27B1, CYP24A1, CYP2R1 and GC genes. Six SNPs in five genes involved in vitamin D metabolism and / or effect were analysed in all participants. The aim of these analyses was to investigate whether individuals with a specific genotype would benefit more from vitamin D_3 supplementation. Genomic DNA was isolated from 200 µl peripheral blood leucocytes using the DNA Blood Mini kit (Qiagen, Hilden Geramany). Allelic discrimination reactions were performed using TaqMan® genotyping assays (Applied Biosystems, Foster City CA USA): C_12060045_20 for VDR (FokI); C_2404008_10 for VDR (TaqI); C_29958084 for CYP24A1; C_2958431_10 for CYP2R1; C_26407519_10 for GC. For the CYP27B1 genotyping, primers and probes described previously were used[5]. The final volume for each reaction was 15 µl consisting of 30 ng DNA and 2xTaqman Universal PCR Master mix (Applied Biosystems). The PCR profile consisted of 95° C for 10 minutes followed by 40 cycles of 92° C for 15 sec and 60° C for 1 minute. The fluorescence signal was measured with an ABI 7500 Sequence detector (Applied Biosystems).

Statistical methods: Sample size calculation

The sample size was based on the assumption that the intervention would reduce the number of days with symptoms from 6 weeks (42 days x 5 points = 210 points) to 4 weeks (28 days x 5 points = 140 points), i.e. a reduction of the infectious burden by 30%. The estimated standard deviation was 3 weeks (21 days x 5 points = 105 points). Given these assumptions, a sample size of 60 patients per study group was predicted to provide the study 90% power at a significance level of p=0.02 (Student's t-test). To compensate for predicted exclusion of participants, the groups were increased to include 70 patients per treatment arm.

Statistical methods: Secondary analyses

The number of bacterial cultures taken in each patient and the number of samples with a positive finding were compared between the two study groups by means of the Mann-Whitney U test. To reduce the influence of patients subjected to very frequent sampling, the odds of having one or more culture taken during the course of the study was also compared by means of Fisher's exact test. Similarly, the frequencies of cultures positive for specific pathogens were compared both as number of positive cultures per patient (Mann-Whitney U test) and as fraction of patients presenting with at least one positive culture (Fisher's exact test). The fraction of nasopharyngeal samples exhibiting bacterial growth was compared between the two groups separately for samples taken at baseline, after six month and after 12 months (Fisher's exact test).

The influence of genetic polymorphisms on the effect of vitamin D_3 treatment was analysed in linear regression models with log-transformed infectious score as dependent variable. Independent variables were study group, genotype and a genotype-study group interaction term. Genotypes were coded as binary variables, based on previous findings reported in the literature[5-10].

In all analyses, P values <0.05 (two-sided) were considered statistically significant (the significance level of 0.02 in the power calculation was chosen to provide an extra safety margin). All statistical analyses were performed using R 2.11.1 (R Development Core Team (2010). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org) and GraphPadPrism, version 5.0, GraphPad Software, La Holla, Calif, USA

Supplementary References

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***Bergman et al*, Vitamin D_3 supplementation in patients with frequent respiratory tract infections - a randomised, double blind intervention study**

Supplementary tables

Table S1. Data on IgG substitution for participants that were included in the per protocol analysis (n=62/arm).

Treatment with IgG per diagnosis	Yes	No	Total
Subclass deficiency	29	17	46
IgA deficiency	6	10	16
CVID	9	0	9
ND	29	24	53
Total:			124

Table S2. Primary endpoint. Unadjusted relative score per day calculated per 3 months periods as indicated. Values are expressed as mean +/- SD. (n=62/arm).

	Month 1-12		Month 1-3		Month 4-6		Month 7-9		Month 10-12	
	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo
Infectious score	0.56(0.58)	0.69(0.54)	0.58(0.66)	0.67(0.70)	0.51(0.61)	0.59(0.57)	0.59(0.67)	0.72(0.65)	0.53(0.56)	0.77(0.61)
Airway symptoms	0.26(0.24)	0.32(0.28)	0.27(0.27)	0.29(0.28)	0.25(0.29)	0.25(0.24)	0.27(0.27)	0.30(0.27)	0.27(0.27)	0.33(0.27)
Malaise	0.16(0.20)	0.18(0.17)	0.16(0.24)	0.18(0.22)	0.14(0.21)	0.15(0.18)	0.17(0.23)	0.19(0.20)	0.15(0.20)	0.22(0.22)
Ear symptoms	0.04(0.09)	0.07(0.15)	0.05(0.12)	0.07(0.17)	0.03(0.09)	0.05(0.14)	0.05(0.10)	0.08(0.18)	0.05(0.10)	0.08(0.18)
Sinus symptoms	0.05(0.12)	0.06(0.10)	0.05(0.12)	0.06(0.13)	0.04(0.10)	0.04(0.10)	0.06(0.15)	0.07(0.13)	0.05(0.14)	0.07(0.12)
Antibiotic use	0.04(0.06)	0.09(0.14)	0.05(0.09)	0.08(0.16)	0.04(0.08)	0.09(0.17)	0.04(0.08)	0.09(0.15)	0.04(0.07)	0.11(0.18)

Table S3: Primary endpoint. Unadjusted score.

	Mean score / patient / year			MWU (unadjusted)
Endpoint	**Vitamin D**	**Placebo**	**Diff.**	**p-value**
Total score	202	249	-47	0,023
Airway	94	101	-7	0,302
Ear*	16	25	-9	0,225
Sinus*	18	21	-3	0,126
Malaise	56	66	-10	0,041
Antibiotics*	16	33	-17	0,024

Table S4: Secondary endpoint. Number of bacterial cultures in relation to immunological diagnosis. Patients can have several bacteria or fungi in the same culture. Values within parentheses indicate the number of patients that provided the positive cultures. **p=0.0065, Fisher's exact test (12/22 vs 22/24).

								Number of patients	Number of patients
Number of bacterial cultures per diagnosis and treatment		(n)	H. infl.	M. Cath	S. pneum.	S. aur	Fungi	>1 culture taken	>1 culture positive
Subclass deficiency	Vitamin D	22	2(1)	3(3)	2(2)	0	0	12**	5
	Placebo	24	15(6)	3(2)	2(2)	13(5)	14(4)	22**	12
IgA deficiency	Vitamin D	9	4(1)	1(1)	0	0	0	4	3
	Placebo	7	1(1)	2(2)	0	1(1)	0	5	2
CVID	Vitamin D	5	1(1)	3(2)	0	0	0	4	1
	Placebo	4	3(1)	0	2(1)	1(1)	3(1)	4	3
ND	Vitamin D	26	21(7)	1(1)	5(2)	6(4)	11(4)	17	12
	Placebo	27	8(5)	1(1)	2(2)	18(7)	36(7)	19	14
Total		124						0	0

Table S5: Secondary endpoint. Microbiological findings in relation to concomitant lung disease. Values within parentheses indicate the number of patients that provided the positive cultures. H. infl: *Haemophilus influenza*; M. cath: *Moraxella catharralis*; S. pne: *Streptococcus pneumonia*; S. aur: *Staphylococcus aureus*; Eba: *Enterobacteriaceae spp*; P. aer: *Pseudomonas aeruginosa*. **Fishers exact test, p=0.0467 (0/27 vs 4/25).

		H. infl.	M. cath	S. pne	S. aur	Eba	P. aer	Fungi	Sum
Asthma	Vit D, n=27	7(4)	4(2)	2(2)	2(2)	1(1)	1(1)	0**	17
	Plac, n=25	9(5)	6(5)	1(1)	4(4)	2(2)	4(1)	11(4)**	37
BE	Vit D, n=5	5(2)	1(19	0	4(1)	2(1)	0	1(1)	13
	Plac, n=7	1(1)	0	0	8(1)	1(1)	0	3(1)	13
COPD	Vit D, n=5	9(2)	1(1)	0	3(1)	2(1)	0	0	15
	Plac, n=4	0	0	0	8(2)	0	0	0	8

Table S6: Genotyping. Influence of genetic factors on the outcome of vitamin D_3 treatment in patients with frequent respiratory tract infections. Mean infectious score (0-12 months) are presented per genotype and study group, along with the number of included patients. P-values refer to an interaction between genotype and study group.

Gene / SNP	Allel-group	Vitamin D_3 Mean inf score (n)	Placebo Mean inf score (n)	$p_{interaction}$
VDR: TaqI	tt tT /TT (reference)	145 (11) 202 (49)	225 (12) 253 (47)	0.757
VDR: FokI	ff fF /FF (reference)	295 (5) 195 (56)	331 (6) 238 (53)	0.575
CYP27B1: Rs10877012	CC AC/CC (reference)	181 (27) 220 (34)	211 (21) 268 (38)	0.194
GC: RS2282679	AA AC/CC (reference)	205 (39) 162 (21)	214 (28) 283 (29)	0.247
CYP2R1: Rs2060793	AA AG/GG(reference)	142 (8) 212 (53)	315 (14) 227 (45)	0.046
CYP24A1: Rs6013897	AA AT /TT(reference)	92 (2) 207 (59)	221 (2) 249 (57)	0.473

Table S7. Detailed description of adverse events.

Group	Adverse event	Vit D	Placebo	Total
CNS	Headache	5		5
	TIA (Transient Ischemic attack)	1		1
	Vertigo	4		4
CNS Total		10		10
Numbness - pain- shakings	Numbness, pain		8	8
	Paresthesias		2	2
	Tremor	1		1
Numbness-pain-shakings Total		1	10	11
Gastrointestinal symptoms	Diarrhoea		2	2
	Diverticulitis		4	4
	Dyspepsia	2	2	4
	Gastroenteritis	2	2	4
	Helicobacter pylori infection		2	2
Gastrointestinal symptoms Total		4	12	16
Heart/ vessels	Congestive heart disease		2	2
	Hypertension		2	2
	Thrombosis		2	2
Heart/ vessels Total			6	6
Infections	Herpes Zoster	1		1
	Pneumonia	1		1
	Sinusitis		2	2
	Urinary tract infection		4	4
	Pyelonephritis		2	2
Infections Total		2	8	10
Body pain – joint pain	Bursitis		2	2
	Body pain	2	2	4
	Joint pain fingers/ hands	1	2	3
	Joint pain hip		4	4
	Pain in feet	1		1
	Back pain	5		5
	Elbow swelling	1		1
Body pain- joint pain Total		10	10	20
Lungs	Asthma exacerbation	1		1
	Pneumothorax		2	2
	Heavy breathing		2	2
Lungs Total		1	4	5
Ears	Hearing problems	1		1
Ears Total		1		1
Other	Shivering	2		2
	Menstruation too often		2	2
	Nose bleeding	1		1
	Toothache	1		1
	Artheritis temporalis		2	2
Other Total		4	4	8
Rash – itch - blisters	Tongue blisters	1		1
	Hand rash	1		1
	Foot rash	1		1
	Facial rash when drinking alcohol		2	2
	Facial rash	1		1
	Chest rash, itching	1		1
Rash – itch – blisters Total		5	2	7
Total		38	56	94

Supplementary figures

Figure S1. The diary that was used for patients to register their daily symptoms.

Symptoms from "airways", "ears" and "sinuses" were calculated as maximum 1 point per anatomical site per day. "Malaise" and "antibiotic consumption" gave maximum 1 point per day. The occurrence of X-ray verified pneumonia resulted in 3 extra points per day for one week. Thus, 8 points was the maximum value that could be obtained per day. These data constituted the primary endpoint of the study. The diaries were filled out by the patient and sent monthly per mail to the study site.

Figure S2. Primary endpoint. Temporal analysis of infectious score components.

The adjusted one-year relative scores presented separately for each 3 month period. (A) airways, (B) malaise, (C) ear symptoms, (D) sinus symptoms and (E) antibiotic consumption. Effects are presented as relative scores (airway and malaise) or odds ratios (ear, sinus and antibiotics). Dashed lines indicate 95% confidence intervals.

Figure S3. Secondary endpoint. Number of bacterial cultures in relation to concomitant lung disease. The number of bacterial cultures taken (A) and positive bacterial cultures (B). Asthma (vit D, n=22; Placebo, n=22). Bronchiectasis, BE (Vit D, n=5; Placebo, n=7). Chronic Obstructive Pulmonary Disease, COPD (Vit D, n=4; Placebo, n=4).

Figure S4. Antimicrobial peptides in nasal fluid. Levels of LL-37 (A) and HNP1-3 (B) were measured in nasal fluid extracts at day 0, 6 and 12 months in a randomly selected group of patients (LL-37, n=12; HNP1-3, n=15). There were no statistically significant differences within or between the groups with regards to peptide levels (Mann-Whitney U test). Bacterial

growth in these samples were also recorded (C) and expressed as either 'no growth/normal flora' or 'growth of a primary pathogen'. The growth pattern of the vitamin D_3 and Placebo groups were compared at each time-point using Fisher's exact test.

Figure S5. Blood chemistry. Plasma levels of calcium (mmol/L), phosphate (mmol/L), albumine (g/L) and creatinine (µmol/L) were measured at the time points 0, 3, 6, 9 and 12 months after inclusion. Values are expressed as mean values.

Figure S1.

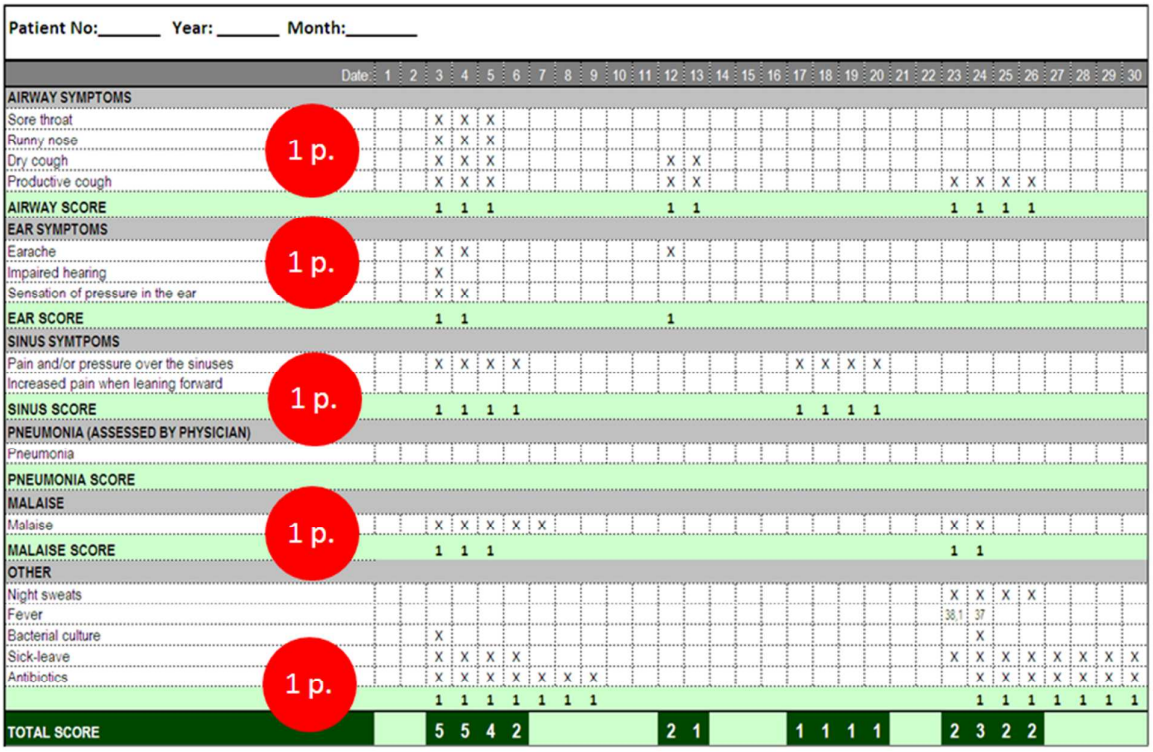


Figure S2

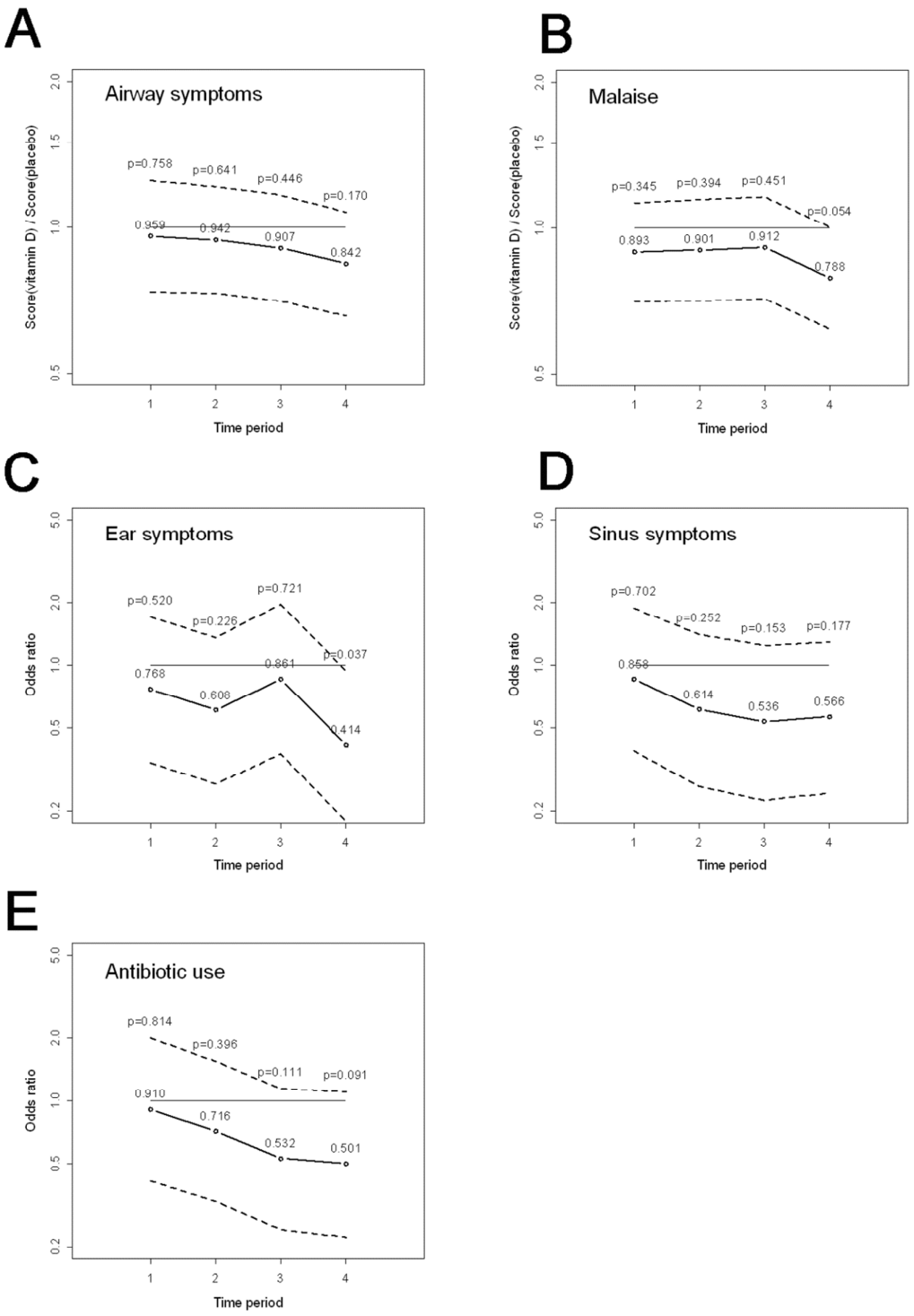

A
Airway symptoms
Score(vitamin D) / Score(placebo)
p=0.758
p=0.641
p=0.446
p=0.170
0.959
0.942
0.907
0.842
Time period
B
Malaise
Score(vitamin D) / Score(placebo)
p=0.345
p=0.394
p=0.451
p=0.054
0.893
0.901
0.912
0.788
Time period
C
Ear symptoms
Odds ratio
p=0.520
p=0.226
p=0.721
p=0.037
0.768
0.608
0.861
0.414
Time period
D
Sinus symptoms
Odds ratio
p=0.702
p=0.252
p=0.153
p=0.177
0.858
0.614
0.536
0.566
Time period
E
Antibiotic use
Odds ratio
p=0.814
p=0.396
p=0.111
p=0.091
0.910
0.716
0.532
0.501
Time period

Figure S3

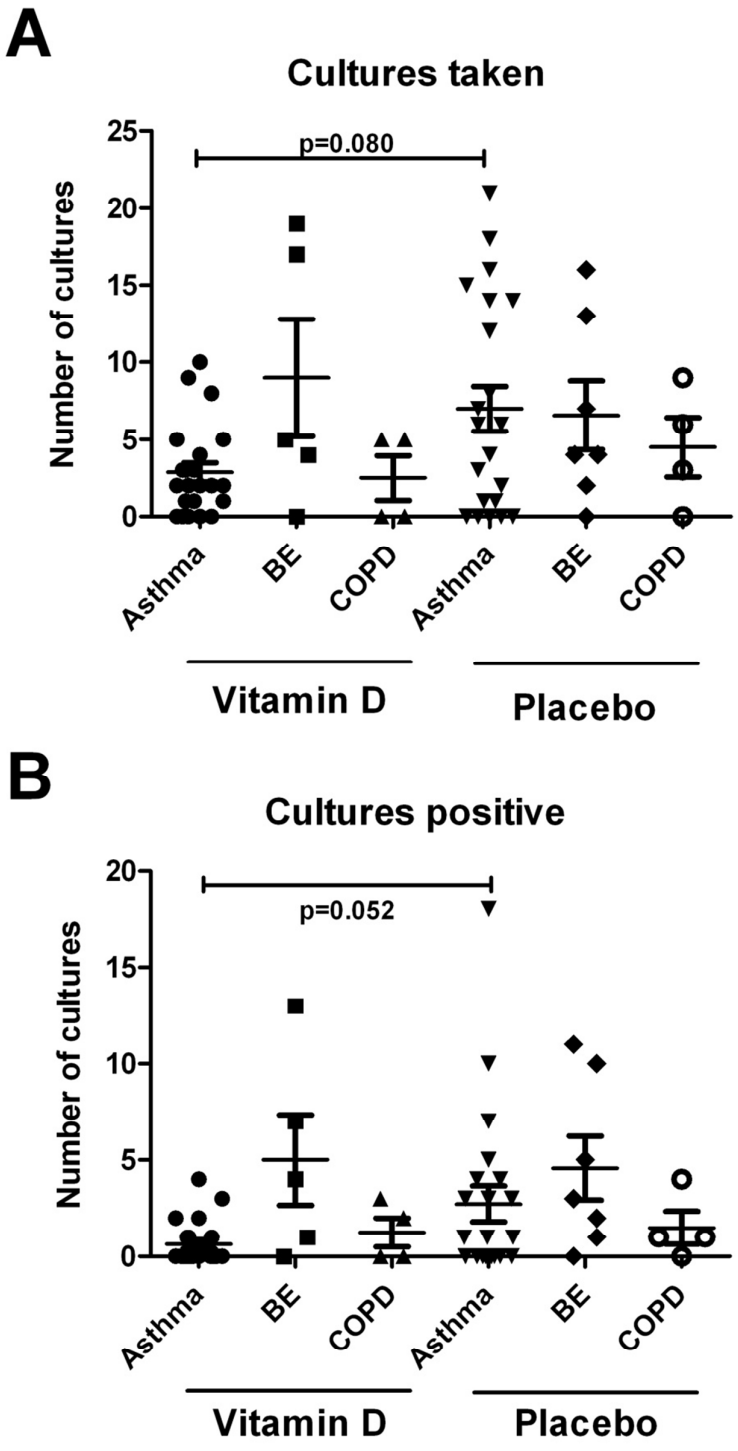


Figure S4

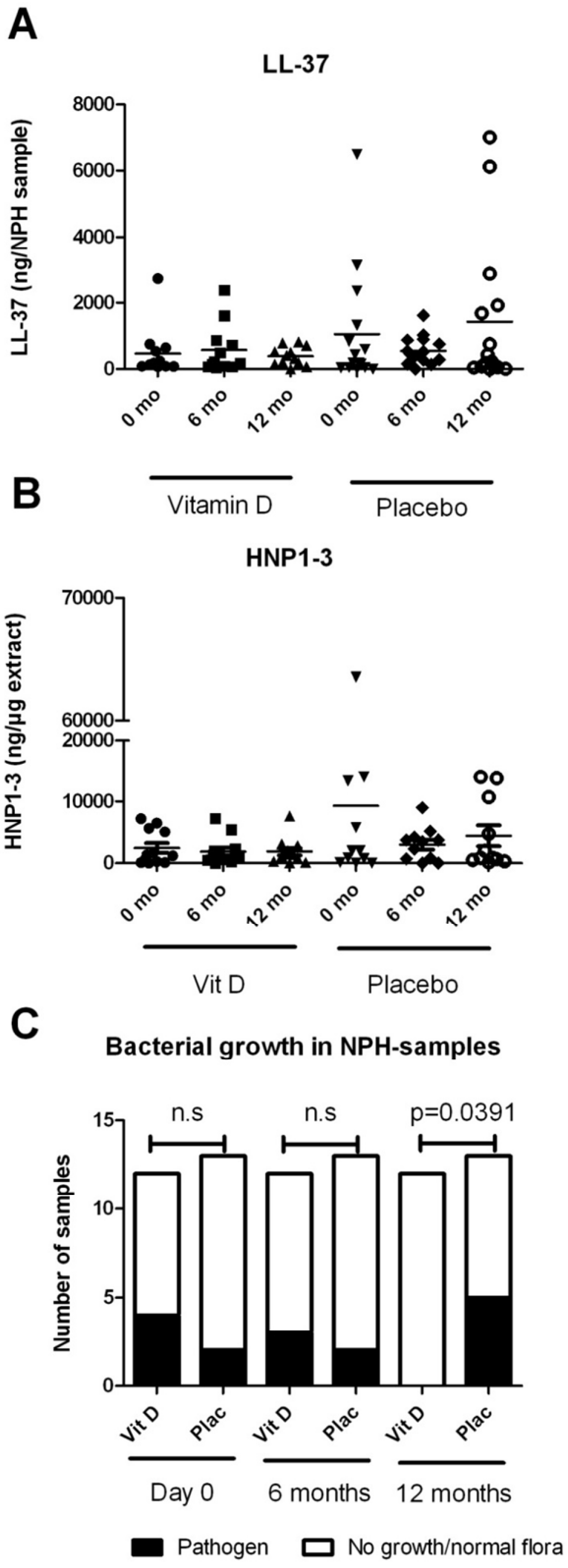
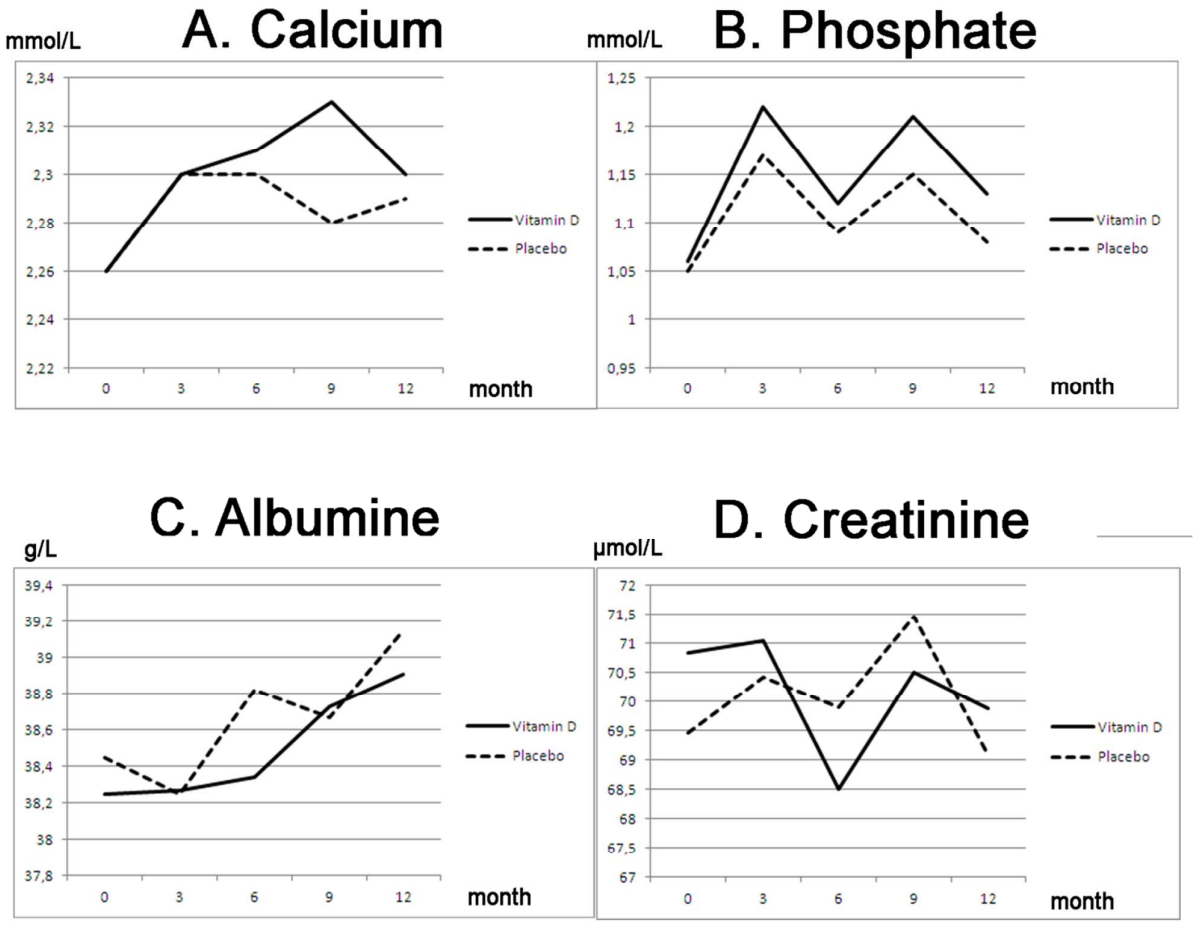


Figure S5



CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	p. 1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	p. 2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	p. 5-6
	2b	Specific objectives or hypotheses	p. 6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	p. 7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	Not Applicable (NA)
Participants	4a	Eligibility criteria for participants	p. 7
	4b	Settings and locations where the data were collected	p. 7
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	p. 8
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	p. 8
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	Suppl meth p4
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	Suppl meth.
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Suppl meth.
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	Suppl meth.

Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	p. 9
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	Suppl meth.
	11b	If relevant, description of the similarity of interventions	NA
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	p. 8-9
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	Suppl meth.
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	p. 10, Fig 1
	13b	For each group, losses and exclusions after randomisation, together with reasons	p. 10, Fig 1
Recruitment	14a	Dates defining the periods of recruitment and follow-up	p. 5
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Yes
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Yes
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Yes
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Yes
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Table 5 +suppl fig/tabl
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Yes
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	Yes
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Yes
Other information			
Registration	23	Registration number and name of trial registry	p. 7
Protocol	24	Where the full trial protocol can be accessed, if available	p. 7
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	p. 20

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Study title:

Study of Vitamin D3 Substitution to Patients With Primary Immunodeficiency (VITAPID)

Date: October 6, 2009

Study site: Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden

Product: Vigantol oil

Substance: Vitamin D3 (cholecalciferol)

Producer: Merck, Germany

EudraCT-number: 2009-011758-16

Sponsor: Professor Jan Andersson, MD, PhD

Co-investigators: Dr Peter Bergman, MD, PhD; Dr Anna-Carin Norlin, MD

Study facts

Protocol identity and aim

EudraCT-number: 2009-011758-16

Protocol title: A placebo-controlled double-blind study of Vitamin D3 supplementation to patients with increased susceptibility to infections.

Aim: To investigate if substitution with vitamin D3 can prevent or ameliorate infectious burden among infection prone patients.

Study drug:

Product:	Vigantol Oil
Pharmaceutic preparation:	Oral mixture (oil)
Administration:	Per os

Methodology

Study design:	Randomized double-blind placebo-controlled
Dose:	Vigantol, 4000 IU/day
Primary endpoint:	Infectious score
Safety parameters:	Plasma levels of calcium, creatinine, albumin and phosphate; serum levels of 25-OH vitamin D_3.
Study population:	Patients with increased susceptibility to respiratory tract infections.
Number:	140

Timeplan:

First patient to be included: Q1, 2010

Last patient to be included: Q4, 2011

Last patient to finish the study: Q4, 2012

Administrative information

Sponsor and Investigators

Professor Jan Andersson, MD, PhD: Sponsor and Principal Investigator

Dr Peter Bergman, MD, PhD: Co-investigator

Dr Anna-Carin Norlin, MD: Co-investigator

Research nurses and study coordinator

Susanne Hansen, Study coordinator, head of Immunodeficiency Unit

Kristina Johansson, Research Nurse

Maria Lindén, Research Nurse

Quality control

Two independent monitors from Karolinska Trial Alliance will monitor the study according to ICH-GCP.

Overview and significance

The innate immune system is depending on antimicrobial peptides, which are potent killers of microbes, such as bacteria, viruses and fungi. These molecules defend epithelial surfaces and are rapidly released after contact with microbes. Vitamin D is a potent inducer of AMPs in epithelial and immune cells. Vitamin D is synthesized in the skin under the influence of UVB-light or can be obtained via the diet. However, in Sweden the UV-radiation has too low intensity during the wintertime and the diet is not enough to maintain adequate levels. Therefore many individuals in Sweden have low levels of vitamin D3, especially during the darker period of the year (October-April). Epidemiological data show a strong association between low vitamin D levels and an increased risk of infection. There is also mechanistic evidence that vitamin D increases the levels of antimicrobial peptides in macrophages and in epithelial cells. However, there are few randomized controlled trials testing the hypothesis that supplementation with vitamin D3 can reduce or ameliorate infections. Therefore, we have designed the study described in this protocol where vitamin D3 will be given to patients with an increased risk of infection. The results may have a great impact on treatment of patients with frequent infections, since vitamin D3 may be used in conjunction with standard care (antibiotics). This may be particularly important in light of the emerging bacterial resistance. Thus, novel strategies to prevent and treat infectious diseases have to be developed and supplementation with Vitamin D3 may constitute one future treatment option.

Aims

To investigate if substitution with vitamin D3 can:

1. Reduce the infectious burden among patients with increased number of infections
2. Increase levels of antimicrobial peptides in nasal fluid
3. Increase serum concentrations of 25-OH vitamin D3

Study design

Participants will be given vitamin D3 or placebo for one year. 140 patients will be recruited and 70 patients will be randomized to placebo or vitamin D3 in a 1:1 randomization. Evaluation of symptoms and antibiotic consumption will be registered by the patient in a diary form that will be sent by mail to the study site on a monthly basis. Patients will be recruited at the tertiary center for primary immune deficiencies. Currently there are 319 patients with IgG-deficiency, 180 patients with selective IgA-deficiency, 90 patients with CVID and 210 patients with an increased susceptibility to infection without a manifest immunological diagnosis. The study patients will be recruited from this group in a nonbiased fashion, ie regardless of diagnosis or IgG-substitution therapy.

Study drug and mechanism

The study drug is cholecalciferol (vitamin D3), which is hydroxylated in the liver to 25-OH vitamin D3 (the storage form in the body). The second hydroxylation step is carried out by 1-alpha hydroxylase. This enzyme is expressed in the kidney but also in immune- and epithelial cells. The kidney is

responsible for the systemic production of 1,25 (OH)2 vitamin D3, which is crucial for skeletal health (endocrine system). The local activation of vitamin D3 in immune- and epithelial cells is described as a paracrine system and is central to the immune effects of vitamin D. The paracrine system is strictly regulated and does not contribute substantially to the systemic levels of 1,25 (OH)2 vitamin D3. This is important since the active and systemically available vitamin D3 is responsible for hypercalcaemia that has been reported as an adverse event for vitamin D3 supplementaton. However, hypercalcaemia is a very rare event and we will strictly follow plasma levels of calcium and 25-OH vitamin D3 during the study period.

Study drug

Vigantol Oil is not a registered drug in Sweden. However, Merck Pharma GmbH has permission to manufacture and sell Vigantol in Germany since many years (permission nr 6154275.00, ATC code A11CC05, mSPC available upon request). The study drug is manufactured according to GMP (GMP certificate from Merck available upon request). The placebo oil is also manufactured by Merck and has identical galenic properties to Vigantol oil. The drugs (vigantol and placebo) will be delivered to Vecura AB at Karolinska University Hospital, Huddinge. Vecura AB is a company specialized in clinical trials and has a GMP-certificate for clinical trials and handling of study drugs. VECURA AB will aliquot the study drug and placebo to the final bottles, carry out randomization and labelling.

Endpoints

Primary endpoint

Infectious score based on patient recorded information registered in a diary-form.

Secondary endpoints

25-OH vitamin D3 in serum

Microbiological findings and numbers of cultures taken

Levels of antimicrobial peptides in nasal fluid

Antibiotic consumption collected from patient records

Design

Evaluations and procedures

Prescreening: Eligible patients fulfilling inclusion criteria will be selected from records and contacted via regular mail. They will be sent a letter of invitation together with information on the study. All these patients will be contacted via telephone one week later and asked for participation.

Visit 1, screening, time=0: Co-investigator (Licenced physician, MD) will meet all patients for screening. Additional information on the study will be given and informed consent will be collected. If the patient is judged to fulfil all criteria for inclusion and all exclusion criteria can be negated, the patient is included in the study. The study drug for 6 months will be given out to the patients as well as diaries and envelopes. The patient will be carefully informed about the procedures with the diaries. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Visit 2, time=6 months +/- 14 days: Co-investigator (Licenced physician, MD) will meet all patients after 6 months. A control for adverse events and compliance will be carried out. Additional bottles of study drug/placebo will be given out for the remaining 6 months of the study. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Visit 3, time= 12 months +/- 14 days: Co-investigator (Licenced physician, MD) will meet all patients after 12 months. A control for adverse events and compliance will be carried out. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Participants

Inclusion Criteria:

- Age 18-75
- Increased number of respiratory tract infections
- At least 42 days of infections during 2008 or 2009
- S-25 OH vitamin D3 < 250 nM
- Not planning a pregnancy during the coming year
- Accepting the use of contraceptives during 1 year

Exclusion Criteria:

- Continuous antibiotic treatment
- Hypercalcaemia
- Sarcoidosis
- Kidney disease
- Tuberculosis
- Pregnancy
- Previous history of kidney stones
- Heart medication (glycosides)

Treatment

Vigantol oil (cholecalciferol). 1 drop contains 500 IU. The patients should take 8 drops per day during the study.

Packing, labelling and handling of study drug: Merck will distribute Vigantol oil and placebo oil to VECURA AB, Karolinska University Hospital, Huddinge, which will handle, pack and label the study drug.

Distribution of the study drug to the participants: At the first visit, the participants will be given study drug for the first 6 months of the study. After 6 months, they will be given the remaining bottles. Oral and written instructions will be given about 8 drops per day .

Blinding and breaking of the code: The design is double-blind. Thus, neither the doctor/nurse nor the patient will have any information on the nature of the study drug. Two monitors will carry out controls of the study. The randomisation list will be stored in such a way that the personnel involved in the study do not have access to it. The Hospital Pharmacy will be given a copy of the list in case of emergency with access via telephone 365 days per year/24 hours per day.

Concomitant medication: All other medication is allowed during the study, including antibiotics. However, recent changes in drug treatments will be documented in the diary and asked for by the study doctors at visits.

Compliance: The compliance to the study drug and diary registration is asked for at visits to the study site.

Control of the study drug: Patients are asked to bring back their empty bottles to the study sites. All bottles will be registered by the study nurses.

Evaluation of efficacy and safety

Evaluation of primary endpoint (clinical endpoint, patient recorded)

The primary endpoint is the infectious score, which is based on the diary form filled out by the patient. The total score is composed of symptoms from airways, sinus and ears together with malaise and antibiotic consumption. The idea is to monitor several aspects of an infectious episode and thus monitor the total infectious burden, rather than a specific symptom.

Evaluation of secondary endpoints (microbiological and biochemical endpoints, collected by the study personnel)

1. 25-OH vitamin D3 in serum
2. Microbiological findings and numbers of cultures taken. This information will be collected from patients' clinical records with a focus on samples taken from the respiratory tract.
3. Levels of antimicrobial peptides in nasal fluid. Every fifth patient (according to a special randomization list) will be asked to leave nasal fluid for analysis of antimicrobial peptides.
4. Antibiotic consumption collected from patient records. Information on how many prescriptions of antibiotics will be collected from patients' records.

Evaluation of clinical safety for participants

Patients will leave blood for analyses of plasma levels of creatinin, calcium, phosphate and albumin as well as serum levels of 25-OH vitamin D3 at times 0, 3, 6, 9 and 12 months. The information regarding all time-points except at inclusion will be sent to an unblinded senior physician who will serve as an external clinical safety monitor. He will contact the study physicians in case of clinically relevant abnormalities in the blood chemistry.

Samples and clinical chemistry

Serum and plasma from the first sampling will be sent to Dept of Clinical Chemistry, Karolinska University Hospital, Huddinge for routine analyses. These answers will be recorded in patients' records. For all other time points samples will be sent to Study Center Karolinska which will coordinate all samples for clinical chemistry and send answers to the unblinded clinical safety monitor. These answers will not appear in patients' records in order to keep the blinded design intact.

Adverse events (AE) and Severe adverse events (SAE)

All adverse events and severe adverse events will be recorded in special forms. They will further be classified for severity (mild, moderate and severe) and for connection with the study drug (probable, possible and unlikely). All SAE will be reported to the sponsor within 24 hours after it has been known by the investigator.

Statistics

Handling of data: All data will be registered in a database especially constructed for the study.

Analysis of excluded patients: Excluded patients will be recorded and followed for adverse events. After the study, special analyses will be performed to understand why these patients did not complete the study.

Statistical analysis and power calculation: The statistical calculation is based on the assumption that the infectious score is reduced with 30 % from 42 days (42x5=240 points) to 28 days (28x5=160 points) with full infectious score. If we include 60 patients per group a significance level of p=0.02 will be reached with a power of 90%. To compensate for expected exclusions, we will increase the number of patients per group to 70. Thus, the total number of patients in the study will be 140.

Quality control

Source data: The information that will be collected from each participant will be: study title, screening number, patient number, written informed consent, main and concomitant diagnoses, study treatment, medication, data on blood chemistry and other investigations carried out.

Monitoring: All study personnel have knowledge on clinical trials and ICH-GCP. The sponsor will sign a contract with Karolinska Trial Alliance for monitoring. The investigator will allocate time for monitoring and supply all available and relevant information to the monitors.

Ethics

The sponsor has applied for ethical approval from the regional Ethical Board. The study will be carried out according to ICH-GCP and the Helsinki-declaration.

Informed consent: The patient will have information sent home via regular mail. One week later the study nurse will call the patient and discuss the study. The first visit will involve the meeting with a physician and time is extended for questions before the written informed consent is signed. One copy will stay at the study site and one copy will go with the patient.

Handling of data

Case Report Forms (CRF) will be used. These will kept at the study site until the end of the study. After completion of the study, all material will be archived at least 10 years.

Insurance

All participants are insured via patient insurance and the Swedisg drug insurance.

Publication of the results

The study group wishes to publish the data in a refereed international scientific journal and to communicate the results at conferences and other venues.

The original protocol is written in Swedish and is available on request.

Vitamin D_3 supplementation in patients with frequent respiratory tract infections - a randomized and double blind intervention study

Peter Bergman*[1,3,6#], Anna-Carin Norlin*[2,3], Susanne Hansen[3], Rokeya Sultana Rekha[4], Birgitta Agerberth[4], Linda Björkhem-Bergman[5], Lena Ekström[5], Jonatan D Lindh[5], Jan Andersson[6]

1. Department of Laboratory Medicine, Clinical Microbiology, Karolinska Institutet
2. Department of Laboratory Medicine, Clinical Immunology, Karolinska Institutet
3. Karolinska University Hospital, Clinical Immunology, Immunodeficiency Unit
4. Department of Medical Biochemistry and Biophysics, Karolinska Institutet
5. Department of Laboratory Medicine, Clinical Pharmacology, Karolinska Institutet
6. Department of Medicine, Center for Infectious Medicine (CIM), Karolinska Institutet

*Equal contribution

Positions:

Peter Bergman, MD, PhD, Resident Physician, Clinical Microbiology

Anna-Carin Norlin, MD, Specialist in Clinical Immunology

Susanne Hansen, RN, Head of Immunedeficiency Unit

Rokeya Sultana Rekha, MSc, PhD-student

Birgitta Agerberth, PhD, Professor, Medical Microbial Pathogenesis

Linda Bjorkhem-Bergman, MD, PhD, Resident Physician, Clinical Pharmacology

Lena Ekström, PhD, Associate Professor, Clinical Pharmacology

Jonatan D Lindh, MD, PhD, Specialist in Clinical Pharmacology

Jan Andersson, MD, PhD, Professor, Infectious Diseases

Running title: Vitamin D_3 supplementation in patients with frequent respiratory tract infections

#Corresponding author:

Peter Bergman, MD, PhD

Department of Laboratory Medicine, Clinical Microbiology F68

Karolinska Institutet and Karolinska University Hospital, Huddinge

SE-141 86 Stockholm, Sweden

peter.bergman@ki.se

Phone: +46858587942

Fax: +46858581305

Competing interest statement

All authors have completed the Unified Competing Interest form (available on request from the corresponding author) and declare no support from any organisation for the submitted work, no financial relationships with any organisations that might have an interest in the submitted work in the previous three years and no other relationships or activities that could appear to have influenced the submitted work.

Article Summary

Article focus

• Recent evidence suggests that vitamin D_3 has potent extra-skeletal effects, such as suppression of inflammation and strengthening of mucosal immunity by induction of antimicrobial peptides.

• Data from observational studies suggest that low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections.

• Results from a limited number of randomized controlled trials on the protective role of vitamin D_3 against respiratory tract infections are inconclusive and thus additional studies are warranted.

Key Messages

• Therefore we designed and carried out a randomized controlled trial where a large dose (4000 IU) of vitamin D_3 was given to patients with an increased susceptibility to infections for one year.

• The main conclusion is that vitamin D_3 supplementation reduces symptoms and antibiotic consumption among patients with an increased frequency of respiratory tract infections. Thus, vitamin D_3 supplementation may be an alternative strategy to reduce antibiotic use among patients with recurrent respiratory tract infections.

Strengths and limitations

• Strengths: A high daily dose of vitamin D3 was used, the study time was a full year covering all seasons and patient with an increased frequency of respiratory tract infections were studied.

• Limitations: A single study center, small sample size (n=140) and a selected group of patients.

Abstract

Background: Low serum levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections (RTIs). Clinical trials with vitamin D_3 against various infections have been carried out but data are so far not conclusive. Thus, there is a need for additional randomized controlled trials of effects of vitamin D_3 on infections.

Objective: To investigate if supplementation with vitamin D_3 could reduce infectious symptoms and antibiotic consumption among patients with antibody deficiency or frequent RTIs.

Design: A double-blind randomized controlled trial.

Setting: Karolinska University Hospital, Huddinge

Participants: 140 patients with antibody deficiency (sIgA-, IgG subclass deficiency, CVID) and patients with increased susceptibility to RTIs (>4 bacterial RTIs/year) but without immunological diagnosis.

Intervention: vitamin D3 (4000 IU) or placebo was given daily for one year

Primary and secondary outcome measures: The primary endpoint was an infectious score based on five parameters: symptoms from respiratory tract, ears and sinuses, malaise and antibiotic consumption. Secondary endpoints were serum levels of 25-hydroxyvitamin D_3, microbiological findings and levels of antimicrobial peptides (LL-37, HNP1-3) in nasal fluid.

Results: The overall infectious score was significantly reduced for patients allocated to the vitamin D-group (202 points) compared with the placebo group (249 points) (adjusted relative score 0.771, 95% CI 0.604-0.985, p=0.04).

Limitations: A single study center, small sample size and a selected group of patients.

Conclusions: Supplementation with vitamin D_3 may reduce disease burden in patients with frequent respiratory tract infections.

Primary funding source: Swedish Foundation for Strategic Research (SSF)

The study was registered at www.clinicaltrials.gov (NCT01131858)

273/275 words

Introduction

Vitamin D was discovered when it was noted that rachitic children were improved by exposure to sunlight [1]. It was later shown by Holick *et al* that vitamin D_3 is synthesized in the skin under the influence of UVB-light [2]. Vitamin D_3 is further hydroxylated in the liver to 25-hydroxyvitamin D_3, which is considered to reflect the vitamin D status of an individual patient [3]. The final activation to the active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)D_3$) requires 1-α-hydroxylase activity. This enzyme (also designated CYP27B1) is expressed in the kidney but also in many other cell-types, including epithelial- and immune-cells [4]. The active vitamin D_3 ($1,25(OH)D_3$) binds to the vitamin D receptor (VDR), which belongs to the nuclear receptor family. Active vitamin D_3 is only present in minute amounts in the circulation and local activation in target cells is crucial for vitamin D-mediated effects on the immune system[5].

Low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of tuberculosis [6-8] and respiratory tract infections [9]. The mechanism is not fully elucidated but vitamin D_3 has been shown to induce antimicrobial peptides in immunecells [10]. In addition, active vitamin D_3 ($1,25(OH)D_3$) has broad anti-inflammatory effects on the adaptive immune system by shifting the T-helper cell pool from a Th1/Th17-response to a Th2/Treg-dominated response [11 12]. Vitamin D_3 has also been shown to suppress the Th2-response in allergic bronchopulmonary aspergillosis [13]. Thus, vitamin D_3 modulates both the adaptive and innate immune system [14]. The bulk of data on vitamin D_3 and infections stems from *in vitro* experiments and retrospective observational studies. Results from randomized controlled trials where the effects of vitamin D_3 on infections have been investigated (reviewed in Yamshchikov et al. [15]) are not conclusive and larger clinical trials are therefore warranted.

We designed a study to test the hypothesis that 4000 IU of vitamin D_3 given daily to patients with antibody deficiency and frequent respiratory tract infections for one year could prevent

or ameliorate infections. In addition, we investigated whether genetic polymorphisms in genes involved in the effect and/or metabolism of vitamin D_3 have an influence on the outcome of vitamin D_3 supplementation.

Methods

Study design

A prospective, randomized, double-blind placebo-controlled study of vitamin D_3 supplementation in patients with an increased susceptibility to respiratory tract infections. The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants. The study was registered at www.clinicaltrials.gov prior to inclusion of the first patient (NCT01131858). The EudraCT number is 2009-011758-16. The full protocol is available from the corresponding author upon request.

Sample size calculation

The sample size was based on the assumption that the intervention would reduce the number of days with symptoms from 42 days (210 points) to 28 days (140 points), i.e. a reduction of the infectious burden by 30%. Given this assumption, a sample size of 60 patients per study group was predicted to provide the study 90% power at a significance level of p=0.02 (Student's t-test). To compensate for predicted exclusion of participants, the groups were increased to include 70 patients per treatment arm. Importantly, the significance level of p=0.02 was chosen in the power calculation to ensure that a sufficient number of patients were recruited in order to avoid a type II error in the primary analysis. However, the widely accepted significance level of p=0.05 was used for statistical analyses of the primary and secondary endpoints. Consequently, p-values are written out together with the 95% confidence interval (CI).

Participants

Patients at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden, were included between March 2010 and June 2010 by the study nurses (SH, ML, KJ). Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infections; i.e. > 42 days with symptoms from the respiratory tract during a 12 months period prior to study inclusion. Patients registered at the Immunodeficiency Unit are closely followed with a diary of symptoms and antibiotic consumption. Thus, the patients are trained and used to apply such an instrument to assess their infectious status. Data from patients' standard diary was used as an instrument in order to assess patients for eligibility, both via telephone and by the responsible physician (PB, ACN) prior to inclusion. Patients with selective IgA-deficiency (**D80.2**), IgG-subclass deficiency (**D80.3**) and common variable immune disorder (CVID, **D83.0**) as well as patients without a defined immunological diagnosis (**D89.9**) were included. Exclusion criteria were prophylactic treatment with antibiotics, history of hypercalcemia or stones in the urinary tract, sarcoidosis, ongoing supplementation with vitamin D_3 exceeding 400 IU/day, HIV-infection and pregnancy.

Interventions

Patients were randomized to 12 months' treatment with Vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. One drop contained 500 IU vitamin D_3 or placebo oil (Miglyol oil®) and the participants were asked to take 8 drops daily. The participants had to mark their daily symptoms of infection in a diary, which was sent via regular mail to the study site every month. The following data was recorded: symptoms from the respiratory tract, ears and sinuses, treatment with antibiotics, numbers of bacterial cultures, times and reasons of visits to hospitals, frequency of travelling abroad and adherence to study drug.

Outcomes

The primary outcome was a composite infectious score, based on a daily patient-reported questionnaire, and included five parameters: symptoms from the respiratory tract, ears and sinuses, malaise and use of antibiotics (Figure S1), each parameter gave 1 point/day. The occurrence of X-ray verified pneumonia gave 3 additional points per day for a period of 7 days. Thus each pneumonia resulted in 3x7 points = 21 extra points. Patients were specifically instructed to record only symptoms related to ongoing respiratory tract infections. Symptoms related to infections at other sites (urinary tract, wounds etc) as well as non-infectious symptoms were reported as adverse events. Secondary outcomes were serum levels of 25-hydroxyvitamin D_3 (at baseline and after 3, 6, 9 and 12 months), numbers of bacterial cultures, microbiological findings and levels of antimicrobial peptides (LL-37 and HNP1-3) in nasal fluid (at baseline and after 6 and 12 months). In addition, 6 *post hoc* genotype analyses were performed in all participants. Analysis of single nucleotide polymorphisms (SNPs) were carried out for VDR (Taq1 and Foq1), CYP27B1, CYP24A1, CYP2R1 and Vitamin D binding protein (GC). Safety tests included plasma levels of creatinine, calcium, phosphate and albumin, measured at baseline and after 3, 6, 9 and 12 months. At inclusion, urine-HCG (human chorionic gonadotropin) in women was measured and p-parathyroid hormone in both genders. The results of the safety tests were reviewed by an independent and un-blinded consultant physician. Two blinded physicians (PB and ACN) were responsible for inclusion and all medical visits to the study site (Immunodeficiency Unit, Karolinska University Hospital, Huddinge).

Randomisation and statistical analysis

Participants were randomised to 12 months' treatment with vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. Block randomization with a block size of ten was used to ascertain equal group sizes. Staff at Karolinska Trial Alliance (KTA) was responsible for randomization procedures. In the statistical analysis, continuous variables were compared using Mann-Whitney U test or linear regression and dichotomous variables by Fisher's exact test or logistic regression. Regressions of log-transformed infectious scores were performed both unadjusted (simple regression) and with adjustment for potential confounders (multiple regression).

Statistical methods: Primary analysis

The distribution of the infectious score was found to be very skewed, thereby violating the normal assumption of the pre-specified t-test analysis. Hence, scores were log-transformed prior to analysis.

Further, the randomisation had resulted in age distributions that were not entirely balanced between the two groups. Since there might be concerns that such imbalance could influence the results of the study, the original analysis plan was extended with a multivariable analysis adjusting for potential confounders. In this linear regression model based on log-transformed values of the primary outcome (the total infectious score) and its individual components, adjustment was made for age, gender, smoking, type of immune deficiency and significant co-morbidities (respiratory or non-respiratory). Due to the transformation procedure, the adjusted effect of vitamin D_3 is expressed as a ratio between the score in the vitamin D_3 and the placebo group. In this multiplicative model, an effect size of 1 indicates identical outcome in the two study groups and statistically non-significant results are recognized by confidence intervals encompassing the value 1.

To explore potential divergent effects on different organ systems, both adjusted and unadjusted analyses were repeated separately for each individual item of the infectious score. In addition, the temporal aspects of the vitamin D_3 effect were investigated by dividing the study period into four 90-day periods (starting on the first day of treatment) and repeating the analyses separately for each time period. “Ear” and “sinus” symptoms as well as “antibiotic use” occurred at low frequencies and for these entities normal distributions could not be achieved despite data transformation. Thus, the adjusted analyses of these individual items were based on multivariable logistic regression, after coding the symptom (or antibiotic therapy) as present or absent during the course of the study. However, this only applies to analysis of the individual items, and not to the primary analysis of the total infectious score, where all item scores were added as originally described.

Most post-randomization exclusions were due to patients failing to fill out the symptoms diary. Hence, no intention to treat (ITT) analysis based on actual outcome data could be performed. However, the potential impact of dropouts was addressed in an ITT analysis based on multiple imputation of missing outcome data. In the imputation process, pooled estimates were derived from 100 datasets created by means of multivariate imputation by chained equations and predictive mean matching for the same covariates as in the adjusted per-protocol analysis

Detailed descriptions of randomisation and blinding, sampling of nasal fluid, measurement of antimicrobial peptides, measurement of 25-hydroxyvitamin D_3, genotyping and statistical analyses of secondary outcomes including sample size calculations are presented in the Supplementary Methods Section.

Results

Baseline data

A total of 286 patients were first assessed for eligibility but 144 were not included because they did not fulfill all inclusion criteria; <42 days with infection/year (n=35), lacked other inclusion criteria (n=42), or declined to participate (n=67). The remaining 142 patients were further screened and 140 patients were included in the study. Of these, 70 were randomized to vitamin D_3 supplementation and 70 to placebo (Figure 1). The groups did not differ with regards to gender, IgG replacement therapy, smoking, baseline 25-hydroxyvitamin D_3 levels, type of immune defect or co-morbidities. Patients with subclass deficiency, selective IgA deficiency (sIgA), common variable immune deficiency (CVID) and patients without a defined immunological diagnosis (ND) but with >4 bacterial respiratory tract infections/year were included. IgG replacement therapy was most common in the CVID-group (100%) and in the subclass deficiency group (63%), but also frequent in the other groups (ND, 54% and sIgA, 38%, table S1). Patients allocated to the placebo group were slightly younger than patients in the treatment group (p=0.025, data not shown). During the course of the study, 16 patients left the study prematurely (8 patients from each study group) and consequently 124 patients were included in the main per-protocol analysis. Reasons for dropout included elevated PTH (n=2), withdrawn consent (n=5), adverse event (n=1), prescription of vitamin D outide the study (n=1), failure to complete diary (n=4) or non-compliance to study medication (n=3) (Figure 1).

Primary endpoint: Infectious score

One year of vitamin D_3 treatment was associated with a significantly reduced total infectious score both in the unadjusted (n=124, p=0.024, Table 2) and the adjusted analyses (n=124,

p=0.040) (Table 2, Figure 2A, B and Table S2). The unadjusted relative score in the intervention group was 0.754 (95% c.i. 0.591-0.963, p=0.024, n=124) corresponding to a 25% reduction and after adjustment for potential confounders, the relative score was 0.771 (95% c.i. 0.604-0.985, p=0.04), corresponding to a 23% reduction (Table 2). According to the temporal analysis, the effect of vitamin D_3 supplementation tended to improve with time (Figure 2A). The absolute unadjusted score per patient was 202 points for the vitamin D group and 249 points for the placebo group, which was a significant reduction of 47 points per patient (p=0.023, Mann Whitney U-test, table S3).

When the individual items of the infectious score were analyzed separately, all point estimates indicated a reduction in the treatment group (Table 2, figure S2), although only antibiotic consumption reached statistical significance (Figure 2B and S2, panel E). The adjusted OR for antibiotic use was 0.365 (95% c.i. 0.153-0.872, p=0.023, n=124), i.e. a 63.5% reduction of the odds of antibiotic use in the intervention group (Table 2). The absolute values were 33 days on antibiotics for the placebo group and 16 days for the vitamin D_3 group, i.e. a reduction of 17 days in the vitamin D_3 group (table S3). The temporal trends for specific symptoms and antibiotic consumption were similar to the total score and reached statistical significance for 'ear'-symptoms (n=124, p=0.041) and for 'malaise' (n=124, p=0.053) in the final quarter of the study (Figure 2S, panels B and C).

Analyzing the primary outcome according to intention-to-treat (n=170) produced results virtually identical to those of the per-protocol analysis. In the unadjusted ITT analysis, vitamin D_3 reduced the total infectious score by 25% (relative score 0.752, 95% c.i. 0.588-0.962, p=0.024) and after adjustment for potential confounders the reduction was 23% (relative score 0.767, 95% c.i. 0.599-0.982, p=0.036).

Serum levels of 25-OH vitamin D_3

Serum 25-hydroxyvitamin D_3 levels did not differ between the groups at baseline (Table 1) but already after 3 months the intervention group had a significantly higher level of 25-hydroxyvitamin D_3 (133.4 nmol/L versus 66.6 nmol/L, p<0.001, Figure 3). This increase remained throughout the study (Figure 3).

Bacterial cultures and microbiology

During the course of the study, 173 microbiological samples were obtained in the vitamin D_3 group (n=62, 2.79/patient) and 301 in the placebo group (n=62, 4.85/patient) (p=0.010, Table 3). The number of samples with at least one positive finding was higher in the placebo group, with close to statistical significance (p=0.052), while the fraction of positive samples was similar for both groups (Table 3). Significantly more patients had a microbiological sample taken from the respiratory tract (≥ 1 sample) during the study period in the placebo group; OR 2.63 (95% CI 1.17-5.92), (Table 3).

In total, the vitamin D_3 group generated 76 positive microbiological findings (bacteria or fungi), compared to 159 in the placebo group (p=0.023). There was no difference between the groups for the traditional respiratory pathogens (*H. influenza, M. catharralis and S. pneumonia*), but there were significantly fewer findings of *S. aureus* (p=0.019) and fungi (p=0.028, *Candida* spp. and *Aspergillus* spp.) in the treatment group (Table 4). Likewise, significantly fewer vitamin D_3-treated patients had a bacterial culture positive for *S. aureus* (p=0.019) or fungal species (p=0.058), although the latter difference did not reach statistical significance (Table 4).

Vitamin D treated patients with sub-class deficiency left significantly fewer bacterial or fungal cultures than placebo-treated patients with this diagnosis; 7 cultures in the vitamin D group (n=22) versus 47 cultures in the placebo group (n=24) (Table S4). Also the number of patients that had ≥1 bacterial culture taken was significantly fewer in the placebo group (12/22 versus 22/24, p=0.0065, table S4). There was no significant effect of other immunological diagnoses on bacterial cultures or microbiology (Table S4).

Since concomitant lung disease may be an important factor for vitamin D mediated effects on respiratory immunity, we performed a detailed analysis of bacterial cultures and microbiology of patients with asthma, bronchiectasis (BE) and chronic obstructive pulmonary disease (COPD). The numbers of patients with these diagnoses were quite small, which preclude any firm conclusions regarding any effect. However, there was a trend – however not significant – that vitamin D treated patients with asthma produced fewer bacterial cultures (average 2.9 cultures/patient versus 7.0 cultures/patients, p=0.080, Figure S3) and fewer positive cultures than placebo treated asthmatics (average 0.6 positive cultures/patients versus 2.7/patient in the placebo group, p=0.052, Figure S3). In addition, vitamin D treated asthma patients showed significantly fewer cultures positive for fungi (candida and aspergillus) compared to placebo treated asthmatics (p=0.0476, table S5). For BE or COPD-patients there was no clear trend or significant effect in bacterial cultures or microbiology.

Levels of antimicrobial peptides (AMPs) in nasal fluid

There was no statistically significant difference between the vitamin D_3 or placebo groups when nasal fluids were analyzed for the presence of AMPs. Initially, levels of both LL-37 and HNP1-3 tended to be higher in the placebo group (Figure S3, panels A and B). However, after 12 months the microbiological pattern was reversed and no primary pathogens could be

detected in nasal swabs from vitamin D_3-treated patients (n=25, p=0.039) (Figure S4, panel C). The placebo-treated patients exhibited the same mix between normal flora and primary pathogens at all three sampling points (0, 6 and 12 months) (Figure S4, panel C).

SNP variants and treatment effect

Most genetic variants did not affect the primary endpoint. However, patients carrying the 'AA' genotype in the CYP2R1-gene, encoding the 25-hydroxylase enzyme, had a larger benefit of vitamin D_3-supplementation (-55%) compared to AG or GG carriers (-6%) (n=124, p=0.046 for interaction, Table S6).

Adverse events

In total, the vitamin D_3 group reported 38 adverse events (AEs) versus 56 AEs in the placebo group. The most common symptoms in the treatment group were headache (n=5) and lumbago (n=5), whereas placebo-treated patients reported paresthesias (n=8), diverticulitis (n=4) and urinary tract infection (n=4) as most frequent AEs (Table 5, Table S7). There was a general trend towards the number of adverse events being higher in the placebo group. Significantly more patients in the placebo group reported cardiovascular problems, such as heart failure, hypertonia and thrombosis (p=0.028). For gastrointestinal and other (non-respiratory) infections there was also a trend favoring the vitamin D_3 group (p=0.058 and p=0.09, respectively). No clinically relevant changes in serum levels of calcium, phosphate, creatinine or albumin could be observed (Figure S5). There was one severe adverse event (SAE) in each group (rabdomyosarcoma in the vitamin D_3 group and lung bleeding in the placebo group), both judged as being unrelated to the study drug.

Discussion

The main conclusion from this long-term randomized controlled trial (RCT) is that vitamin D_3 supplementation reduces the total burden of respiratory tract infections. The primary endpoint was composed of five different parameters that patients recorded daily throughout the study year. All point estimates favoured the vitamin D_3 group and a statistically significant effect was seen on both the total score and on the probability of receiving antibiotics. The effect on the infectious score was evident both in analysis per-protocol and according to intention-to-treat, and withstood adjustment for potential confounders. In addition, the number of bacterial cultures and microbiological findings was significantly reduced in the intervention group. These findings are potentially important and support that Vitamin D_3 supplementation may prevent respiratory tract infections and reduce antibiotic consumption, particularly in patients with hypogammaglobulinemia or with an increased frequency of respiratory tract infections.

However, our study has several limitations: Firstly, the choice of primary endpoint may be questioned since it relies solely on patient-reported information. To compensate for inherent problems with patient-reported data, the evaluation instrument was designed to cover many aspects of an infectious episode, including various symptoms as well as antibiotic consumption. Together the reported data formed an "infectious score", which constituted the primary endpoint of the study. Similar composite scores have successfully been applied to different diseases, such as tuberculosis (TB-score [16]), pneumonia (CURB-65 [17]) and bacterial meningitis (BMS-score [18]). Notably, vitamin D supplementation had a major effect on the odds of taking antibiotics during the study period (a reduction by 63.5%). In addition, the absolute number of days on antibiotics was reduced by 50% (from 33 days in the placebo group to 16 days in the intervention group), which was statistically significant both in the adjusted and unadjusted analyses (table 2). However, despite the relatively modest reduction for the other components of the primary endpoint the overall infectious score was

significantly reduced – mainly as a result of the large effect on the antibiotic parameter - both in the unadjusted and in the adjusted analyses (table 2, figure 2). It is important to interpret the statistical significance in the light of our power calculation, which was based on a significance level of p=0.02. This unusual significance level was chosen as a means of accounting for uncertainties in the power calculation assumptions, thereby asserting a sample size large enough to produce results significant at the p=0.05 without an unacceptably high risk of a type II error. However, we have used the widely accepted and conventionally used significance level p=0.05 in the statistical analyses, although this is not in full accordance with the significance level mentioned in the power calculation. Another potential problem was that the patient population was very heterogeneous with regards to immune deficiency and concomitant diseases. We adjusted for these factors in the multivariable analyses of the primary endpoint, but the sample sizes in each subgroup were too small to draw any conclusions of effects in specific disease groups. However, a detailed *post-hoc* analysis of the relation between immunological diagnosis, concomitant lung-disease and the secondary endpoints "taken bacterial cultures", "positive bacterial" cultures and "microbiological findings" was performed. There was a clear trend that Vitamin D treated patients with subclass deficiency and/or asthma produced fewer bacterial cultures, fewer positive cultures and fewer fungal cultures (tables S4 and S5, Figure S3). Although this analysis may lack precision by the small number of patients included, it could have clinical implications regarding target groups for vitamin D supplementation.

Nevertheless, our double-blind RCT has several strengths. For example, we chose a high daily dose of vitamin D_3 based on published calculations on metabolism and effects on immunity [14 19]. Other RCTs using lower doses of vitamin D_3, 400-2000 IU/day, have mainly been negative with regards to prevention of infections [20 21]. However, one study using 1200 IU/day showed a significant reduction of influenza among school children in Japan [22]. Notably, also studies

using higher doses of vitamin D_3 have been negative. Martineau *et al* used 400,000 IU vitamin D_3 during 42 days (9523 IU/day) with the aim of shortening time to sputum conversion in tuberculosis. No significant effect on the primary endpoint could be observed in that study, except in a subgroup with the *tt* genotype in the vitamin D receptor gene [23]. A recent study investigated whether 100,000 IU vitamin D_3/month (3333 IU/day) could reduce the incidence of chronic obstructive pulmonary disease (COPD) exacerbations. There was no significant effect on the primary endpoint, although a *post hoc* analysis revealed that patients with a low vitamin D_3 level at baseline had a significant effect of Vitamin D_3 supplementation[24].

Importantly, our study is the first to utilize high daily doses for an extended period of one full year. Thus, we covered all four seasons, which was important in Sweden with a known seasonal variation in 25-hydroxyvitamin D_3 levels [25]. Two previous RCTs were performed during the winter season – when vitamin D levels are low – but only during 4 [22] and 6 months [20], respectively. Previous RCTs have been conducted during shorter periods; 42 days [23], 6 weeks [26] and 12 weeks [21], respectively. Interestingly, we observed a clear time dependent effect suggesting that a long term supplementation approach (> 6 months) may be necessary to affect immunity. To expand on the results of a previous study in healthy individuals where no difference between the intervention and placebo groups was observed [21], we chose a study population with frequent RTIs and at least 42 days with infection during the year prior to inclusion. Notably, patients in the study represent a selected group of individuals with frequent RTI, although the immune disorders that they represent (IgA-, IgG-subclass and patients with no defined immune disorder) are generally mild in character and dominated by mucosal RTIs. We also included a small number of CVID-patients, which can be considered to be a more severe immune disorder, but all these patients are treated with IgG replacement therapy and thus well controlled. Hence, the results from this study cannot directly be applied

to the general healthy population. Nevertheless, the results provide solid support for additional interventional studies of vitamin D_3, especially in groups consuming large amounts of antibiotics.

The mechanism of the observed effects remains largely unknown. Vitamin D_3 modulates the immune response at many levels, such as induction of AMPs, skewing of T-cells from Th1/Th17 to Tregs as well as general anti-inflammatory effects [14]. Here, we investigated the role of AMPs in nasal fluid. However, we could not detect any significant changes of LL-37 or HNP1-3 during the study period, but noted that placebo-treated patients tended to have higher levels of AMPs after one year of treatment. This was paralleled by a shift of the microflora in the nasal compartment that could explain the unexpected finding of higher AMP-levels in the placebo group. Recently, it was shown that 1,25 $(OH)_2$-vitamin D_3 induces both HNP1-3 and LL-37 in nasal fluid of healthy volunteers (Hiemstra et al, abstract, European Respiratory Society, 2011), supporting that LL-37 may indeed be induced *in vivo*. However, our study design did not allow such conclusions but rather support that vitamin D_3 affect mucosal immunity, leading to a shift of the microflora. Recently, we showed that the bacterial composition in nasal swabs is an important determinant of AMP-levels in nasal fluid [27].

Given that vitamin D_3 induces LL-37 in epithelial cells and that LL-37 kills bacteria *in vitro,* we expected a reduction of the classical bacterial pathogens *H. influenza*, *M. catharralis* and *S. pneumonia* in the intervention group. However, the frequency of these bacteria was not reduced but a reduction of *S. aureus* and fungal species that often colonise the airways was observed. This could be explained by specific effects by vitamin D_3 on immunity against *S. aureus*. In fact, vitamin D induces human beta defensin-2 (HBD-2) with bactericidal activity against *S. aureus* [28]. A recent study showed that low vitamin D levels were associated with an increased risk of being colonised by this bacterium [29]. Further, vitamin D affects immunity

against *C. albicans*, which indicates direct effects of vitamin D on human immunity [30]. Alternatively, it is possible that vitamin D_3 may have prevented symptomatic viral infections, which prompted patients to leave a bacterial sample from the airways. Interestingly, there is both mechanistic and clinical evidence that vitamin D_3 can prevent viral infections [31-33], although we did not address this in the current study.

Notably, we observed a prominent increase in the serum concentration of 25-hydroxyvitamin D_3, which indicated good compliance and tolerability of the study drug. In fact, there was a trend towards adverse events being reported more often in the placebo group, suggesting that vitamin D_3 possibly could be efficient against other diseases, but this observation requires further studies. No clinically relevant changes of blood chemistry (calcium, phosphate, albumin or creatinine) were observed. Despite few adverse events and high tolerability, 16 exclusions occurred during the study year. The main reason was problems to adhere to the protocol and 6/16 patients dropped out of the study after a few weeks. The rest failed to send in the diaries, did not leave blood for monitoring of safety parameters or did not take the study drug. One patient was excluded based on symptoms that could be attributed to vitamin D_3 (facial paraesthesia). However, this patient was later confirmed to have been allocated to placebo.

In summary, we found that supplementation with vitamin D_3 reduced the total infectious score with 47 points per patient (23% reduction in the adjusted analysis) during the study year. The observed reduction was lower than the assumed reduction of 70 points per patient (predefined assumption: 210 points => 140 points; a reduction of 30%) that formed the basis for the power calculation. However, despite the predefined level of a reduction of infectious score by 30% as a clinically meaningful effect, we believe that effects lower than this also could be relevant for the individual patient. We base this line of reasoning on the fact that a reduction of 47 points per patient can be translated into 47 days with cough (47 points), 23 days with ear

and sinus symptoms (23×2=46 points) or 9 days with cough, sinus and ear symptoms together with malaise and antibiotics (9×5=45 points). In addition, our data indicate that vitamin D_3 supplementation reduces the odds of taking antibiotics by approximately 60% in patients with frequent respiratory tract infections. Thus, supplementation with vitamin D_3 could provide a novel strategy to reduce antibiotic use among high consumers and indirectly prevent the emerging epidemic of bacterial resistance.

Acknowledgment

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Data sharing statement:

There is no additional data available.

Statements

Author contributions:

Peter Bergman, designed the study, collected, analysed and interpreted data, wrote the paper.

Anna-Carin Norlin, designed the study, collected and interpreted data, wrote the paper.

Susanne Hansen, designed and coordinated the study, collected and interpreted data.

Rokeya Sultana Rekha, carried out experimental work, analysed data

Birgitta Agerberth, analysed and interpreted data, wrote the paper.

Linda Björkhem-Bergman, analysed and interpreted data, wrote the paper.

Lena Ekström, analysed and interpreted data

Jonatan Lindh, analysed and interpreted data, wrote the paper.

Jan Andersson, designed the study, interpreted data, wrote the paper.

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Merck GmbH provided the study drug (Vigantol) but did not have any influence on study design, analysis of data, writing or decision to publish.

Ethics statement: The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants.

Conflicts of interest: There are no conflicts of interest.

Legends to figures

Figure 1. Study outline.

Figure 2. Primary endpoint. The adjusted total relative infectious score (A) is expressed 'per quarter' (3 month periods). The adjusted one-year scores (total score, airway, malaise, ear, sinus and antibiotics) are depicted in a Forrest-plot (B) together with 95% confidence intervals. Effects are presented as relative scores (total score, airway, malaise) or odds ratios (ear, sinus, antibiotics, indicated with asterisks).

Figure 3. Secondary endpoint. Vitamin D-levels. Serum was collected at day 0, 3, 6, 9 and 12 months and levels of 25-hydroxyvitamin D_3 were measured. Values are expressed as mean +/- 95% confidence interval.

Tables

Table 1. Baseline data. Mann Whitney U-test was used for comparisons of age and 25-OH vitamin D_3. Fisher's exact test was used for all other comparisons. 1) "other disease" includes hypertension, body pain, hypothyroidism and gastritis as most common diagnoses. CVID, common variable immuno deficiency; ND, increased susceptibility to infections without a defined immunological disorder; BE, bronchiectasis; COPD, chronic obstructive pulmonary disease.

	Vitamin D_3	Placebo
Number	70	70
Age (mean)	55,4	50,8
Female	52/70	50/70
Male	18/70	20/70
IgG-replacem.	39/70	42/70
Smoking	4/70	6/70
25-OH levels (mean)	51,5 nmol/L	46,9 nmol/L
Immunological diagnosis		
sIgA-deficiency	9/70	9/70
IgG subclass	27/70	30/70
CVID	6/70	4/70
ND	28/70	27/70
Concomitant disease		
No other disease	16/70	18/70
Lung: Asthma	27/70	25/70
Lung: BE	5/70	7/70
Lung: COPD	5/70	4/70
Other disease[1]	17/70	16/70

Table 2. Primary Endpoint. Treatment effect calculated as the ratio between infectious scores in the vitamin D_3 and the placebo groups. Due to low frequencies, endpoints marked with asterisks were coded as binary outcomes (i.e. present or absent in each patient) and compared by means of logistic regression. In these cases, the effect refers to odds ratios of experiencing the outcome at least once during the course of the study. (The data are based on n=124 patients).

	Univariable regression model (unadjusted values)			Multiple regression model (adjusted values)		
Endpoint	**Effect**	**95% CI**	**p-value**	**Effect**	**95% CI**	**p-value**
Total score	0.754	0.591-0.963	**0.024**	0.771	0.604-0.985	**0.040**
Airway	0.857	0.697-1.053	0.141	0.871	0.706-1.074	0.200
Ear*	0.721	0.352-1.465	0.367	0.695	0.320-1.501	0.357
Sinus*	0.583	0.280-1.198	0.144	0.594	0.265-1.328	0.204
Malaise	0.845	0.692-1.032	0.098	0.845	0.689-1.036	0.108
Antibiotics*	0.355	0.154-0.784	**0.012**	0.365	0.153-0.872	**0.023**

Table 3. Bacterial cultures. [1]Mann-Whitney U-test, [2]Fisher's exact test.

	Vitamin D_3	Placebo	Significance
Number of samples per patient (mean, n=62/62)	2.79	4.85	p=0.010[1]
Number of positive samples per patient (mean, n=62/62)	1.01	2.02	p=0.052[1]
Fraction positive cultures (%)	63/173 (36%)	125/301 (41%)	P=0.28[2]
Patients with ≥ 1 sample taken	38/62 (61%)	50/62 (81%)	p=0.029[2]

Table 4. Microbiological findings. Mann-Whitney U-test was used to analyze the total number of findings, whereas Fisher's exact test was used for analysis of the number of patients (fraction) with a specific finding.

	Number of findings (total)			Number of patients		
Microorganism	Vitamin D_3	Placebo	MW-U	Vitamin D_3	Placebo	Fisher
H. influenzae	28	27	P=0.46	10/62	13/62	P=0.64
M. catharralis	8	17	P=0.39	7/62	10/62	P=0.60
S. pneumoniae	7	6	P=0.74	4/62	5/62	P=1.00
S. aureus	6	33	P=0.010	4/62	14/62	p=0.019
Enterobacteriacae	8	8	P=0.39	4/62	7/62	P=0.53
P. aeruginosa	8	15	P=0.68	3/62	4/62	P=1.00
Fungal infection	11	53	P=0.028	4/62	12/62	p=0.058
Total	76	159	P=0.023			

Table 5. Adverse events. Number of reports. Fisher's exact test was used for between group comparison. (The data are based on AE-reports from n=62 patients/arm).

Organ	Vitamin D_3 n (%)	Placebo n (%)	P-value
CNS	11 (29)	10 (18)	1.00
Gastrointestinal	4 (11)	12 (21)	**0.058**
Cardiovascular	0 (0)	6 (11)	**0.028**
Infections (other than RTI)	2 (5)	8 (14)	**0.09**
Musculoskeletal	10 (26)	10 (18)	1.00
Respiratory (non-infectious)	2 (5)	4 (7)	0.68
Skin	5 (13)	2 (4)	0.44
Other	4 (10)	4 (7)	1.00
Total	**38**	**56**	

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Vitamin D_3 supplementation in patients with frequent respiratory tract infections

- A randomized and double blind intervention study

Peter Bergman*[1,3,6#], Anna-Carin Norlin*[2,3], Susanne Hansen[3], Rokeya Sultana Rekha[4], Birgitta Agerberth[4], Linda Björkhem-Bergman[5], Lena Ekström[5], Jonatan D Lindh[5], Jan Andersson[6]

1. Department of Laboratory Medicine, Clinical Microbiology, Karolinska Institutet
2. Department of Laboratory Medicine, Clinical Immunology, Karolinska Institutet
3. Karolinska University Hospital, Clinical Immunology, Immunodeficiency Unit
4. Department of Medical Biochemistry and Biophysics, Karolinska Institutet
5. Department of Laboratory Medicine, Clinical Pharmacology, Karolinska Institutet
6. Department of Medicine, Center for Infectious Medicine (CIM), Karolinska Institutet

*Equal contribution

Positions:

Peter Bergman, MD, PhD, Resident Physician, Clinical Microbiology

Anna-Carin Norlin, MD, Specialist in Clinical Immunology

Susanne Hansen, RN, Head of the Immunodeficiency Unit

Rokeya Sultana Rekha, MSc, PhD-student

Birgitta Agerberth, PhD, Professor, Medical Microbial Pathogenesis

Linda Bjorkhem-Bergman, MD, PhD, Resident Physician, Clinical Pharmacology

Lena Ekström, PhD, Associate Professor, Clinical Pharmacology

Jonatan D Lindh, MD, PhD, Specialist in Clinical Pharmacology

Jan Andersson, MD, PhD, Professor, Infectious Diseases

Running title: Vitamin D_3 supplementation in patients with frequent respiratory tract infections

#Corresponding author:

Peter Bergman, MD, PhD

Department of Laboratory Medicine, Clinical Microbiology F68

Karolinska Institutet and Karolinska University Hospital, Huddinge

SE-141 86 Stockholm, Sweden

peter.bergman@ki.se

Phone: +46858587942

Fax: +46858581305

Competing interest statement

All authors have completed the Unified Competing Interest form (available on request from the corresponding author) and declare no support from any organisation for the submitted work, no financial relationships with any organisations that might have an interest in the submitted work in the previous three years and no other relationships or activities that could appear to have influenced the submitted work.

Article Summary

Article focus

• Recent evidence suggests that vitamin D_3 has potent extra-skeletal effects, such as suppression of inflammation and strengthening of mucosal immunity by induction of antimicrobial peptides.

• Data from observational studies suggest that low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections.

• Results from a limited number of randomized controlled trials on the protective role of vitamin D_3 against respiratory tract infections are inconclusive and thus additional studies are warranted.

Key Messages

• Therefore we designed and carried out a randomized controlled trial where a large dose (4000 IU) of vitamin D_3 was given to patients with an increased susceptibility to infections for one year.

• The main conclusion is that vitamin D_3 supplementation reduces symptoms and antibiotic consumption among patients with an increased frequency of respiratory tract infections. Thus, vitamin D_3 supplementation may be an alternative strategy to reduce antibiotic use among patients with recurrent respiratory tract infections.

Strengths and limitations

• Strengths: A high daily dose of vitamin D3 was used, the study time was a full year covering all seasons and patient with an increased frequency of respiratory tract infections were studied.

• Limitations: A single study center, small sample size (n=140) and a selected group of patients.

Abstract

Background: Low serum levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections (RTIs). Clinical trials with vitamin D_3 against various infections have been carried out but data are so far not conclusive. Thus, there is a need for additional randomized controlled trials of effects of vitamin D_3 on infections.

Objective: To investigate if supplementation with vitamin D_3 could reduce infectious symptoms and antibiotic consumption among patients with antibody deficiency or frequent RTIs.

Design: A double-blind randomized controlled trial.

Setting: Karolinska University Hospital, Huddinge

Participants: 140 patients with antibody deficiency (sIgA-, IgG subclass deficiency, CVID) and patients with increased susceptibility to RTIs (>4 bacterial RTIs/year) but without immunological diagnosis.

Intervention: vitamin D3 (4000 IU) or placebo was given daily for one year

Primary and secondary outcome measures: The primary endpoint was an infectious score based on five parameters: symptoms from respiratory tract, ears and sinuses, malaise and antibiotic consumption. Secondary endpoints were serum levels of 25-hydroxyvitamin D_3, microbiological findings and levels of antimicrobial peptides (LL-37, HNP1-3) in nasal fluid.

Results: The overall infectious score was significantly reduced for patients allocated to the vitamin D-group (202 points) compared with the placebo group (249 points) (adjusted relative score 0.771, 95% CI 0.604-0.985, p=0.04).

Limitations: A single study center, small sample size and a selected group of patients. The sample size calculation was performed using p=0.02 as the significance level whereas the primary and secondary endpoints were analyzed using the conventional p=0.05 as the significance level.

Conclusions: Supplementation with vitamin D_3 may reduce disease burden in patients with frequent respiratory tract infections.

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Introduction

Vitamin D was discovered when it was noted that rachitic children were improved by exposure to sunlight [1]. It was later shown by Holick *et al* that vitamin D_3 is synthesized in the skin under the influence of UVB-light [2]. Vitamin D_3 is further hydroxylated in the liver to 25-hydroxyvitamin D_3, which is considered to reflect the vitamin D status of an individual patient [3]. The final activation to the active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)D_3$) requires 1-α-hydroxylase activity. This enzyme (also designated CYP27B1) is expressed in the kidney but also in many other cell-types, including epithelial- and immune-cells [4]. The active vitamin D_3 ($1,25(OH)D_3$) binds to the vitamin D receptor (VDR), which belongs to the nuclear receptor family. Active vitamin D_3 is only present in minute amounts in the circulation and local activation in target cells is crucial for vitamin D-mediated effects on the immune system[5].

Low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of tuberculosis [6-8] and respiratory tract infections [9]. The mechanism is not fully elucidated but vitamin D_3 has been shown to induce antimicrobial peptides in immune cells [10]. In addition, active vitamin D_3 ($1,25(OH)D_3$) has broad anti-inflammatory effects on the adaptive immune system by shifting the T-helper cell pool from a Th1/Th17-response to a Th2/Treg-dominated response [11 12]. Vitamin D_3 has also been shown to suppress the Th2-response in allergic bronchopulmonary aspergillosis [13]. Thus, vitamin D_3 modulates both the adaptive and innate immune system [14]. The bulk of data on vitamin D_3 and infections stems from *in vitro* experiments and retrospective observational studies. Results from randomized controlled trials where the effects of vitamin D_3 on infections have been investigated (reviewed in Yamshchikov et al. [15]) are not conclusive and larger clinical trials are therefore warranted.

We designed a study to test the hypothesis that 4000 IU of vitamin D_3 given daily to patients with antibody deficiency and frequent respiratory tract infections for one year could prevent

or ameliorate infections. In addition, we investigated whether genetic polymorphisms in genes involved in the effect and/or metabolism of vitamin D_3 have an influence on the outcome of vitamin D_3 supplementation.

Methods

Study design

A prospective, randomized, double-blind placebo-controlled study of vitamin D_3 supplementation in patients with an increased susceptibility to respiratory tract infections. The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants. The study was registered at www.clinicaltrials.gov prior to inclusion of the first patient (NCT01131858). The EudraCT number is 2009-011758-16. The full protocol is available from the corresponding author upon request.

Sample size calculation

The sample size was based on the assumption that the intervention would reduce the number of days with symptoms from 42 days (210 points) to 28 days (140 points), i.e. a reduction of the infectious burden by 30%. Given this assumption, a sample size of 60 patients per study group was predicted to provide the study 90% power at a significance level of p=0.02 (Student's t-test). To compensate for predicted exclusion of participants, the groups were increased to include 70 patients per treatment arm. Importantly, the significance level of p=0.02 was chosen in the power calculation to ensure that a sufficient number of patients were recruited in order to avoid a type II error in the primary analysis. However, the conventional and widely accepted significance level of p=0.05 was used for statistical analyses of the primary and secondary endpoints.

Participants

Patients at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden, were included between March 2010 and June 2010 by the study nurses (SH, ML, KJ). Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infections; i.e. > 42 days with symptoms from the respiratory tract during a 12 months period prior to study inclusion. Patients registered at the Immunodeficiency Unit are closely followed with a diary of symptoms and antibiotic consumption. Thus, the patients are trained and used to apply such an instrument to assess their infectious status. Data from patients' standard diary was used as an instrument in order to assess patients for eligibility, both via telephone and by the responsible physician (PB, ACN) prior to inclusion. Patients with selective IgA-deficiency (**D80.2**), IgG-subclass deficiency (**D80.3**) and common variable immune disorder (CVID, **D83.0**) as well as patients without a defined immunological diagnosis (**D89.9**) were included. Exclusion criteria were prophylactic treatment with antibiotics, history of hypercalcemia or stones in the urinary tract, sarcoidosis, ongoing supplementation with vitamin D_3 exceeding 400 IU/day, HIV-infection and pregnancy.

Interventions

Patients were randomized to 12 months' treatment with Vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. One drop contained 500 IU vitamin D_3 or placebo oil (Miglyol oil®) and the participants were asked to take 8 drops daily. The participants had to mark their daily symptoms of infection in a diary, which was sent via regular mail to the study site every month. The following data was recorded: symptoms from the respiratory tract, ears and sinuses, treatment with antibiotics, numbers of bacterial cultures, times and reasons of visits to hospitals, frequency of travelling abroad and adherence to study drug.

Outcomes

The primary outcome was a composite infectious score, based on a daily patient-reported questionnaire, and included five parameters: symptoms from the respiratory tract, ears and sinuses, malaise and use of antibiotics (Figure S1), each parameter gave 1 point/day. The occurrence of X-ray verified pneumonia gave 3 additional points per day for a period of 7 days. Thus a pneumonia resulted in 3x7 points = 21 extra points. Patients were specifically instructed to record only symptoms related to ongoing respiratory tract infections. Symptoms related to infections at other sites (urinary tract, wounds etc) as well as non-infectious symptoms were reported as adverse events. Secondary outcomes were serum levels of 25-hydroxyvitamin D_3 (at baseline and after 3, 6, 9 and 12 months), numbers of bacterial cultures, microbiological findings and levels of antimicrobial peptides (LL-37 and HNP1-3) in nasal fluid (at baseline and after 6 and 12 months). In addition, 6 *post hoc* genotype analyses were performed in all participants. Analysis of single nucleotide polymorphisms (SNPs) were carried out for VDR (Taq1 and Foq1), CYP27B1, CYP24A1, CYP2R1 and Vitamin D binding protein (GC). Safety tests included plasma levels of creatinine, calcium, phosphate and albumin, measured at baseline and after 3, 6, 9 and 12 months. At inclusion, urine-HCG (human chorionic gonadotropin) in women was measured and p-parathyroid hormone in both genders. The results of the safety tests were reviewed by an independent and un-blinded consultant physician. Two blinded physicians (PB and ACN) were responsible for inclusion and all medical visits to the study site (Immunodeficiency Unit, Karolinska University Hospital, Huddinge).

Randomisation and statistical analysis

Participants were randomised to 12 months' treatment with vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. Block randomization with a block size of ten was used to ascertain equal group sizes. Staff at Karolinska Trial Alliance (KTA) was responsible for randomization procedures. In the statistical analysis, continuous variables were compared

using Mann-Whitney U test or linear regression and dichotomous variables by Fisher's exact test or logistic regression. Regressions of log-transformed infectious scores were performed both unadjusted (simple regression) and with adjustment for potential confounders (multiple regression).

Statistical methods: Primary analysis

The distribution of the infectious score was found to be skewed, thereby violating the normal assumption of the pre-specified t-test analysis. Hence, scores were log-transformed prior to analysis. Further, the randomisation had resulted in age distributions that were not entirely balanced between the two groups. Since there might be concerns that such imbalance could influence the results of the study, the original analysis plan was extended with a multivariable analysis adjusting for potential confounders. In this linear regression model based on log-transformed values of the primary outcome (the total infectious score) and its individual components, adjustment was made for age, gender, smoking, type of immune deficiency and significant co-morbidities (respiratory or non-respiratory). Due to the transformation procedure, the adjusted effect of vitamin D_3 is expressed as a ratio between the score in the vitamin D_3 and the placebo group. In this multiplicative model, an effect size of 1 indicates identical outcome in the two study groups and statistically non-significant results are recognized by confidence intervals encompassing the value 1.

To explore potential divergent effects on different organ systems, both adjusted and unadjusted analyses were repeated separately for each individual item of the infectious score. In addition, the temporal aspects of the vitamin D_3 effect were investigated by dividing the

study period into four 90-day periods (starting on the first day of treatment) and repeating the analyses separately for each time period. “Ear” and “sinus” symptoms as well as “antibiotic use” occurred at low frequencies and for these entities normal distributions could not be achieved despite data transformation. Thus, the adjusted analyses of these individual items were based on multivariable logistic regression, after coding the symptom (or antibiotic therapy) as present or absent during the course of the study. However, this only applies to analysis of the individual items, and not to the primary analysis of the total infectious score, where all item scores were added as originally described.

Most post-randomization exclusions were due to patients failing to fill out the symptoms diary. Hence, no intention to treat (ITT) analysis based on actual outcome data could be performed. However, the potential impact of dropouts was addressed in an ITT analysis based on multiple imputation of missing outcome data. In the imputation process, pooled estimates were derived from 100 datasets created by means of multivariate imputation by chained equations and predictive mean matching for the same covariates as in the adjusted per-protocol analysis

Detailed descriptions of randomisation and blinding, sampling of nasal fluid, measurement of antimicrobial peptides, measurement of 25-hydroxyvitamin D_3, genotyping and statistical analyses of secondary outcomes including sample size calculations are presented in the Supplementary Methods Section.

Results

Baseline data

A total of 286 patients were first assessed for eligibility but 144 were not included because they did not fulfill all inclusion criteria; <42 days with infection/year (n=35), lacked other inclusion criteria (n=42), or declined to participate (n=67). The remaining 142 patients were further screened and 140 patients were included in the study. Of these, 70 were randomized to vitamin D_3 supplementation and 70 to placebo (Figure 1). The groups did not differ with regards to gender, IgG replacement therapy, smoking, baseline 25-hydroxyvitamin D_3 levels, type of immune defect or co-morbidities. Patients with subclass deficiency, selective IgA deficiency (sIgA), common variable immune deficiency (CVID) and patients without a defined immunological diagnosis (ND) but with >4 bacterial respiratory tract infections/year were included. IgG replacement therapy was most common in the CVID-group (100%) and in the subclass deficiency group (63%), but also frequent in the other groups (ND, 54% and sIgA, 38%, table S1). Patients allocated to the placebo group were slightly younger than patients in the treatment group (p=0.025, data not shown). During the course of the study, 16 patients left the study prematurely (8 patients from each study group) and consequently 124 patients were included in the main per-protocol analysis. Reasons for dropout included elevated PTH (n=2), withdrawn consent (n=5), adverse event (n=1), prescription of vitamin D outside the study (n=1), failure to complete diary (n=4) or non-compliance to study medication (n=3) (Figure 1).

Primary endpoint: Infectious score

One year of vitamin D_3 treatment was associated with a significantly reduced total infectious score both in the unadjusted (n=124, p=0.024, Table 2) and the adjusted analyses (n=124,

p=0.040) (Table 2, Figure 2A, B and Table S2). The unadjusted relative score in the intervention group was 0.754 (95% c.i. 0.591-0.963, p=0.024, n=124) corresponding to a 25% reduction and after adjustment for potential confounders, the relative score was 0.771 (95% c.i. 0.604-0.985, p=0.04), corresponding to a 23% reduction (Table 2). According to the temporal analysis, the effect of vitamin D_3 supplementation tended to improve with time (Figure 2A). The absolute unadjusted score per patient was 202 points for the vitamin D group and 249 points for the placebo group, which was a significant reduction of 47 points per patient (p=0.023, Mann Whitney U-test, table S3).

When the individual items of the infectious score were analyzed separately, all point estimates indicated a reduction in the treatment group (Table 2, figure S2), although only antibiotic consumption reached statistical significance (Figure 2B and S2, panel E). The adjusted OR for antibiotic use was 0.365 (95% c.i. 0.153-0.872, p=0.023, n=124), i.e. a 63.5% reduction of the odds of antibiotic use in the intervention group (Table 2). The absolute values were 33 days on antibiotics for the placebo group and 16 days for the vitamin D_3 group, i.e. a reduction of 17 days in the vitamin D_3 group (table S3). The temporal trends for specific symptoms and antibiotic consumption were similar to the total score and reached statistical significance for 'ear'-symptoms (n=124, p=0.041) and for 'malaise' (n=124, p=0.053) in the final quarter of the study (Figure 2S, panels B and C).

Analyzing the primary outcome according to intention-to-treat (n=170) produced results virtually identical to those of the per-protocol analysis. In the unadjusted ITT analysis, vitamin D_3 reduced the total infectious score by 25% (relative score 0.752, 95% c.i. 0.588-0.962, p=0.024) and after adjustment for potential confounders the reduction was 23% (relative score 0.767, 95% c.i. 0.599-0.982, p=0.036).

Serum levels of 25-OH vitamin D_3

Serum 25-hydroxyvitamin D_3 levels did not differ between the groups at baseline (Table 1) but already after 3 months the intervention group had a significantly higher level of 25-hydroxyvitamin D_3 (133.4 nmol/L versus 66.6 nmol/L, p<0.001, Figure 3). This increase remained throughout the study (Figure 3).

Bacterial cultures and microbiology

During the course of the study, 173 microbiological samples were obtained in the vitamin D_3 group (n=62, 2.79/patient) and 301 in the placebo group (n=62, 4.85/patient) (p=0.010, Table 3). The number of samples with at least one positive finding was higher in the placebo group, with close to statistical significance (p=0.052), while the fraction of positive samples was similar for both groups (Table 3). Significantly more patients had a microbiological sample taken from the respiratory tract (≥ 1 sample) during the study period in the placebo group; OR 2.63 (95% CI 1.17-5.92), (Table 3).

In total, the vitamin D_3 group generated 76 positive microbiological findings (bacteria or fungi), compared to 159 in the placebo group (p=0.023). There was no difference between the groups for the traditional respiratory pathogens (*H. influenza, M. catharralis and S. pneumonia*), but there were significantly fewer findings of *S. aureus* (p=0.019) and fungi (p=0.028, *Candida* spp. and *Aspergillus* spp.) in the treatment group (Table 4). Likewise, significantly fewer vitamin D_3-treated patients had a bacterial culture positive for *S. aureus* (p=0.019) or fungal species (p=0.058), although the latter difference did not reach statistical significance (Table 4).

Vitamin D treated patients with sub-class deficiency left significantly fewer bacterial or fungal cultures than placebo-treated patients with this diagnosis; 7 cultures in the vitamin D group (n=22) versus 47 cultures in the placebo group (n=24) (Table S4). Also the number of patients that had ≥1 bacterial culture taken was significantly fewer in the placebo group (12/22 versus 22/24, p=0.0065, table S4). There was no significant effect of other immunological diagnoses on bacterial cultures or microbiology (Table S4).

Since concomitant lung disease may be an important factor for vitamin D mediated effects on respiratory immunity, we performed a detailed analysis of bacterial cultures and microbiology of patients with asthma, bronchiectasis (BE) and chronic obstructive pulmonary disease (COPD). The numbers of patients with these diagnoses were quite small, which preclude any firm conclusions regarding any effect. However, there was a trend – however not significant – that vitamin D treated patients with asthma produced fewer bacterial cultures (average 2.9 cultures/patient versus 7.0 cultures/patients, p=0.080, Figure S3) and fewer positive cultures than placebo treated asthmatics (average 0.6 positive cultures/patients versus 2.7/patient in the placebo group, p=0.052, Figure S3). In addition, vitamin D treated asthma patients showed significantly fewer cultures positive for fungi (candida and aspergillus) compared to placebo treated asthmatics (p=0.0476, table S5). For BE or COPD-patients there was no clear trend or significant effect in bacterial cultures or microbiology.

Levels of antimicrobial peptides (AMPs) in nasal fluid

There was no statistically significant difference between the vitamin D_3 or placebo groups when nasal fluids were analyzed for the presence of AMPs. Initially, levels of both LL-37 and HNP1-3 tended to be higher in the placebo group (Figure S3, panels A and B). However, after 12 months the microbiological pattern was reversed and no primary pathogens could be

detected in nasal swabs from vitamin D_3-treated patients (n=25, p=0.039) (Figure S4, panel C). The placebo-treated patients exhibited the same mix between normal flora and primary pathogens at all three sampling points (0, 6 and 12 months) (Figure S4, panel C).

SNP variants and treatment effect

Most genetic variants did not affect the primary endpoint. However, patients carrying the 'AA' genotype in the CYP2R1-gene, encoding the 25-hydroxylase enzyme, had a larger benefit of vitamin D_3-supplementation (-55%) compared to AG or GG carriers (-6%) (n=124, p=0.046 for interaction, Table S6).

Adverse events

In total, the vitamin D_3 group reported 38 adverse events (AEs) versus 56 AEs in the placebo group. The most common symptoms in the treatment group were headache (n=5) and lumbago (n=5), whereas placebo-treated patients reported paresthesias (n=8), diverticulitis (n=4) and urinary tract infection (n=4) as most frequent AEs (Table 5, Table S7). There was a general trend towards the number of adverse events being higher in the placebo group. Significantly more patients in the placebo group reported cardiovascular problems, such as heart failure, hypertonia and thrombosis (p=0.028). For gastrointestinal and other (non-respiratory) infections there was also a trend favoring the vitamin D_3 group (p=0.058 and p=0.09, respectively). No clinically relevant changes in serum levels of calcium, phosphate, creatinine or albumin could be observed (Figure S5). There was one severe adverse event (SAE) in each group (rabdomyosarcoma in the vitamin D_3 group and lung bleeding in the placebo group), both judged as being unrelated to the study drug.

Discussion

The main conclusion from this long-term randomized controlled trial (RCT) is that vitamin D_3 supplementation reduces the total burden of respiratory tract infections. The primary endpoint was composed of five different parameters that patients recorded daily throughout the study year. All point estimates favoured the vitamin D_3 group and a statistically significant effect was seen on both the total score and on the probability of receiving antibiotics ($p<0.05$). The effect on the infectious score was evident both in analysis per-protocol and according to intention-to-treat, and withstood adjustment for potential confounders. In addition, the number of bacterial cultures and microbiological findings was significantly reduced in the intervention group. These findings are potentially important and support that Vitamin D_3 supplementation may prevent respiratory tract infections and reduce antibiotic consumption, particularly in patients with hypogammaglobulinemia or with an increased frequency of respiratory tract infections.

However, our study has several limitations: Firstly, the choice of primary endpoint may be questioned since it relies solely on patient-reported information. To compensate for inherent problems with patient-reported data, the evaluation instrument was designed to cover many aspects of an infectious episode, including various symptoms as well as antibiotic consumption. Together the reported data formed an "infectious score", which constituted the primary endpoint of the study. Similar composite scores have successfully been applied to different diseases, such as tuberculosis (TB-score [16]), pneumonia (CURB-65 [17]) and bacterial meningitis (BMS-score [18]). Notably, vitamin D supplementation had a major effect on the odds of taking antibiotics during the study period (a reduction by 63.5%). In addition, the absolute number of days on antibiotics was reduced by 50% (from 33 days in the placebo group to 16 days in the intervention group), which was statistically significant both in the adjusted and unadjusted analyses (table 2). However, despite the relatively modest reduction

for the other components of the primary endpoint the overall infectious score was significantly reduced – mainly as a result of the large effect on the antibiotic parameter - both in the unadjusted and in the adjusted analyses (table 2, figure 2). It is important to interpret the statistical significance in light of our power calculation, which was based on a significance level of p=0.02. In the power calculation, the significance level was reduced from 0.05 to 0.02 in order to increase the statistical power at the p=0.05 level. This approach was incorrect, and the targeted power (at the p=0.05 level) should instead have been increased without altering the p-value threshold. However, we have used the widely accepted significance level p=0.05 in the statistical analyses for both the primary and secondary endpoints, respectively. Another potential problem was that the patient population was very heterogeneous with regards to immune deficiency and concomitant diseases. We adjusted for these factors in the multivariable analyses of the primary endpoint, but the sample sizes in each subgroup were too small to draw any conclusions of effects in specific disease groups. However, a detailed *post-hoc* analysis of the relation between immunological diagnosis, concomitant lung-disease and the secondary endpoints "taken bacterial cultures", "positive bacterial" cultures and "microbiological findings" was performed. There was a clear trend that Vitamin D treated patients with subclass deficiency and/or asthma produced fewer bacterial cultures, fewer positive cultures and fewer fungal cultures (tables S4 and S5, Figure S3). Although this analysis may lack precision by the small number of patients included, it could have clinical implications regarding target groups for vitamin D supplementation.

Nevertheless, our double-blind RCT has several strengths. For example, we chose a high daily dose of vitamin D_3 based on published calculations on metabolism and effects on immunity [14 19]. Other RCTs using lower doses of vitamin D_3, 400-2000 IU/day, have mainly been negative with regards to prevention of infections [20 21]. However, one study using 1200 IU/day showed a significant reduction of influenza among school children in Japan [22]. Notably, also studies

using higher doses of vitamin D_3 have been negative. Martineau *et al* used 400,000 IU vitamin D_3 during 42 days (9523 IU/day) with the aim of shortening time to sputum conversion in tuberculosis. No significant effect on the primary endpoint could be observed in that study, except in a subgroup with the *tt* genotype in the vitamin D receptor gene [23]. A recent study investigated whether 100,000 IU vitamin D_3/month (3333 IU/day) could reduce the incidence of chronic obstructive pulmonary disease (COPD) exacerbations. There was no significant effect on the primary endpoint, although a *post hoc* analysis revealed that patients with a low vitamin D_3 level at baseline had a significant effect of Vitamin D_3 supplementation[24].

Importantly, our study is the first to utilize high daily doses for an extended period of one full year. Thus, we covered all four seasons, which was important in Sweden with a known seasonal variation in 25-hydroxyvitamin D_3 levels [25]. Two previous RCTs were performed during the winter season – when vitamin D levels are low – but only during 4 [22] and 6 months [20], respectively. Previous RCTs have been conducted during shorter periods; 42 days [23], 6 weeks [26] and 12 weeks [21], respectively. Interestingly, we observed a clear time dependent effect suggesting that a long term supplementation approach (> 6 months) may be necessary to affect immunity. To expand on the results of a previous study in healthy individuals where no difference between the intervention and placebo groups was observed [21], we chose a study population with frequent RTIs and at least 42 days with infection during the year prior to inclusion. Notably, patients in the study represent a selected group of individuals with frequent RTI, although the immune disorders that they represent (IgA-, IgG-subclass and patients with no defined immune disorder) are generally mild in character and dominated by mucosal RTIs. We also included a small number of CVID-patients, which can be considered to be a more severe immune disorder, but all these patients are treated with IgG replacement therapy and thus well controlled. Hence, the results from this study cannot directly be applied

to the general healthy population. Nevertheless, the results provide solid support for additional interventional studies of vitamin D_3, especially in groups consuming large amounts of antibiotics.

The mechanism of the observed effects remains largely unknown. Vitamin D_3 modulates the immune response at many levels, such as induction of AMPs, skewing of T-cells from Th1/Th17 to Tregs as well as general anti-inflammatory effects [14]. Here, we investigated the role of AMPs in nasal fluid. However, we could not detect any significant changes of LL-37 or HNP1-3 during the study period, but noted that placebo-treated patients tended to have higher levels of AMPs after one year of treatment. This was paralleled by a shift of the microflora in the nasal compartment that could explain the unexpected finding of higher AMP-levels in the placebo group. Recently, it was shown that 1,25 $(OH)_2$-vitamin D_3 induces both HNP1-3 and LL-37 in nasal fluid of healthy volunteers (Hiemstra et al, abstract, European Respiratory Society, 2011), supporting that LL-37 may indeed be induced *in vivo*. However, our study design did not allow such conclusions but rather support that vitamin D_3 affect mucosal immunity, leading to a shift of the microflora. Recently, we showed that the bacterial composition in nasal swabs is an important determinant of AMP-levels in nasal fluid [27].

Given that vitamin D_3 induces LL-37 in epithelial cells and that LL-37 kills bacteria *in vitro,* we expected a reduction of the classical bacterial pathogens *H. influenza*, *M. catharralis* and *S. pneumonia* in the intervention group. However, the frequency of these bacteria was not reduced but a reduction of *S. aureus* and fungal species that often colonise the airways was observed. This could be explained by specific effects by vitamin D_3 on immunity against *S. aureus*. In fact, vitamin D induces human beta defensin-2 (HBD-2) with bactericidal activity against *S. aureus* [28]. A recent study showed that low vitamin D levels were associated with an increased risk of being colonised by this bacterium [29]. Further, vitamin D affects immunity

against *C. albicans*, which indicates direct effects of vitamin D on human immunity [30]. Alternatively, it is possible that vitamin D_3 may have prevented symptomatic viral infections, which prompted patients to leave a bacterial sample from the airways. Interestingly, there is both mechanistic and clinical evidence that vitamin D_3 can prevent viral infections [31-33], although we did not address this in the current study.

Notably, we observed a prominent increase in the serum concentration of 25-hydroxyvitamin D_3, which indicated good compliance and tolerability of the study drug. In fact, there was a trend towards adverse events being reported more often in the placebo group, suggesting that vitamin D_3 possibly could be efficient against other diseases, but this observation requires further studies. No clinically relevant changes of blood chemistry (calcium, phosphate, albumin or creatinine) were observed. Despite few adverse events and high tolerability, 16 exclusions occurred during the study year. The main reason was problems to adhere to the protocol and 6/16 patients dropped out of the study after a few weeks. The rest failed to send in the diaries, did not leave blood for monitoring of safety parameters or did not take the study drug. One patient was excluded based on symptoms that could be attributed to vitamin D_3 (facial paraesthesia). However, this patient was later confirmed to have been allocated to placebo.

In summary, we found that supplementation with vitamin D_3 reduced the total infectious score with 47 points per patient (23% reduction in the adjusted analysis) during the study year. The observed reduction was lower than the assumed reduction of 70 points per patient (predefined assumption: 210 points => 140 points; a reduction of 30%) that formed the basis for the power calculation. However, despite the predefined level of a reduction of infectious score by 30% as a clinically meaningful effect, we believe that effects lower than this also could be relevant for the individual patient. We base this line of reasoning on the fact that a reduction of 47 points per patient can be translated into 47 days with cough (47 points), 23 days with ear

and sinus symptoms (23×2=46 points) or 9 days with cough, sinus and ear symptoms together with malaise and antibiotics (9×5=45 points). In addition, our data indicate that vitamin D_3 supplementation reduces the odds of taking antibiotics by approximately 60% in patients with frequent respiratory tract infections. Thus, supplementation with vitamin D_3 could provide a novel strategy to reduce antibiotic use among high consumers and indirectly prevent the emerging epidemic of bacterial resistance.

Acknowledgment

The study was registered at www.clinicaltrials.gov (NCT01131858), prior to the start of the study. The entire process of study design and protocol, data monitoring and analyses was performed by academic authors; there was no industry involvement in the study except that vitamin D_3 (Vigantol®) and placebo oil (Miglyol®) were provided by Merck KGaA (Darmstadt, Germany). Merck did not have any influence on study design, analysis of data, writing or decision to publish. We extend our gratitude to Ilona Skilving, Karolinska Trial Alliance for invaluable help with the protocol. Further, we thank registered nurses Maria Lindén and Kristina Johansson for skillful work with patients. Thanks also to Jenny Lindén and Alicia Hansson for registration of data and to professor Mats Remberger, KI for discussions on statistical methods. Professor Lars Lindqvist, Department of Infectious Diseases, Karolinska University Hospital is gratefully acknowledged for serving as the monitor of the study. PB, LBB and JDL are holding PostDoc-positions financed by Karolinska Institutet and Stockholm County Council (KI/SLL).

Data sharing statement:

There is no additional data available.

Statements

Author contributions:

Peter Bergman, designed the study, collected, analysed and interpreted data, wrote the paper.

Anna-Carin Norlin, designed the study, collected and interpreted data, wrote the paper.

Susanne Hansen, designed and coordinated the study, collected and interpreted data.

Rokeya Sultana Rekha, carried out experimental work, analysed data

Birgitta Agerberth, analysed and interpreted data, wrote the paper.

Linda Björkhem-Bergman, analysed and interpreted data, wrote the paper.

Lena Ekström, analysed and interpreted data

Jonatan Lindh, analysed and interpreted data, wrote the paper.

Jan Andersson, designed the study, interpreted data, wrote the paper.

Role of the funding source

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Merck GmbH provided the study drug (Vigantol) but did not have any influence on study design, analysis of data, writing or decision to publish.

Ethics statement: The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants.

Conflicts of interest: There are no conflicts of interest.

Legends to figures

Figure 1. Study outline.

Figure 2. Primary endpoint. The adjusted total relative infectious score (A) is expressed 'per quarter' (3 month periods). The adjusted one-year scores (total score, airway, malaise, ear, sinus and antibiotics) are depicted in a Forrest-plot (B) together with 95% confidence intervals. Effects are presented as relative scores (total score, airway, malaise) or odds ratios (ear, sinus, antibiotics, indicated with asterisks).

Figure 3. Secondary endpoint. Vitamin D-levels. Serum was collected at day 0, 3, 6, 9 and 12 months and levels of 25-hydroxyvitamin D_3 were measured. Values are expressed as mean +/- 95% confidence interval.

Tables

Table 1. Baseline data. Mann Whitney U-test was used for comparisons of age and 25-OH vitamin D_3. Fisher's exact test was used for all other comparisons. 1) "other disease" includes hypertension, body pain, hypothyroidism and gastritis as most common diagnoses. CVID, common variable immuno deficiency; ND, increased susceptibility to infections without a defined immunological disorder; BE, bronchiectasis; COPD, chronic obstructive pulmonary disease.

	Vitamin D_3	Placebo
Number	70	70
Age (mean)	55,4	50,8
Female	52/70	50/70
Male	18/70	20/70
IgG-replacem.	39/70	42/70
Smoking	4/70	6/70
25-OH levels (mean)	51,5 nmol/L	46,9 nmol/L
Immunological diagnosis		
sIgA-deficiency	9/70	9/70
IgG subclass	27/70	30/70
CVID	6/70	4/70
ND	28/70	27/70
Concomitant disease		
No other disease	16/70	18/70
Lung: Asthma	27/70	25/70
Lung: BE	5/70	7/70
Lung: COPD	5/70	4/70
Other disease[1]	17/70	16/70

Table 2. Primary Endpoint. Treatment effect calculated as the ratio between infectious scores in the vitamin D_3 and the placebo groups. Due to low frequencies, endpoints marked with asterisks were coded as binary outcomes (i.e. present or absent in each patient) and compared by means of logistic regression. In these cases, the effect refers to odds ratios of experiencing the outcome at least once during the course of the study. (The data are based on n=124 patients).

	Univariable regression model (unadjusted values)			Multiple regression model (adjusted values)		
Endpoint	**Effect**	**95% CI**	**p-value**	**Effect**	**95% CI**	**p-value**
Total score	0.754	0.591-0.963	**0.024**	0.771	0.604-0.985	**0.040**
Airway	0.857	0.697-1.053	0.141	0.871	0.706-1.074	0.200
Ear*	0.721	0.352-1.465	0.367	0.695	0.320-1.501	0.357
Sinus*	0.583	0.280-1.198	0.144	0.594	0.265-1.328	0.204
Malaise	0.845	0.692-1.032	0.098	0.845	0.689-1.036	0.108
Antibiotics*	0.355	0.154-0.784	**0.012**	0.365	0.153-0.872	**0.023**

Table 3. Bacterial cultures. [1]Mann-Whitney U-test, [2]Fisher's exact test.

	Vitamin D_3	Placebo	Significance
Number of samples per patient (mean, n=62/62)	2.79	4.85	p=0.010[1]
Number of positive samples per patient (mean, n=62/62)	1.01	2.02	p=0.052[1]
Fraction positive cultures (%)	63/173 (36%)	125/301 (41%)	P=0.28[2]
Patients with ≥ 1 sample taken	38/62 (61%)	50/62 (81%)	p=0.029[2]

Table 4. Microbiological findings. Mann-Whitney U-test was used to analyze the total number of findings, whereas Fisher's exact test was used for analysis of the number of patients (fraction) with a specific finding.

	Number of findings (total)			Number of patients		
Microorganism	Vitamin D_3	Placebo	MW-U	Vitamin D_3	Placebo	Fisher
H. influenzae	28	27	P=0.46	10/62	13/62	P=0.64
M. catharralis	8	17	P=0.39	7/62	10/62	P=0.60
S. pneumoniae	7	6	P=0.74	4/62	5/62	P=1.00
S. aureus	6	33	P=0.010	4/62	14/62	p=0.019
Enterobacteriacae	8	8	P=0.39	4/62	7/62	P=0.53
P. aeruginosa	8	15	P=0.68	3/62	4/62	P=1.00
Fungal infection	11	53	P=0.028	4/62	12/62	p=0.058
Total	76	159	P=0.023			

Table 5. Adverse events. Number of reports. Fisher's exact test was used for between group comparison. (The data are based on AE-reports from n=62 patients/arm).

Organ	Vitamin D_3 n (%)	Placebo n (%)	P-value
CNS	11 (29)	10 (18)	1.00
Gastrointestinal	4 (11)	12 (21)	**0.058**
Cardiovascular	0 (0)	6 (11)	**0.028**
Infections (other than RTI)	2 (5)	8 (14)	**0.09**
Musculoskeletal	10 (26)	10 (18)	1.00
Respiratory (non-infectious)	2 (5)	4 (7)	0.68
Skin	5 (13)	2 (4)	0.44
Other	4 (10)	4 (7)	1.00
Total	**38**	**56**	

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Vitamin D_3 supplementation in patients with frequent respiratory tract infections

- A randomized and double blind intervention study

Peter Bergman*[1,3,6#], Anna-Carin Norlin*[2,3], Susanne Hansen[3], Rokeya Sultana Rekha[4], Birgitta Agerberth[4], Linda Björkhem-Bergman[5], Lena Ekström[5], Jonatan D Lindh[5], Jan Andersson[6]

1. Department of Laboratory Medicine, Clinical Microbiology, Karolinska Institutet
2. Department of Laboratory Medicine, Clinical Immunology, Karolinska Institutet
3. Karolinska University Hospital, Clinical Immunology, Immunodeficiency Unit
4. Department of Medical Biochemistry and Biophysics, Karolinska Institutet
5. Department of Laboratory Medicine, Clinical Pharmacology, Karolinska Institutet
6. Department of Medicine, Center for Infectious Medicine (CIM), Karolinska Institutet

*Equal contribution

Positions:

Peter Bergman, MD, PhD, Resident Physician, Clinical Microbiology

Anna-Carin Norlin, MD, Specialist in Clinical Immunology

Susanne Hansen, RN, Head of the Immunodeficiency Unit

Rokeya Sultana Rekha, MSc, PhD-student

Birgitta Agerberth, PhD, Professor, Medical Microbial Pathogenesis

Linda Bjorkhem-Bergman, MD, PhD, Resident Physician, Clinical Pharmacology

Lena Ekström, PhD, Associate Professor, Clinical Pharmacology

Jonatan D Lindh, MD, PhD, Specialist in Clinical Pharmacology

Jan Andersson, MD, PhD, Professor, Infectious Diseases

Running title: Vitamin D_3 supplementation in patients with frequent respiratory tract infections

#Corresponding author:

Peter Bergman, MD, PhD

Department of Laboratory Medicine, Clinical Microbiology F68

Karolinska Institutet and Karolinska University Hospital, Huddinge

SE-141 86 Stockholm, Sweden

peter.bergman@ki.se

Phone: +46858587942

Fax: +46858581305

Competing interest statement

All authors have completed the Unified Competing Interest form (available on request from the corresponding author) and declare no support from any organisation for the submitted work, no financial relationships with any organisations that might have an interest in the submitted work in the previous three years and no other relationships or activities that could appear to have influenced the submitted work.

Article Summary

Article focus

• Recent evidence suggests that vitamin D_3 has potent extra-skeletal effects, such as suppression of inflammation and strengthening of mucosal immunity by induction of antimicrobial peptides.

• Data from observational studies suggest that low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections.

• Results from a limited number of randomized controlled trials on the protective role of vitamin D_3 against respiratory tract infections are inconclusive and thus additional studies are warranted.

Key Messages

• Therefore we designed and carried out a randomized controlled trial where a large dose (4000 IU) of vitamin D_3 was given to patients with an increased susceptibility to infections for one year.

• The main conclusion is that vitamin D_3 supplementation reduces symptoms and antibiotic consumption among patients with an increased frequency of respiratory tract infections. Thus, vitamin D_3 supplementation may be an alternative strategy to reduce antibiotic use among patients with recurrent respiratory tract infections.

Strengths and limitations

• Strengths: A high daily dose of vitamin D3 was used, the study time was a full year covering all seasons and patient with an increased frequency of respiratory tract infections were studied.

• Limitations: A single study center, small sample size (n=140) and a selected group of patients.

Abstract

Background: Low serum levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections (RTIs). Clinical trials with vitamin D_3 against various infections have been carried out but data are so far not conclusive. Thus, there is a need for additional randomized controlled trials of effects of vitamin D_3 on infections.

Objective: To investigate if supplementation with vitamin D_3 could reduce infectious symptoms and antibiotic consumption among patients with antibody deficiency or frequent RTIs.

Design: A double-blind randomized controlled trial.

Setting: Karolinska University Hospital, Huddinge

Participants: 140 patients with antibody deficiency (sIgA-, IgG subclass deficiency, CVID) and patients with increased susceptibility to RTIs (>4 bacterial RTIs/year) but without immunological diagnosis.

Intervention: vitamin D3 (4000 IU) or placebo was given daily for one year

Primary and secondary outcome measures: The primary endpoint was an infectious score based on five parameters: symptoms from respiratory tract, ears and sinuses, malaise and antibiotic consumption. Secondary endpoints were serum levels of 25-hydroxyvitamin D_3, microbiological findings and levels of antimicrobial peptides (LL-37, HNP1-3) in nasal fluid.

Results: The overall infectious score was significantly reduced for patients allocated to the vitamin D-group (202 points) compared with the placebo group (249 points) (adjusted relative score 0.771, 95% CI 0.604-0.985, p=0.04).

Limitations: A single study center, small sample size and a selected group of patients. The sample size calculation was performed using p=0.02 as the significance level whereas the primary and secondary endpoints were analyzed using the conventional p=0.05 as the significance level.

Conclusions: Supplementation with vitamin D_3 may reduce disease burden in patients with frequent respiratory tract infections.

Primary funding source: Swedish Foundation for Strategic Research (SSF)

The study was registered at www.clinicaltrials.gov (NCT01131858)

Introduction

Vitamin D was discovered when it was noted that rachitic children were improved by exposure to sunlight [1]. It was later shown by Holick *et al* that vitamin D_3 is synthesized in the skin under the influence of UVB-light [2]. Vitamin D_3 is further hydroxylated in the liver to 25-hydroxyvitamin D_3, which is considered to reflect the vitamin D status of an individual patient [3]. The final activation to the active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)D_3$) requires 1-α-hydroxylase activity. This enzyme (also designated CYP27B1) is expressed in the kidney but also in many other cell-types, including epithelial- and immune-cells [4]. The active vitamin D_3 ($1,25(OH)D_3$) binds to the vitamin D receptor (VDR), which belongs to the nuclear receptor family. Active vitamin D_3 is only present in minute amounts in the circulation and local activation in target cells is crucial for vitamin D-mediated effects on the immune system[5].

Low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of tuberculosis [6-8] and respiratory tract infections [9]. The mechanism is not fully elucidated but vitamin D_3 has been shown to induce antimicrobial peptides in immune cells [10]. In addition, active vitamin D_3 ($1,25(OH)D_3$) has broad anti-inflammatory effects on the adaptive immune system by shifting the T-helper cell pool from a Th1/Th17-response to a Th2/Treg-dominated response [11 12]. Vitamin D_3 has also been shown to suppress the Th2-response in allergic bronchopulmonary aspergillosis [13]. Thus, vitamin D_3 modulates both the adaptive and innate immune system [14]. The bulk of data on vitamin D_3 and infections stems from *in vitro* experiments and retrospective observational studies. Results from randomized controlled trials where the effects of vitamin D_3 on infections have been investigated (reviewed in Yamshchikov et al. [15]) are not conclusive and larger clinical trials are therefore warranted.

We designed a study to test the hypothesis that 4000 IU of vitamin D_3 given daily to patients with antibody deficiency and frequent respiratory tract infections for one year could prevent

or ameliorate infections. In addition, we investigated whether genetic polymorphisms in genes involved in the effect and/or metabolism of vitamin D_3 have an influence on the outcome of vitamin D_3 supplementation.

Methods

Study design

A prospective, randomized, double-blind placebo-controlled study of vitamin D_3 supplementation in patients with an increased susceptibility to respiratory tract infections. The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants. The study was registered at www.clinicaltrials.gov prior to inclusion of the first patient (NCT01131858). The EudraCT number is 2009-011758-16. The full protocol is available from the corresponding author upon request.

Sample size calculation

The sample size was based on the assumption that the intervention would reduce the number of days with symptoms from 42 days (210 points) to 28 days (140 points), i.e. a reduction of the infectious burden by 30%. Given this assumption, a sample size of 60 patients per study group was predicted to provide the study 90% power at a significance level of p=0.02 (Student's t-test). To compensate for predicted exclusion of participants, the groups were increased to include 70 patients per treatment arm. Importantly, the significance level of p=0.02 was chosen in the power calculation to ensure that a sufficient number of patients were recruited in order to avoid a type II error in the primary analysis. However, the conventional and widely accepted significance level of p=0.05 was used for statistical analyses of the primary and secondary endpoints.

Participants

Patients at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden, were included between March 2010 and June 2010 by the study nurses (SH, ML, KJ). Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infections; i.e. > 42 days with symptoms from the respiratory tract during a 12 months period prior to study inclusion. Patients registered at the Immunodeficiency Unit are closely followed with a diary of symptoms and antibiotic consumption. Thus, the patients are trained and used to apply such an instrument to assess their infectious status. Data from patients' standard diary was used as an instrument in order to assess patients for eligibility, both via telephone and by the responsible physician (PB, ACN) prior to inclusion. Patients with selective IgA-deficiency (**D80.2**), IgG-subclass deficiency (**D80.3**) and common variable immune disorder (CVID, **D83.0**) as well as patients without a defined immunological diagnosis (**D89.9**) were included. Exclusion criteria were prophylactic treatment with antibiotics, history of hypercalcemia or stones in the urinary tract, sarcoidosis, ongoing supplementation with vitamin D_3 exceeding 400 IU/day, HIV-infection and pregnancy.

Interventions

Patients were randomized to 12 months' treatment with Vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. One drop contained 500 IU vitamin D_3 or placebo oil (Miglyol oil®) and the participants were asked to take 8 drops daily. The participants had to mark their daily symptoms of infection in a diary, which was sent via regular mail to the study site every month. The following data was recorded: symptoms from the respiratory tract, ears and sinuses, treatment with antibiotics, numbers of bacterial cultures, times and reasons of visits to hospitals, frequency of travelling abroad and adherence to study drug.

Outcomes

The primary outcome was a composite infectious score, based on a daily patient-reported questionnaire, and included five parameters: symptoms from the respiratory tract, ears and sinuses, malaise and use of antibiotics (Figure S1), each parameter gave 1 point/day. The occurrence of X-ray verified pneumonia gave 3 additional points per day for a period of 7 days. Thus a pneumonia resulted in 3x7 points = 21 extra points. Patients were specifically instructed to record only symptoms related to ongoing respiratory tract infections. Symptoms related to infections at other sites (urinary tract, wounds etc) as well as non-infectious symptoms were reported as adverse events. Secondary outcomes were serum levels of 25-hydroxyvitamin D_3 (at baseline and after 3, 6, 9 and 12 months), numbers of bacterial cultures, microbiological findings and levels of antimicrobial peptides (LL-37 and HNP1-3) in nasal fluid (at baseline and after 6 and 12 months). In addition, 6 *post hoc* genotype analyses were performed in all participants. Analysis of single nucleotide polymorphisms (SNPs) were carried out for VDR (Taq1 and Foq1), CYP27B1, CYP24A1, CYP2R1 and Vitamin D binding protein (GC). Safety tests included plasma levels of creatinine, calcium, phosphate and albumin, measured at baseline and after 3, 6, 9 and 12 months. At inclusion, urine-HCG (human chorionic gonadotropin) in women was measured and p-parathyroid hormone in both genders. The results of the safety tests were reviewed by an independent and un-blinded consultant physician. Two blinded physicians (PB and ACN) were responsible for inclusion and all medical visits to the study site (Immunodeficiency Unit, Karolinska University Hospital, Huddinge).

Randomisation and statistical analysis

Participants were randomised to 12 months' treatment with vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. Block randomization with a block size of ten was used to ascertain equal group sizes. Staff at Karolinska Trial Alliance (KTA) was responsible for randomization procedures. In the statistical analysis, continuous variables were compared

using Mann-Whitney U test or linear regression and dichotomous variables by Fisher's exact test or logistic regression. Regressions of log-transformed infectious scores were performed both unadjusted (simple regression) and with adjustment for potential confounders (multiple regression).

Statistical methods: Primary analysis

The distribution of the infectious score was found to be skewed, thereby violating the normal assumption of the pre-specified t-test analysis. Hence, scores were log-transformed prior to analysis. Further, the randomisation had resulted in age distributions that were not entirely balanced between the two groups. Since there might be concerns that such imbalance could influence the results of the study, the original analysis plan was extended with a multivariable analysis adjusting for potential confounders. In this linear regression model based on log-transformed values of the primary outcome (the total infectious score) and its individual components, adjustment was made for age, gender, smoking, type of immune deficiency and significant co-morbidities (respiratory or non-respiratory). Due to the transformation procedure, the adjusted effect of vitamin D_3 is expressed as a ratio between the score in the vitamin D_3 and the placebo group. In this multiplicative model, an effect size of 1 indicates identical outcome in the two study groups and statistically non-significant results are recognized by confidence intervals encompassing the value 1.

To explore potential divergent effects on different organ systems, both adjusted and unadjusted analyses were repeated separately for each individual item of the infectious score. In addition, the temporal aspects of the vitamin D_3 effect were investigated by dividing the

study period into four 90-day periods (starting on the first day of treatment) and repeating the analyses separately for each time period. “Ear” and “sinus” symptoms as well as “antibiotic use” occurred at low frequencies and for these entities normal distributions could not be achieved despite data transformation. Thus, the adjusted analyses of these individual items were based on multivariable logistic regression, after coding the symptom (or antibiotic therapy) as present or absent during the course of the study. However, this only applies to analysis of the individual items, and not to the primary analysis of the total infectious score, where all item scores were added as originally described.

Most post-randomization exclusions were due to patients failing to fill out the symptoms diary. Hence, no intention to treat (ITT) analysis based on actual outcome data could be performed. However, the potential impact of dropouts was addressed in an ITT analysis based on multiple imputation of missing outcome data. In the imputation process, pooled estimates were derived from 100 datasets created by means of multivariate imputation by chained equations and predictive mean matching for the same covariates as in the adjusted per-protocol analysis

Detailed descriptions of randomisation and blinding, sampling of nasal fluid, measurement of antimicrobial peptides, measurement of 25-hydroxyvitamin D_3, genotyping and statistical analyses of secondary outcomes including sample size calculations are presented in the Supplementary Methods Section.

Results

Baseline data

A total of 286 patients were first assessed for eligibility but 144 were not included because they did not fulfill all inclusion criteria; <42 days with infection/year (n=35), lacked other inclusion criteria (n=42), or declined to participate (n=67). The remaining 142 patients were further screened and 140 patients were included in the study. Of these, 70 were randomized to vitamin D_3 supplementation and 70 to placebo (Figure 1). The groups did not differ with regards to gender, IgG replacement therapy, smoking, baseline 25-hydroxyvitamin D_3 levels, type of immune defect or co-morbidities. Patients with subclass deficiency, selective IgA deficiency (sIgA), common variable immune deficiency (CVID) and patients without a defined immunological diagnosis (ND) but with >4 bacterial respiratory tract infections/year were included. IgG replacement therapy was most common in the CVID-group (100%) and in the subclass deficiency group (63%), but also frequent in the other groups (ND, 54% and sIgA, 38%, table S1). Patients allocated to the placebo group were slightly younger than patients in the treatment group (p=0.025, data not shown). During the course of the study, 16 patients left the study prematurely (8 patients from each study group) and consequently 124 patients were included in the main per-protocol analysis. Reasons for dropout included elevated PTH (n=2), withdrawn consent (n=5), adverse event (n=1), prescription of vitamin D outside the study (n=1), failure to complete diary (n=4) or non-compliance to study medication (n=3) (Figure 1).

Primary endpoint: Infectious score

One year of vitamin D_3 treatment was associated with a significantly reduced total infectious score both in the unadjusted (n=124, p=0.024, Table 2) and the adjusted analyses (n=124,

p=0.040) (Table 2, Figure 2A, B and Table S2). The unadjusted relative score in the intervention group was 0.754 (95% c.i. 0.591-0.963, p=0.024, n=124) corresponding to a 25% reduction and after adjustment for potential confounders, the relative score was 0.771 (95% c.i. 0.604-0.985, p=0.04), corresponding to a 23% reduction (Table 2). According to the temporal analysis, the effect of vitamin D_3 supplementation tended to improve with time (Figure 2A). The absolute unadjusted score per patient was 202 points for the vitamin D group and 249 points for the placebo group, which was a significant reduction of 47 points per patient (p=0.023, Mann Whitney U-test, table S3).

When the individual items of the infectious score were analyzed separately, all point estimates indicated a reduction in the treatment group (Table 2, figure S2), although only antibiotic consumption reached statistical significance (Figure 2B and S2, panel E). The adjusted OR for antibiotic use was 0.365 (95% c.i. 0.153-0.872, p=0.023, n=124), i.e. a 63.5% reduction of the odds of antibiotic use in the intervention group (Table 2). The absolute values were 33 days on antibiotics for the placebo group and 16 days for the vitamin D_3 group, i.e. a reduction of 17 days in the vitamin D_3 group (table S3). The temporal trends for specific symptoms and antibiotic consumption were similar to the total score and reached statistical significance for 'ear'-symptoms (n=124, p=0.041) and for 'malaise' (n=124, p=0.053) in the final quarter of the study (Figure 2S, panels B and C).

Analyzing the primary outcome according to intention-to-treat (n=170) produced results virtually identical to those of the per-protocol analysis. In the unadjusted ITT analysis, vitamin D_3 reduced the total infectious score by 25% (relative score 0.752, 95% c.i. 0.588-0.962, p=0.024) and after adjustment for potential confounders the reduction was 23% (relative score 0.767, 95% c.i. 0.599-0.982, p=0.036).

Serum levels of 25-OH vitamin D_3

Serum 25-hydroxyvitamin D_3 levels did not differ between the groups at baseline (Table 1) but already after 3 months the intervention group had a significantly higher level of 25-hydroxyvitamin D_3 (133.4 nmol/L versus 66.6 nmol/L, p<0.001, Figure 3). This increase remained throughout the study (Figure 3).

Bacterial cultures and microbiology

During the course of the study, 173 microbiological samples were obtained in the vitamin D_3 group (n=62, 2.79/patient) and 301 in the placebo group (n=62, 4.85/patient) (p=0.010, Table 3). The number of samples with at least one positive finding was higher in the placebo group, with close to statistical significance (p=0.052), while the fraction of positive samples was similar for both groups (Table 3). Significantly more patients had a microbiological sample taken from the respiratory tract (≥ 1 sample) during the study period in the placebo group; OR 2.63 (95% CI 1.17-5.92), (Table 3).

In total, the vitamin D_3 group generated 76 positive microbiological findings (bacteria or fungi), compared to 159 in the placebo group (p=0.023). There was no difference between the groups for the traditional respiratory pathogens (*H. influenza, M. catharralis and S. pneumonia*), but there were significantly fewer findings of *S. aureus* (p=0.019) and fungi (p=0.028, *Candida* spp. and *Aspergillus* spp.) in the treatment group (Table 4). Likewise, significantly fewer vitamin D_3-treated patients had a bacterial culture positive for *S. aureus* (p=0.019) or fungal species (p=0.058), although the latter difference did not reach statistical significance (Table 4).

Vitamin D treated patients with sub-class deficiency left significantly fewer bacterial or fungal cultures than placebo-treated patients with this diagnosis; 7 cultures in the vitamin D group (n=22) versus 47 cultures in the placebo group (n=24) (Table S4). Also the number of patients that had ≥1 bacterial culture taken was significantly fewer in the placebo group (12/22 versus 22/24, p=0.0065, table S4). There was no significant effect of other immunological diagnoses on bacterial cultures or microbiology (Table S4).

Since concomitant lung disease may be an important factor for vitamin D mediated effects on respiratory immunity, we performed a detailed analysis of bacterial cultures and microbiology of patients with asthma, bronchiectasis (BE) and chronic obstructive pulmonary disease (COPD). The numbers of patients with these diagnoses were quite small, which preclude any firm conclusions regarding any effect. However, there was a trend – however not significant – that vitamin D treated patients with asthma produced fewer bacterial cultures (average 2.9 cultures/patient versus 7.0 cultures/patients, p=0.080, Figure S3) and fewer positive cultures than placebo treated asthmatics (average 0.6 positive cultures/patients versus 2.7/patient in the placebo group, p=0.052, Figure S3). In addition, vitamin D treated asthma patients showed significantly fewer cultures positive for fungi (candida and aspergillus) compared to placebo treated asthmatics (p=0.0476, table S5). For BE or COPD-patients there was no clear trend or significant effect in bacterial cultures or microbiology.

Levels of antimicrobial peptides (AMPs) in nasal fluid

There was no statistically significant difference between the vitamin D_3 or placebo groups when nasal fluids were analyzed for the presence of AMPs. Initially, levels of both LL-37 and HNP1-3 tended to be higher in the placebo group (Figure S3, panels A and B). However, after 12 months the microbiological pattern was reversed and no primary pathogens could be

detected in nasal swabs from vitamin D_3-treated patients (n=25, p=0.039) (Figure S4, panel C). The placebo-treated patients exhibited the same mix between normal flora and primary pathogens at all three sampling points (0, 6 and 12 months) (Figure S4, panel C).

SNP variants and treatment effect

Most genetic variants did not affect the primary endpoint. However, patients carrying the 'AA' genotype in the CYP2R1-gene, encoding the 25-hydroxylase enzyme, had a larger benefit of vitamin D_3-supplementation (-55%) compared to AG or GG carriers (-6%) (n=124, p=0.046 for interaction, Table S6).

Adverse events

In total, the vitamin D_3 group reported 38 adverse events (AEs) versus 56 AEs in the placebo group. The most common symptoms in the treatment group were headache (n=5) and lumbago (n=5), whereas placebo-treated patients reported paresthesias (n=8), diverticulitis (n=4) and urinary tract infection (n=4) as most frequent AEs (Table 5, Table S7). There was a general trend towards the number of adverse events being higher in the placebo group. Significantly more patients in the placebo group reported cardiovascular problems, such as heart failure, hypertonia and thrombosis (p=0.028). For gastrointestinal and other (non-respiratory) infections there was also a trend favoring the vitamin D_3 group (p=0.058 and p=0.09, respectively). No clinically relevant changes in serum levels of calcium, phosphate, creatinine or albumin could be observed (Figure S5). There was one severe adverse event (SAE) in each group (rabdomyosarcoma in the vitamin D_3 group and lung bleeding in the placebo group), both judged as being unrelated to the study drug.

Discussion

The main conclusion from this long-term randomized controlled trial (RCT) is that vitamin D_3 supplementation reduces the total burden of respiratory tract infections. The primary endpoint was composed of five different parameters that patients recorded daily throughout the study year. All point estimates favoured the vitamin D_3 group and a statistically significant effect was seen on both the total score and on the probability of receiving antibiotics ($p<0.05$). The effect on the infectious score was evident both in analysis per-protocol and according to intention-to-treat, and withstood adjustment for potential confounders. In addition, the number of bacterial cultures and microbiological findings was significantly reduced in the intervention group. These findings are potentially important and support that Vitamin D_3 supplementation may prevent respiratory tract infections and reduce antibiotic consumption, particularly in patients with hypogammaglobulinemia or with an increased frequency of respiratory tract infections.

However, our study has several limitations: Firstly, the choice of primary endpoint may be questioned since it relies solely on patient-reported information. To compensate for inherent problems with patient-reported data, the evaluation instrument was designed to cover many aspects of an infectious episode, including various symptoms as well as antibiotic consumption. Together the reported data formed an "infectious score", which constituted the primary endpoint of the study. Similar composite scores have successfully been applied to different diseases, such as tuberculosis (TB-score [16]), pneumonia (CURB-65 [17]) and bacterial meningitis (BMS-score [18]). Notably, vitamin D supplementation had a major effect on the odds of taking antibiotics during the study period (a reduction by 63.5%). In addition, the absolute number of days on antibiotics was reduced by 50% (from 33 days in the placebo group to 16 days in the intervention group), which was statistically significant both in the adjusted and unadjusted analyses (table 2). However, despite the relatively modest reduction

for the other components of the primary endpoint the overall infectious score was significantly reduced – mainly as a result of the large effect on the antibiotic parameter - both in the unadjusted and in the adjusted analyses (table 2, figure 2). It is important to interpret the statistical significance in light of our power calculation, which was based on a significance level of p=0.02. In the power calculation, the significance level was reduced from 0.05 to 0.02 in order to increase the statistical power at the p=0.05 level. This approach was incorrect, and the targeted power (at the p=0.05 level) should instead have been increased without altering the p-value threshold. However, we have used the widely accepted significance level p=0.05 in the statistical analyses for both the primary and secondary endpoints, respectively. Another potential problem was that the patient population was very heterogeneous with regards to immune deficiency and concomitant diseases. We adjusted for these factors in the multivariable analyses of the primary endpoint, but the sample sizes in each subgroup were too small to draw any conclusions of effects in specific disease groups. However, a detailed *post-hoc* analysis of the relation between immunological diagnosis, concomitant lung-disease and the secondary endpoints "taken bacterial cultures", "positive bacterial" cultures and "microbiological findings" was performed. There was a clear trend that Vitamin D treated patients with subclass deficiency and/or asthma produced fewer bacterial cultures, fewer positive cultures and fewer fungal cultures (tables S4 and S5, Figure S3). Although this analysis may lack precision by the small number of patients included, it could have clinical implications regarding target groups for vitamin D supplementation.

Nevertheless, our double-blind RCT has several strengths. For example, we chose a high daily dose of vitamin D_3 based on published calculations on metabolism and effects on immunity [14 19]. Other RCTs using lower doses of vitamin D_3, 400-2000 IU/day, have mainly been negative with regards to prevention of infections [20 21]. However, one study using 1200 IU/day showed a significant reduction of influenza among school children in Japan [22]. Notably, also studies

using higher doses of vitamin D_3 have been negative. Martineau *et al* used 400,000 IU vitamin D_3 during 42 days (9523 IU/day) with the aim of shortening time to sputum conversion in tuberculosis. No significant effect on the primary endpoint could be observed in that study, except in a subgroup with the *tt* genotype in the vitamin D receptor gene [23]. A recent study investigated whether 100,000 IU vitamin D_3/month (3333 IU/day) could reduce the incidence of chronic obstructive pulmonary disease (COPD) exacerbations. There was no significant effect on the primary endpoint, although a *post hoc* analysis revealed that patients with a low vitamin D_3 level at baseline had a significant effect of Vitamin D_3 supplementation[24].

Importantly, our study is the first to utilize high daily doses for an extended period of one full year. Thus, we covered all four seasons, which was important in Sweden with a known seasonal variation in 25-hydroxyvitamin D_3 levels [25]. Two previous RCTs were performed during the winter season – when vitamin D levels are low – but only during 4 [22] and 6 months [20], respectively. Previous RCTs have been conducted during shorter periods; 42 days [23], 6 weeks [26] and 12 weeks [21], respectively. Interestingly, we observed a clear time dependent effect suggesting that a long term supplementation approach (> 6 months) may be necessary to affect immunity. To expand on the results of a previous study in healthy individuals where no difference between the intervention and placebo groups was observed [21], we chose a study population with frequent RTIs and at least 42 days with infection during the year prior to inclusion. Notably, patients in the study represent a selected group of individuals with frequent RTI, although the immune disorders that they represent (IgA-, IgG-subclass and patients with no defined immune disorder) are generally mild in character and dominated by mucosal RTIs. We also included a small number of CVID-patients, which can be considered to be a more severe immune disorder, but all these patients are treated with IgG replacement therapy and thus well controlled. Hence, the results from this study cannot directly be applied

to the general healthy population. Nevertheless, the results provide solid support for additional interventional studies of vitamin D_3, especially in groups consuming large amounts of antibiotics.

The mechanism of the observed effects remains largely unknown. Vitamin D_3 modulates the immune response at many levels, such as induction of AMPs, skewing of T-cells from Th1/Th17 to Tregs as well as general anti-inflammatory effects [14]. Here, we investigated the role of AMPs in nasal fluid. However, we could not detect any significant changes of LL-37 or HNP1-3 during the study period, but noted that placebo-treated patients tended to have higher levels of AMPs after one year of treatment. This was paralleled by a shift of the microflora in the nasal compartment that could explain the unexpected finding of higher AMP-levels in the placebo group. Recently, it was shown that 1,25 $(OH)_2$-vitamin D_3 induces both HNP1-3 and LL-37 in nasal fluid of healthy volunteers (Hiemstra et al, abstract, European Respiratory Society, 2011), supporting that LL-37 may indeed be induced *in vivo*. However, our study design did not allow such conclusions but rather support that vitamin D_3 affect mucosal immunity, leading to a shift of the microflora. Recently, we showed that the bacterial composition in nasal swabs is an important determinant of AMP-levels in nasal fluid [27].

Given that vitamin D_3 induces LL-37 in epithelial cells and that LL-37 kills bacteria *in vitro,* we expected a reduction of the classical bacterial pathogens *H. influenza*, *M. catharralis* and *S. pneumonia* in the intervention group. However, the frequency of these bacteria was not reduced but a reduction of *S. aureus* and fungal species that often colonise the airways was observed. This could be explained by specific effects by vitamin D_3 on immunity against *S. aureus*. In fact, vitamin D induces human beta defensin-2 (HBD-2) with bactericidal activity against *S. aureus* [28]. A recent study showed that low vitamin D levels were associated with an increased risk of being colonised by this bacterium [29]. Further, vitamin D affects immunity

against *C. albicans*, which indicates direct effects of vitamin D on human immunity [30]. Alternatively, it is possible that vitamin D_3 may have prevented symptomatic viral infections, which prompted patients to leave a bacterial sample from the airways. Interestingly, there is both mechanistic and clinical evidence that vitamin D_3 can prevent viral infections [31-33], although we did not address this in the current study.

Notably, we observed a prominent increase in the serum concentration of 25-hydroxyvitamin D_3, which indicated good compliance and tolerability of the study drug. In fact, there was a trend towards adverse events being reported more often in the placebo group, suggesting that vitamin D_3 possibly could be efficient against other diseases, but this observation requires further studies. No clinically relevant changes of blood chemistry (calcium, phosphate, albumin or creatinine) were observed. Despite few adverse events and high tolerability, 16 exclusions occurred during the study year. The main reason was problems to adhere to the protocol and 6/16 patients dropped out of the study after a few weeks. The rest failed to send in the diaries, did not leave blood for monitoring of safety parameters or did not take the study drug. One patient was excluded based on symptoms that could be attributed to vitamin D_3 (facial paraesthesia). However, this patient was later confirmed to have been allocated to placebo.

In summary, we found that supplementation with vitamin D_3 reduced the total infectious score with 47 points per patient (23% reduction in the adjusted analysis) during the study year. The observed reduction was lower than the assumed reduction of 70 points per patient (predefined assumption: 210 points => 140 points; a reduction of 30%) that formed the basis for the power calculation. However, despite the predefined level of a reduction of infectious score by 30% as a clinically meaningful effect, we believe that effects lower than this also could be relevant for the individual patient. We base this line of reasoning on the fact that a reduction of 47 points per patient can be translated into 47 days with cough (47 points), 23 days with ear

and sinus symptoms (23×2=46 points) or 9 days with cough, sinus and ear symptoms together with malaise and antibiotics (9×5=45 points). In addition, our data indicate that vitamin D_3 supplementation reduces the odds of taking antibiotics by approximately 60% in patients with frequent respiratory tract infections. Thus, supplementation with vitamin D_3 could provide a novel strategy to reduce antibiotic use among high consumers and indirectly prevent the emerging epidemic of bacterial resistance.

Acknowledgment

The study was registered at www.clinicaltrials.gov (NCT01131858), prior to the start of the study. The entire process of study design and protocol, data monitoring and analyses was performed by academic authors; there was no industry involvement in the study except that vitamin D_3 (Vigantol®) and placebo oil (Miglyol®) were provided by Merck KGaA (Darmstadt, Germany). Merck did not have any influence on study design, analysis of data, writing or decision to publish. We extend our gratitude to Ilona Skilving, Karolinska Trial Alliance for invaluable help with the protocol. Further, we thank registered nurses Maria Lindén and Kristina Johansson for skillful work with patients. Thanks also to Jenny Lindén and Alicia Hansson for registration of data and to professor Mats Remberger, KI for discussions on statistical methods. Professor Lars Lindqvist, Department of Infectious Diseases, Karolinska University Hospital is gratefully acknowledged for serving as the monitor of the study. PB, LBB and JDL are holding PostDoc-positions financed by Karolinska Institutet and Stockholm County Council (KI/SLL).

Data sharing statement:

There is no additional data available.

Statements

Author contributions:

Peter Bergman, designed the study, collected, analysed and interpreted data, wrote the paper.

Anna-Carin Norlin, designed the study, collected and interpreted data, wrote the paper.

Susanne Hansen, designed and coordinated the study, collected and interpreted data.

Rokeya Sultana Rekha, carried out experimental work, analysed data

Birgitta Agerberth, analysed and interpreted data, wrote the paper.

Linda Björkhem-Bergman, analysed and interpreted data, wrote the paper.

Lena Ekström, analysed and interpreted data

Jonatan Lindh, analysed and interpreted data, wrote the paper.

Jan Andersson, designed the study, interpreted data, wrote the paper.

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Merck GmbH provided the study drug (Vigantol) but did not have any influence on study design, analysis of data, writing or decision to publish.

Ethics statement: The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants.

Conflicts of interest: There are no conflicts of interest.

Legends to figures

Figure 1. Study outline.

Figure 2. Primary endpoint. The adjusted total relative infectious score (A) is expressed 'per quarter' (3 month periods). The adjusted one-year scores (total score, airway, malaise, ear, sinus and antibiotics) are depicted in a Forrest-plot (B) together with 95% confidence intervals. Effects are presented as relative scores (total score, airway, malaise) or odds ratios (ear, sinus, antibiotics, indicated with asterisks).

Figure 3. Secondary endpoint. Vitamin D-levels. Serum was collected at day 0, 3, 6, 9 and 12 months and levels of 25-hydroxyvitamin D_3 were measured. Values are expressed as mean +/- 95% confidence interval.

Tables

Table 1. Baseline data. Mann Whitney U-test was used for comparisons of age and 25-OH vitamin D_3. Fisher's exact test was used for all other comparisons. 1) "other disease" includes hypertension, body pain, hypothyroidism and gastritis as most common diagnoses. CVID, common variable immuno deficiency; ND, increased susceptibility to infections without a defined immunological disorder; BE, bronchiectasis; COPD, chronic obstructive pulmonary disease.

	Vitamin D_3	Placebo
Number	70	70
Age (mean)	55,4	50,8
Female	52/70	50/70
Male	18/70	20/70
IgG-replacem.	39/70	42/70
Smoking	4/70	6/70
25-OH levels (mean)	51,5 nmol/L	46,9 nmol/L
Immunological diagnosis		
sIgA-deficiency	9/70	9/70
IgG subclass	27/70	30/70
CVID	6/70	4/70
ND	28/70	27/70
Concomitant disease		
No other disease	16/70	18/70
Lung: Asthma	27/70	25/70
Lung: BE	5/70	7/70
Lung: COPD	5/70	4/70
Other disease[1]	17/70	16/70

Table 2. Primary Endpoint. Treatment effect calculated as the ratio between infectious scores in the vitamin D_3 and the placebo groups. Due to low frequencies, endpoints marked with asterisks were coded as binary outcomes (i.e. present or absent in each patient) and compared by means of logistic regression. In these cases, the effect refers to odds ratios of experiencing the outcome at least once during the course of the study. (The data are based on n=124 patients).

	Univariable regression model (unadjusted values)			Multiple regression model (adjusted values)		
Endpoint	**Effect**	**95% CI**	**p-value**	**Effect**	**95% CI**	**p-value**
Total score	0.754	0.591-0.963	**0.024**	0.771	0.604-0.985	**0.040**
Airway	0.857	0.697-1.053	0.141	0.871	0.706-1.074	0.200
Ear*	0.721	0.352-1.465	0.367	0.695	0.320-1.501	0.357
Sinus*	0.583	0.280-1.198	0.144	0.594	0.265-1.328	0.204
Malaise	0.845	0.692-1.032	0.098	0.845	0.689-1.036	0.108
Antibiotics*	0.355	0.154-0.784	**0.012**	0.365	0.153-0.872	**0.023**

Table 3. Bacterial cultures. [1]Mann-Whitney U-test, [2]Fisher's exact test.

	Vitamin D_3	Placebo	Significance
Number of samples per patient (mean, n=62/62)	2.79	4.85	p=0.010[1]
Number of positive samples per patient (mean, n=62/62)	1.01	2.02	p=0.052[1]
Fraction positive cultures (%)	63/173 (36%)	125/301 (41%)	P=0.28[2]
Patients with ≥ 1 sample taken	38/62 (61%)	50/62 (81%)	p=0.029[2]

Table 4. Microbiological findings. Mann-Whitney U-test was used to analyze the total number of findings, whereas Fisher's exact test was used for analysis of the number of patients (fraction) with a specific finding.

	Number of findings (total)			Number of patients		
Microorganism	Vitamin D_3	Placebo	MW-U	Vitamin D_3	Placebo	Fisher
H. influenzae	28	27	P=0.46	10/62	13/62	P=0.64
M. catharralis	8	17	P=0.39	7/62	10/62	P=0.60
S. pneumoniae	7	6	P=0.74	4/62	5/62	P=1.00
S. aureus	6	33	P=0.010	4/62	14/62	p=0.019
Enterobacteriacae	8	8	P=0.39	4/62	7/62	P=0.53
P. aeruginosa	8	15	P=0.68	3/62	4/62	P=1.00
Fungal infection	11	53	P=0.028	4/62	12/62	p=0.058
Total	76	159	P=0.023			

Table 5. Adverse events. Number of reports. Fisher's exact test was used for between group comparison. (The data are based on AE-reports from n=62 patients/arm).

Organ	Vitamin D_3 n (%)	Placebo n (%)	P-value
CNS	11 (29)	10 (18)	1.00
Gastrointestinal	4 (11)	12 (21)	**0.058**
Cardiovascular	0 (0)	6 (11)	**0.028**
Infections (other than RTI)	2 (5)	8 (14)	**0.09**
Musculoskeletal	10 (26)	10 (18)	1.00
Respiratory (non-infectious)	2 (5)	4 (7)	0.68
Skin	5 (13)	2 (4)	0.44
Other	4 (10)	4 (7)	1.00
Total	**38**	**56**	

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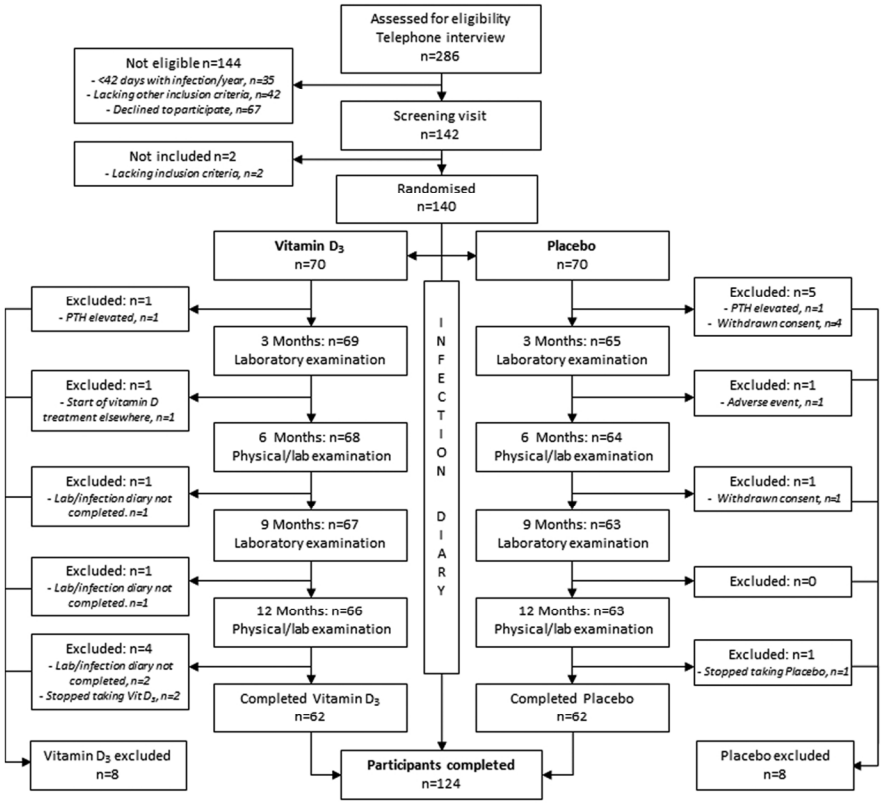

Figure 1
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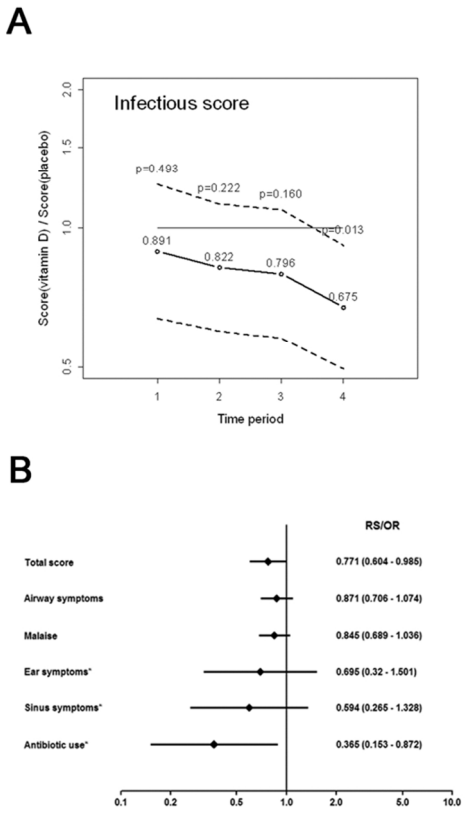

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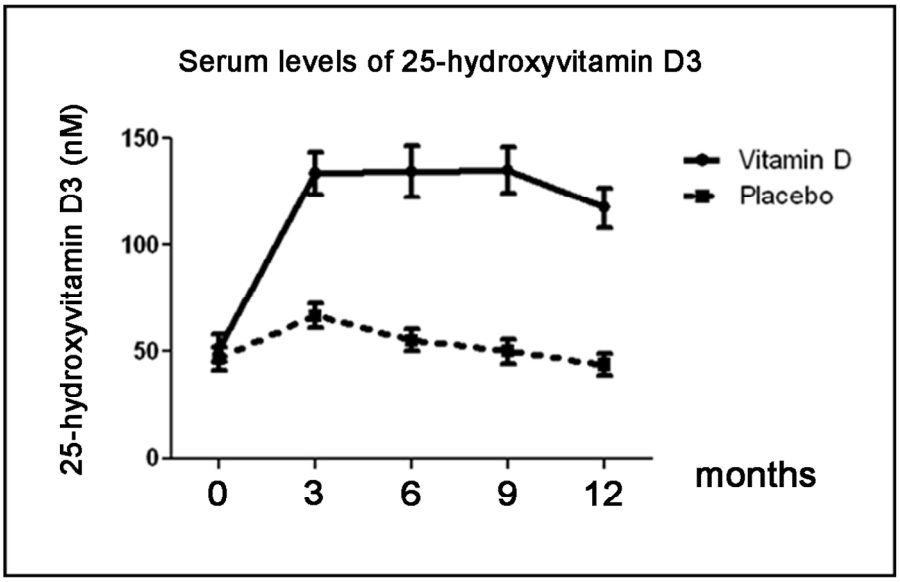

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Supplementary Methods

Randomisation and Blinding

A computer-generated list of random numbers was used for patient allocation. Randomization sequence was created using Randomization.com (http://www.randomization.com) and was stratified with a 1:1 allocation using a fixed block size of 10. Within each block two participants were randomly assigned to provide samples of nasal fluid, one for each treatment group.

The vitamin D_3 and placebo were in liquid form and identical in appearance. They were prepacked in bottles and consecutively numbered for each participant according to the randomization schedule. In connection with the inclusion each participant was sequentially assigned a number by the responsible physician and received the corresponding prepacked bottles.

Participants, investigators and staff were kept blinded to the allocation throughout the trial. It was not necessary to un-blind information on any participant during the trial.

Sampling of nasal fluid, NPH swabs

Since vitamin D_3 can induce antimicrobial peptides both in macrophages and in epithelial cells[1], we measured levels of LL-37 and α-defensins (HNP1-3) in nasal fluid (Figure 4A and B). For logistical reasons we limited patients for nasal fluid collection and only 36/140 patients (20%) were randomised to this procedure. Nasopharyngeal swabs were taken from

one nostril and sent to the Clinical Microbiology Laboratory at Karolinska University Hospital, Huddinge for bacterial culture. The bacterial content was evaluated as "no growth of bacteria", "normal flora" (typical findings include α-haemolytic streptococci, *Corynebacteria* spp, *Neisseria* spp. and other nonpathogenic strains) or "pathogenic growth" (defined here as *H. influenzae, S. aureus, S. pneumoniae, M. catharralis* and *Enterobacteriacae* spp). Subsequently, nasal fluid was collected through a thin plastic tube that was carefully placed in the back of the nose using the other nostril as entry port (10-12 cm from the nostril meatus). 5-10 ml of saline was administered into the nose prior to sampling in order to make the epithelial lining moist and to dissolve mucus depositions. A gentle vacuum was applied and 3-5 ml nasal fluid was collected and stored at -20°C, as described in Cederlund et al, PLoS One, 2011[2].

Extraction of peptides and proteins from nasal fluid

Nasal fluid (3-5 ml) was extracted in an equal volume (1:1) of 60% acetonitrile (AcN) in 1% trifluoroacetic acid (TFA) over night at 4°C. The extract was centrifuged at 3500g and the supernatant was lyophilized. The lyophilized extract was resuspended in 0.1% TFA and enriched for polypeptides using solid phase extraction as described in[2]. The lyophilized polypeptide extract was reconstituted in 0.1% TFA to a concentration of 5 µg/µl as determined spectrophotometrically using a Nanodrop-system (Thermo Scientific, Wilmington, U.S.).

Analysis of antimicrobial peptides in nasal fluid

The concentrated and lyophilized extract (25 µg) was dissolved in lithium dodecyl sulphate (LDS) sample buffer, 50 mM Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 70°C for 10 min. The samples were then separated using LDS-PAGE and blotted onto PVDF membranes, as described in[3]. Antibodies used were a LL-37 monoclonal[4] and a HNP1-3 goat polyclonal (sc-22916, Santa Cruz, Santa Cruz, Calif., USA). Proteins and peptides were visualized on chemiluminescence film with ECL plus Western blot detection system (GE Healthcare, Buckinghamshire, United Kingdom). LL-37 and HNP1-3 concentration in nasal fluid were determined by densitometry using the software ImageJ (http://rsbweb.nih.gov/ij/). The intensity of each band was normalized to an external standard on each membrane and the total amount of LL-37 and HNP1-3 was determined by multiplying the densitometric result (ng peptide/µg extract) with the total amount of polypeptide-extract (µg). Thus, the values represent the total amount of LL-37 and HNP1-3 from each nasal fluid sample.

Analysis of 25-OH vitamin D_3 in serum

Levels of 25-hydroxyvitamin D_3 in serum were determined by using DiaSorin immunochemical method (DiaSorin S.p.A, Saluggia, Italy) at the Department of Clinical Chemistry, Karolinska University Hospital.

Genotyping

Specific single nucleotide polymorphisms (SNPs) in key genes for vitamin D metabolism might influence the outcome of vitamin D_3 supplementation. Therefore, all patients were

genotyped for 6 SNPs in the VDR (TaqI and FokI), CYP27B1, CYP24A1, CYP2R1 and GC genes. Six SNPs in five genes involved in vitamin D metabolism and / or effect were analysed in all participants. The aim of these analyses was to investigate whether individuals with a specific genotype would benefit more from vitamin D_3 supplementation. Genomic DNA was isolated from 200 µl peripheral blood leucocytes using the DNA Blood Mini kit (Qiagen, Hilden Geramany). Allelic discrimination reactions were performed using TaqMan® genotyping assays (Applied Biosystems, Foster City CA USA): C_12060045_20 for VDR (FokI); C_2404008_10 for VDR (TaqI); C_29958084 for CYP24A1; C_2958431_10 for CYP2R1; C_26407519_10 for GC. For the CYP27B1 genotyping, primers and probes described previously were used[5]. The final volume for each reaction was 15 µl consisting of 30 ng DNA and 2xTaqman Universal PCR Master mix (Applied Biosystems). The PCR profile consisted of 95° C for 10 minutes followed by 40 cycles of 92° C for 15 sec and 60° C for 1 minute. The fluorescence signal was measured with an ABI 7500 Sequence detector (Applied Biosystems).

Statistical methods: Sample size calculation

The sample size was based on the assumption that the intervention would reduce the number of days with symptoms from 6 weeks (42 days x 5 points = 210 points) to 4 weeks (28 days x 5 points = 140 points), i.e. a reduction of the infectious burden by 30%. The estimated standard deviation was 3 weeks (21 days x 5 points = 105 points). Given these assumptions, a sample size of 60 patients per study group was predicted to provide the study 90% power at a significance level of p=0.02 (Student's t-test). To compensate for predicted exclusion of participants, the groups were increased to include 70 patients per treatment arm.

Statistical methods: Secondary analyses

The number of bacterial cultures taken in each patient and the number of samples with a positive finding were compared between the two study groups by means of the Mann-Whitney U test. To reduce the influence of patients subjected to very frequent sampling, the odds of having one or more culture taken during the course of the study was also compared by means of Fisher's exact test. Similarly, the frequencies of cultures positive for specific pathogens were compared both as number of positive cultures per patient (Mann-Whitney U test) and as fraction of patients presenting with at least one positive culture (Fisher's exact test). The fraction of nasopharyngeal samples exhibiting bacterial growth was compared between the two groups separately for samples taken at baseline, after six month and after 12 months (Fisher's exact test).

The influence of genetic polymorphisms on the effect of vitamin D_3 treatment was analysed in linear regression models with log-transformed infectious score as dependent variable. Independent variables were study group, genotype and a genotype-study group interaction term. Genotypes were coded as binary variables, based on previous findings reported in the literature[5-10].

In all analyses, P values <0.05 (two-sided) were considered statistically significant (the significance level of 0.02 in the power calculation was chosen to provide an extra safety margin). All statistical analyses were performed using R 2.11.1 (R Development Core Team (2010). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org) and GraphPadPrism, version 5.0, GraphPad Software, La Holla, Calif, USA

Supplementary References

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Supplementary tables

Table S1. Data on IgG substitution for participants that were included in the per protocol analysis (n=62/arm).

Treatment with IgG per diagnosis	Yes	No	Total
Subclass deficiency	29	17	46
IgA deficiency	6	10	16
CVID	9	0	9
ND	29	24	53
Total:			124

Table S2. Primary endpoint. Unadjusted relative score per day calculated per 3 months periods as indicated. Values are expressed as mean +/- SD. (n=62/arm).

	Month 1-12		Month 1-3		Month 4-6		Month 7-9		Month 10-12	
	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo	Vitamin D	Placebo
Infectious score	0.56(0.58)	0.69(0.54)	0.58(0.66)	0.67(0.70)	0.51(0.61)	0.59(0.57)	0.59(0.67)	0.72(0.65)	0.53(0.56)	0.77(0.61)
Airway symptoms	0.26(0.24)	0.32(0.28)	0.27(0.27)	0.29(0.28)	0.25(0.29)	0.25(0.24)	0.27(0.27)	0.30(0.27)	0.27(0.27)	0.33(0.27)
Malaise	0.16(0.20)	0.18(0.17)	0.16(0.24)	0.18(0.22)	0.14(0.21)	0.15(0.18)	0.17(0.23)	0.19(0.20)	0.15(0.20)	0.22(0.22)
Ear symptoms	0.04(0.09)	0.07(0.15)	0.05(0.12)	0.07(0.17)	0.03(0.09)	0.05(0.14)	0.05(0.10)	0.08(0.18)	0.05(0.10)	0.08(0.18)
Sinus symptoms	0.05(0.12)	0.06(0.10)	0.05(0.12)	0.06(0.13)	0.04(0.10)	0.04(0.10)	0.06(0.15)	0.07(0.13)	0.05(0.14)	0.07(0.12)
Antibiotic use	0.04(0.06)	0.09(0.14)	0.05(0.09)	0.08(0.16)	0.04(0.08)	0.09(0.17)	0.04(0.08)	0.09(0.15)	0.04(0.07)	0.11(0.18)

Table S3: Primary endpoint. Unadjusted score.

	Mean score / patient / year			MWU (unadjusted)
Endpoint	**Vitamin D**	**Placebo**	**Diff.**	**p-value**
Total score	202	249	-47	0,023
Airway	94	101	-7	0,302
Ear*	16	25	-9	0,225
Sinus*	18	21	-3	0,126
Malaise	56	66	-10	0,041
Antibiotics*	16	33	-17	0,024

Table S4: Secondary endpoint. Number of bacterial cultures in relation to immunological diagnosis. Patients can have several bacteria or fungi in the same culture. Values within parentheses indicate the number of patients that provided the positive cultures. **p=0.0065, Fisher's exact test (12/22 vs 22/24).

								Number of patients	Number of patients
Number of bacterial cultures per diagnosis and treatment		(n)	H. infl.	M. Cath	S. pneum.	S. aur	Fungi	>1 culture taken	>1 culture positive
Subclass deficiency	Vitamin D	22	2(1)	3(3)	2(2)	0	0	12**	5
	Placebo	24	15(6)	3(2)	2(2)	13(5)	14(4)	22**	12
IgA deficiency	Vitamin D	9	4(1)	1(1)	0	0	0	4	3
	Placebo	7	1(1)	2(2)	0	1(1)	0	5	2
CVID	Vitamin D	5	1(1)	3(2)	0	0	0	4	1
	Placebo	4	3(1)	0	2(1)	1(1)	3(1)	4	3
ND	Vitamin D	26	21(7)	1(1)	5(2)	6(4)	11(4)	17	12
	Placebo	27	8(5)	1(1)	2(2)	18(7)	36(7)	19	14
Total		124						0	0

Table S5: Secondary endpoint. Microbiological findings in relation to concomitant lung disease. Values within parentheses indicate the number of patients that provided the positive cultures. H. infl: *Haemophilus influenza*; M. cath: *Moraxella catharralis*; S. pne: *Streptococcus pneumonia*; S. aur: *Staphylococcus aureus*; Eba: *Enterobacteriaceae spp*; P. aer: *Pseudomonas aeruginosa*. **Fishers exact test, p=0.0467 (0/27 vs 4/25).

		H. infl.	M. cath	S. pne	S. aur	Eba	P. aer	Fungi	Sum
Asthma	Vit D, n=27	7(4)	4(2)	2(2)	2(2)	1(1)	1(1)	0**	17
	Plac, n=25	9(5)	6(5)	1(1)	4(4)	2(2)	4(1)	11(4)**	37
BE	Vit D, n=5	5(2)	1(19	0	4(1)	2(1)	0	1(1)	13
	Plac, n=7	1(1)	0	0	8(1)	1(1)	0	3(1)	13
COPD	Vit D, n=5	9(2)	1(1)	0	3(1)	2(1)	0	0	15
	Plac, n=4	0	0	0	8(2)	0	0	0	8

Table S6: Genotyping. Influence of genetic factors on the outcome of vitamin D_3 treatment in patients with frequent respiratory tract infections. Mean infectious score (0-12 months) are presented per genotype and study group, along with the number of included patients. P-values refer to an interaction between genotype and study group.

Gene / SNP	Allel-group	Vitamin D_3 Mean inf score (n)	Placebo Mean inf score (n)	$p_{interaction}$
VDR: TaqI	tt tT /TT (reference)	145 (11) 202 (49)	225 (12) 253 (47)	0.757
VDR: FoqI	ff fF /FF (reference)	295 (5) 195 (56)	331 (6) 238 (53)	0.575
CYP27B1: Rs10877012	CC AC/CC (reference)	181 (27) 220 (34)	211 (21) 268 (38)	0.194
GC: RS2282679	AA AC/CC (reference)	205 (39) 162 (21)	214 (28) 283 (29)	0.247
CYP2R1: Rs2060793	AA AG/GG(reference)	142 (8) 212 (53)	315 (14) 227 (45)	0.046
CYP24A1: Rs6013897	AA AT /TT(reference)	92 (2) 207 (59)	221 (2) 249 (57)	0.473

Table S7. Detailed description of adverse events.

Group	Adverse event	Vit D	Placebo	Total
CNS	Headache	5		5
	TIA (Transient Ischemic attack)	1		1
	Vertigo	4		4
CNS Total		10		10
Numbness - pain- shakings	Numbness, pain		8	8
	Paresthesias		2	2
	Tremor	1		1
Numbness-pain-shakings Total		1	10	11
Gastrointestinal symptoms	Diarrhoea		2	2
	Diverticulitis		4	4
	Dyspepsia	2	2	4
	Gastroenteritis	2	2	4
	Helicobacter pylori infection		2	2
Gastrointestinal symptoms Total		4	12	16
Heart/ vessels	Congestive heart disease		2	2
	Hypertension		2	2
	Thrombosis		2	2
Heart/ vessels Total			6	6
Infections	Herpes Zoster	1		1
	Pneumonia	1		1
	Sinusitis		2	2
	Urinary tract infection		4	4
	Pyelonephritis		2	2
Infections Total		2	8	10
Body pain – joint pain	Bursitis		2	2
	Body pain	2	2	4
	Joint pain fingers/ hands	1	2	3
	Joint pain hip		4	4
	Pain in feet	1		1
	Back pain	5		5
	Elbow swelling	1		1
Body pain- joint pain Total		10	10	20
Lungs	Asthma exacerbation	1		1
	Pneumothorax		2	2
	Heavy breathing		2	2
Lungs Total		1	4	5
Ears	Hearing problems	1		1
Ears Total		1		1
Other	Shivering	2		2
	Menstruation too often		2	2
	Nose bleeding	1		1
	Toothache	1		1
	Artheritis temporalis		2	2
Other Total		4	4	8
Rash – itch - blisters	Tongue blisters	1		1
	Hand rash	1		1
	Foot rash	1		1
	Facial rash when drinking alcohol		2	2
	Facial rash	1		1
	Chest rash, itching	1		1
Rash – itch – blisters Total		5	2	7
Total		38	56	94

Supplementary figures

Figure S1. The diary that was used for patients to register their daily symptoms.

Symptoms from "airways", "ears" and "sinuses" were calculated as maximum 1 point per anatomical site per day. "Malaise" and "antibiotic consumption" gave maximum 1 point per day. The occurrence of X-ray verified pneumonia resulted in 3 extra points per day for one week. Thus, 8 points was the maximum value that could be obtained per day. These data constituted the primary endpoint of the study. The diaries were filled out by the patient and sent monthly per mail to the study site.

Figure S2. Primary endpoint. Temporal analysis of infectious score components.

The adjusted one-year relative scores presented separately for each 3 month period. (A) airways, (B) malaise, (C) ear symptoms, (D) sinus symptoms and (E) antibiotic consumption. Effects are presented as relative scores (airway and malaise) or odds ratios (ear, sinus and antibiotics). Dashed lines indicate 95% confidence intervals.

Figure S3. Secondary endpoint. Number of bacterial cultures in relation to concomitant lung disease. The number of bacterial cultures taken (A) and positive bacterial cultures (B). Asthma (vit D, n=22; Placebo, n=22). Bronchiectasis, BE (Vit D, n=5; Placebo, n=7). Chronic Obstructive Pulmonary Disease, COPD (Vit D, n=4; Placebo, n=4).

Figure S4. Antimicrobial peptides in nasal fluid. Levels of LL-37 (A) and HNP1-3 (B) were measured in nasal fluid extracts at day 0, 6 and 12 months in a randomly selected group of patients (LL-37, n=12; HNP1-3, n=15). There were no statistically significant differences within or between the groups with regards to peptide levels (Mann-Whitney U test). Bacterial

growth in these samples were also recorded (C) and expressed as either ‘no growth/normal flora’ or ‘growth of a primary pathogen’. The growth pattern of the vitamin D_3 and Placebo groups were compared at each time-point using Fisher’s exact test.

Figure S5. Blood chemistry. Plasma levels of calcium (mmol/L), phosphate (mmol/L), albumine (g/L) and creatinine (µmol/L) were measured at the time points 0, 3, 6, 9 and 12 months after inclusion. Values are expressed as mean values.

Figure S1.

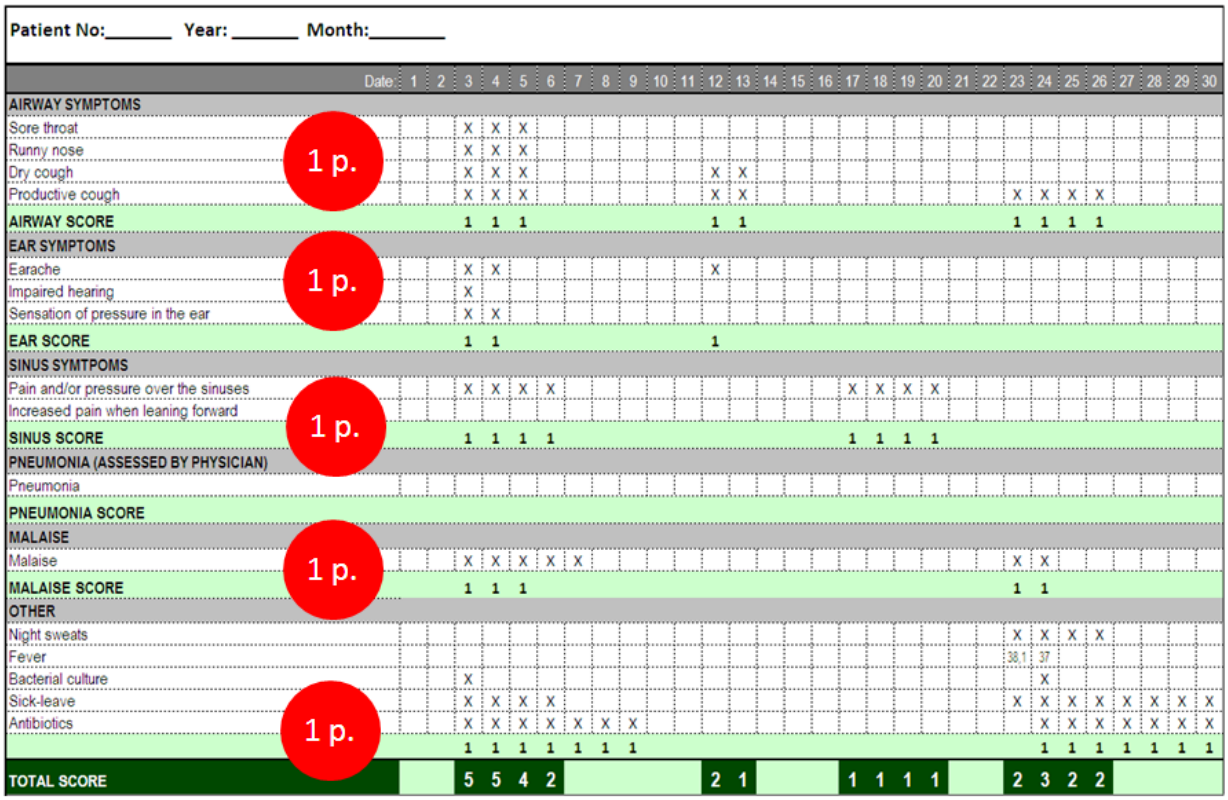


Figure S2

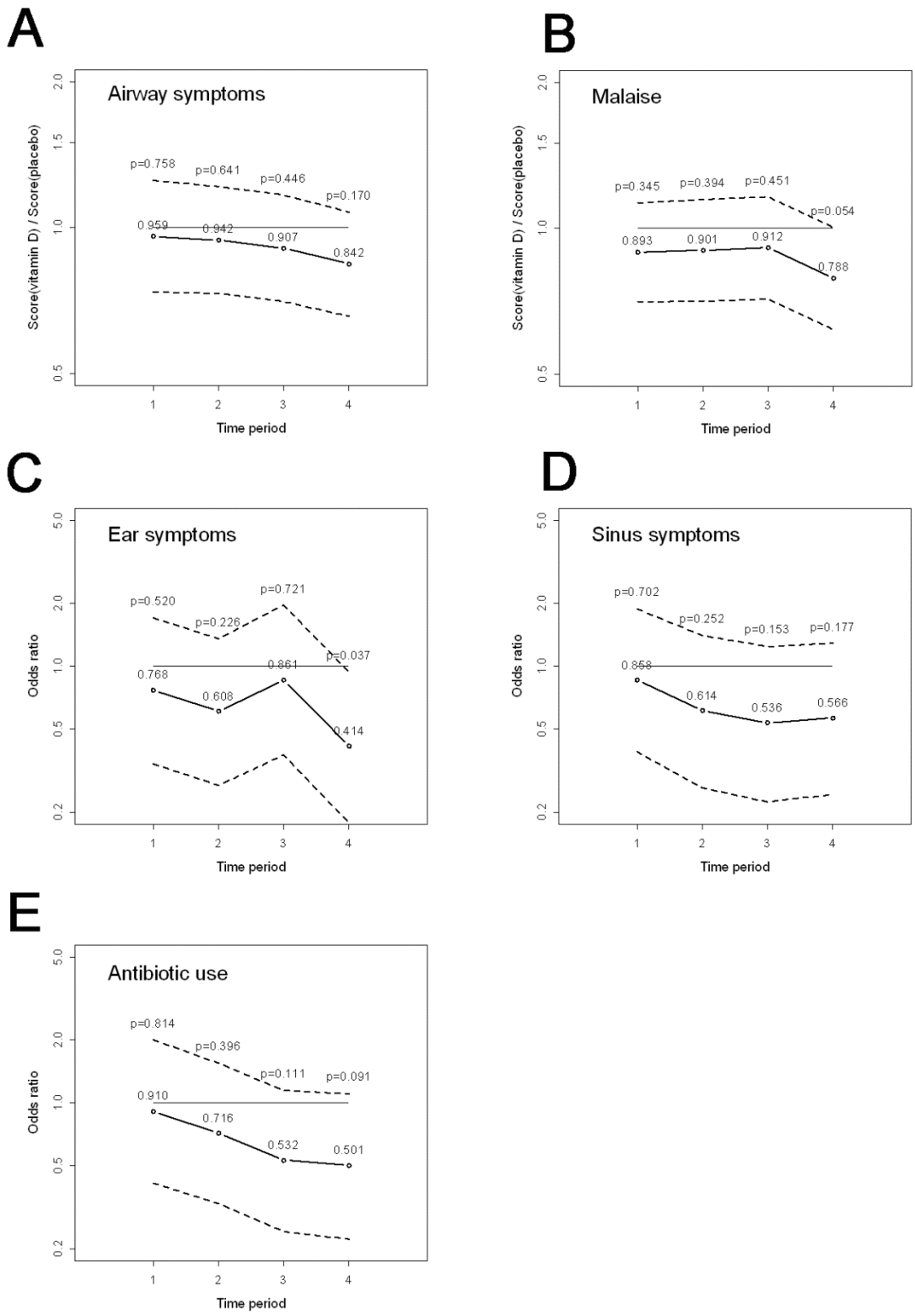

A
Airway symptoms
Score(vitamin D) / Score(placebo)
p=0.758
p=0.641
p=0.446
p=0.170
0.959
0.942
0.907
0.842
Time period
B
Malaise
Score(vitamin D) / Score(placebo)
p=0.345
p=0.394
p=0.451
p=0.054
0.893
0.901
0.912
0.788
Time period
C
Ear symptoms
Odds ratio
p=0.520
p=0.226
p=0.721
p=0.037
0.768
0.608
0.861
0.414
Time period
D
Sinus symptoms
Odds ratio
p=0.702
p=0.252
p=0.153
p=0.177
0.858
0.614
0.536
0.566
Time period
E
Antibiotic use
Odds ratio
p=0.814
p=0.396
p=0.111
p=0.091
0.910
0.716
0.532
0.501
Time period

Figure S3

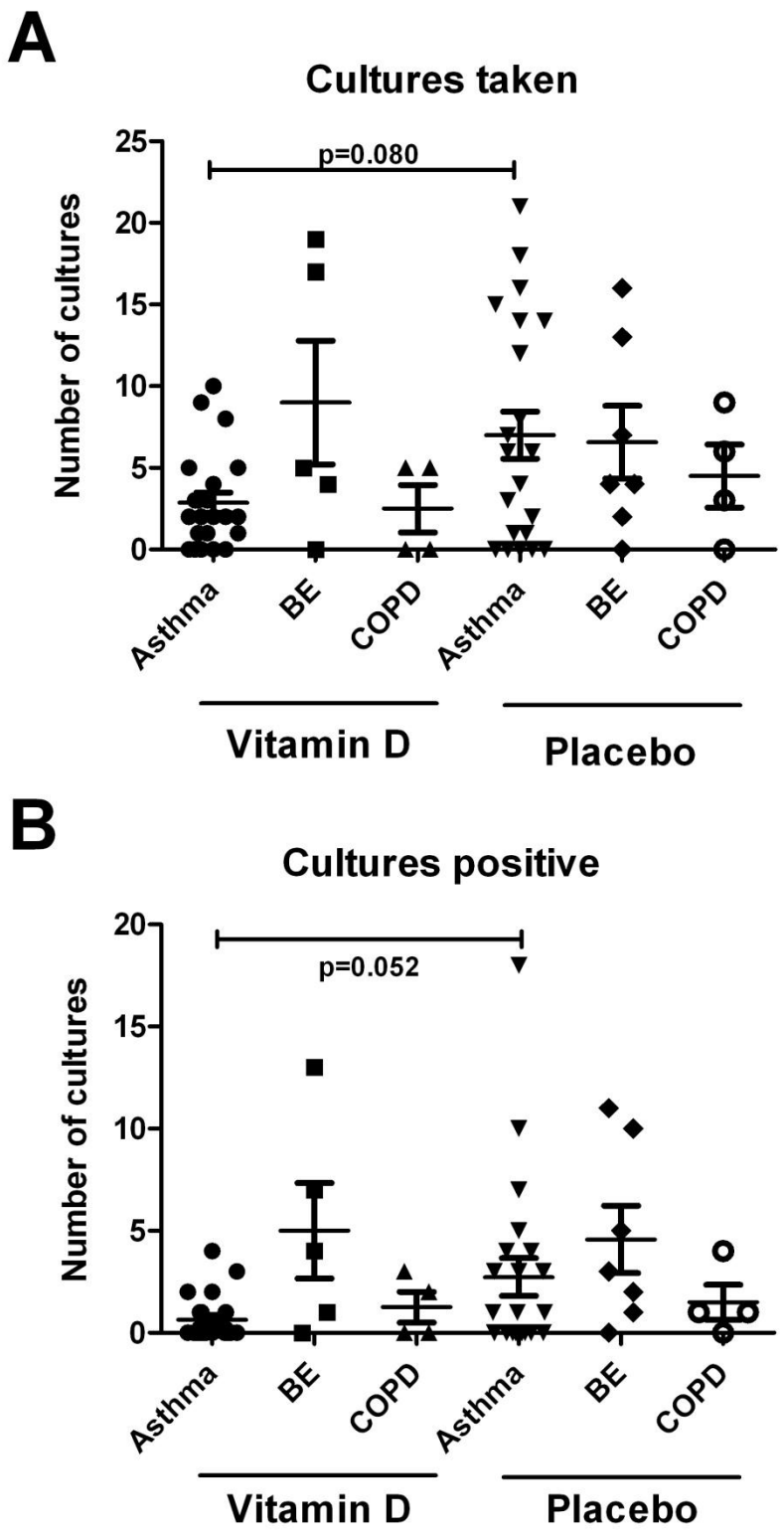

A
Cultures taken
p=0.080
Number of cultures
25
20
15
10
5
0
Asthma
BE
COPD
Asthma
BE
COPD
Vitamin D
Placebo
B
Cultures positive
p=0.052
Number of cultures
20
15
10
5
0
Asthma
BE
COPD
Asthma
BE
COPD
Vitamin D
Placebo

Figure S4

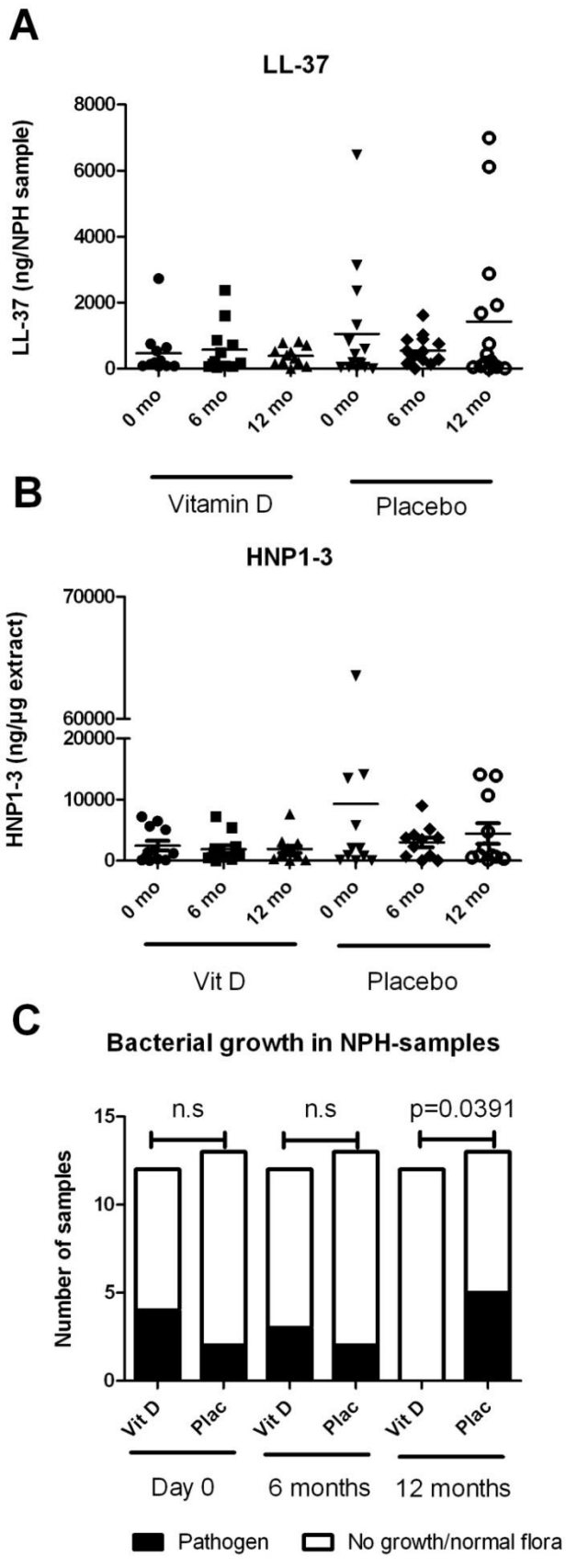
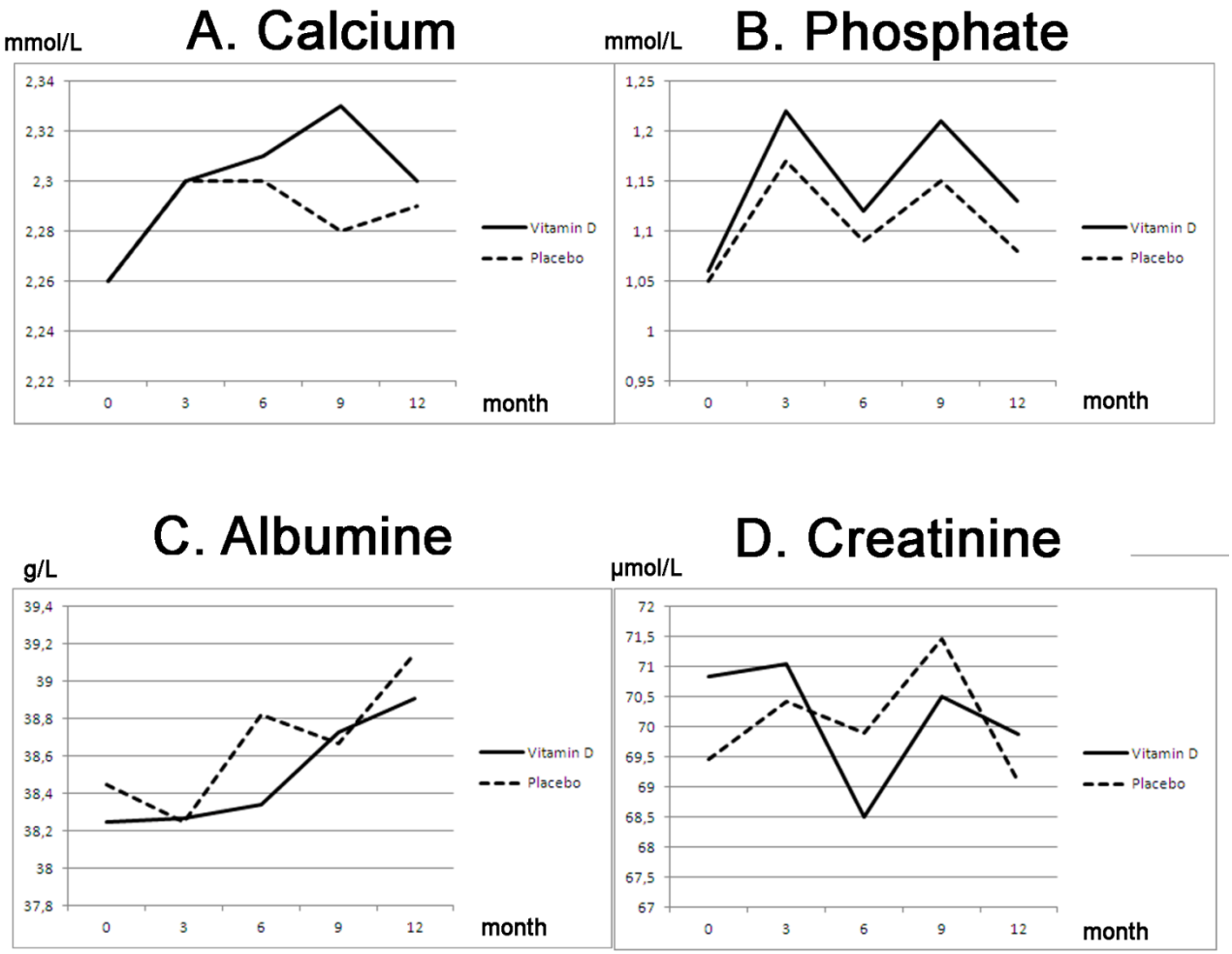


Figure S5



CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	p. 1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	p. 2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	p. 5-6
	2b	Specific objectives or hypotheses	p. 6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	p. 7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	Not Applicable (NA)
Participants	4a	Eligibility criteria for participants	p. 7
	4b	Settings and locations where the data were collected	p. 7
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	p. 8
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	p. 8
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	Suppl meth p4
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	Suppl meth.
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Suppl meth.
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	Suppl meth.

Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	p. 9
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	Suppl meth.
	11b	If relevant, description of the similarity of interventions	NA
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	p. 8-9
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	Suppl meth.
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	p. 10, Fig 1
	13b	For each group, losses and exclusions after randomisation, together with reasons	p. 10, Fig 1
Recruitment	14a	Dates defining the periods of recruitment and follow-up	p. 5
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Yes
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Yes
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Yes
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Yes
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Table 5 +suppl fig/tabl
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Yes
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	Yes
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Yes
Other information			
Registration	23	Registration number and name of trial registry	p. 7
Protocol	24	Where the full trial protocol can be accessed, if available	p. 7
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	p. 20

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Study title:

Study of Vitamin D3 Substitution to Patients With Primary Immunodeficiency (VITAPID)

Date: October 6, 2009

Study site: Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden

Product: Vigantol oil

Substance: Vitamin D3 (cholecalciferol)

Producer: Merck, Germany

EudraCT-number: 2009-011758-16

Sponsor: Professor Jan Andersson, MD, PhD

Co-investigators: Dr Peter Bergman, MD, PhD; Dr Anna-Carin Norlin, MD

Study facts

Protocol identity and aim

EudraCT-number: 2009-011758-16

Protocol title: A placebo-controlled double-blind study of Vitamin D3 supplementation to patients with increased susceptibility to infections.

Aim: To investigate if substitution with vitamin D3 can prevent or ameliorate infectious burden among infection prone patients.

Study drug:

Product:	Vigantol Oil
Pharmaceutic preparation:	Oral mixture (oil)
Administration:	Per os

Methodology

Study design:	Randomized double-blind placebo-controlled
Dose:	Vigantol, 4000 IU/day
Primary endpoint:	Infectious score
Safety parameters:	Plasma levels of calcium, creatinine, albumin and phosphate; serum levels of 25-OH vitamin D_3.
Study population:	Patients with increased susceptibility to respiratory tract infections.
Number:	140

Timeplan:

First patient to be included: Q1, 2010

Last patient to be included: Q4, 2011

Last patient to finish the study: Q4, 2012

Administrative information

Sponsor and Investigators

Professor Jan Andersson, MD, PhD: Sponsor and Principal Investigator

Dr Peter Bergman, MD, PhD: Co-investigator

Dr Anna-Carin Norlin, MD: Co-investigator

Research nurses and study coordinator

Susanne Hansen, Study coordinator, head of Immunodeficiency Unit

Kristina Johansson, Research Nurse

Maria Lindén, Research Nurse

Quality control

Two independent monitors from Karolinska Trial Alliance will monitor the study according to ICH-GCP.

Overview and significance

The innate immune system is depending on antimicrobial peptides, which are potent killers of microbes, such as bacteria, viruses and fungi. These molecules defend epithelial surfaces and are rapidly released after contact with microbes. Vitamin D is a potent inducer of AMPs in epithelial and immune cells. Vitamin D is synthesized in the skin under the influence of UVB-light or can be obtained via the diet. However, in Sweden the UV-radiation has too low intensity during the wintertime and the diet is not enough to maintain adequate levels. Therefore many individuals in Sweden have low levels of vitamin D3, especially during the darker period of the year (October-April). Epidemiological data show a strong association between low vitamin D levels and an increased risk of infection. There is also mechanistic evidence that vitamin D increases the levels of antimicrobial peptides in macrophages and in epithelial cells. However, there are few randomized controlled trials testing the hypothesis that supplementation with vitamin D3 can reduce or ameliorate infections. Therefore, we have designed the study described in this protocol where vitamin D3 will be given to patients with an increased risk of infection. The results may have a great impact on treatment of patients with frequent infections, since vitamin D3 may be used in conjunction with standard care (antibiotics). This may be particularly important in light of the emerging bacterial resistance. Thus, novel strategies to prevent and treat infectious diseases have to be developed and supplementation with Vitamin D3 may constitute one future treatment option.

Aims

To investigate if substitution with vitamin D3 can:

1. Reduce the infectious burden among patients with increased number of infections
2. Increase levels of antimicrobial peptides in nasal fluid
3. Increase serum concentrations of 25-OH vitamin D3

Study design

Participants will be given vitamin D3 or placebo for one year. 140 patients will be recruited and 70 patients will be randomized to placebo or vitamin D3 in a 1:1 randomization. Evaluation of symptoms and antibiotic consumption will be registered by the patient in a diary form that will be sent by mail to the study site on a monthly basis. Patients will be recruited at the tertiary center for primary immune deficiencies. Currently there are 319 patients with IgG-deficiency, 180 patients with selective IgA-deficiency, 90 patients with CVID and 210 patients with an increased susceptibility to infection without a manifest immunological diagnosis. The study patients will be recruited from this group in a nonbiased fashion, ie regardless of diagnosis or IgG-substitution therapy.

Study drug and mechanism

The study drug is cholecalciferol (vitamin D3), which is hydroxylated in the liver to 25-OH vitamin D3 (the storage form in the body). The second hydroxylation step is carried out by 1-alpha hydroxylase. This enzyme is expressed in the kidney but also in immune- and epithelial cells. The kidney is

responsible for the systemic production of 1,25 (OH)2 vitamin D3, which is crucial for skeletal health (endocrine system). The local activation of vitamin D3 in immune- and epithelial cells is described as a paracrine system and is central to the immune effects of vitamin D. The paracrine system is strictly regulated and does not contribute substantially to the systemic levels of 1,25 (OH)2 vitamin D3. This is important since the active and systemically available vitamin D3 is responsible for hypercalcaemia that has been reported as an adverse event for vitamin D3 supplementaton. However, hypercalcaemia is a very rare event and we will strictly follow plasma levels of calcium and 25-OH vitamin D3 during the study period.

Study drug

Vigantol Oil is not a registered drug in Sweden. However, Merck Pharma GmbH has permission to manufacture and sell Vigantol in Germany since many years (permission nr 6154275.00, ATC code A11CC05, mSPC available upon request). The study drug is manufactured according to GMP (GMP certificate from Merck available upon request). The placebo oil is also manufactured by Merck and has identical galenic properties to Vigantol oil. The drugs (vigantol and placebo) will be delivered to Vecura AB at Karolinska University Hospital, Huddinge. Vecura AB is a company specialized in clinical trials and has a GMP-certificate for clinical trials and handling of study drugs. VECURA AB will aliquot the study drug and placebo to the final bottles, carry out randomization and labelling.

Endpoints

Primary endpoint

Infectious score based on patient recorded information registered in a diary-form.

Secondary endpoints

25-OH vitamin D3 in serum

Microbiological findings and numbers of cultures taken

Levels of antimicrobial peptides in nasal fluid

Antibiotic consumption collected from patient records

Design

Evaluations and procedures

Prescreening: Eligible patients fulfilling inclusion criteria will be selected from records and contacted via regular mail. They will be sent a letter of invitation together with information on the study. All these patients will be contacted via telephone one week later and asked for participation.

Visit 1, screening, time=0: Co-investigator (Licenced physician, MD) will meet all patients for screening. Additional information on the study will be given and informed consent will be collected. If the patient is judged to fulfil all criteria for inclusion and all exclusion criteria can be negated, the patient is included in the study. The study drug for 6 months will be given out to the patients as well as diaries and envelopes. The patient will be carefully informed about the procedures with the diaries. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Visit 2, time=6 months +/- 14 days: Co-investigator (Licenced physician, MD) will meet all patients after 6 months. A control for adverse events and compliance will be carried out. Additional bottles of study drug/placebo will be given out for the remaining 6 months of the study. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Visit 3, time= 12 months +/- 14 days: Co-investigator (Licenced physician, MD) will meet all patients after 12 months. A control for adverse events and compliance will be carried out. Blood will be drawn: serum 25-Oh vitamin D3, plasma samples for calcium, creatinine, phosphate and albumine. Every fifth patient, according to a special randomization list, will leave a sample for nasal fluid.

Participants

Inclusion Criteria:

- Age 18-75
- Increased number of respiratory tract infections
- At least 42 days of infections during 2008 or 2009
- S-25 OH vitamin D3 < 250 nM
- Not planning a pregnancy during the coming year
- Accepting the use of contraceptives during 1 year

Exclusion Criteria:

- Continuous antibiotic treatment
- Hypercalcaemia
- Sarcoidosis
- Kidney disease
- Tuberculosis
- Pregnancy
- Previous history of kidney stones
- Heart medication (glycosides)

Treatment

Vigantol oil (cholecalciferol). 1 drop contains 500 IU. The patients should take 8 drops per day during the study.

Packing, labelling and handling of study drug: Merck will distribute Vigantol oil and placebo oil to VECURA AB, Karolinska University Hospital, Huddinge, which will handle, pack and label the study drug.

Distribution of the study drug to the participants: At the first visit, the participants will be given study drug for the first 6 months of the study. After 6 months, they will be given the remaining bottles. Oral and written instructions will be given about 8 drops per day .

Blinding and breaking of the code: The design is double-blind. Thus, neither the doctor/nurse nor the patient will have any information on the nature of the study drug. Two monitors will carry out controls of the study. The randomisation list will be stored in such a way that the personnel involved in the study do not have access to it. The Hospital Pharmacy will be given a copy of the list in case of emergency with access via telephone 365 days per year/24 hours per day.

Concomitant medication: All other medication is allowed during the study, including antibiotics. However, recent changes in drug treatments will be documented in the diary and asked for by the study doctors at visits.

Compliance: The compliance to the study drug and diary registration is asked for at visits to the study site.

Control of the study drug: Patients are asked to bring back their empty bottles to the study sites. All bottles will be registered by the study nurses.

Evaluation of efficacy and safety

Evaluation of primary endpoint (clinical endpoint, patient recorded)

The primary endpoint is the infectious score, which is based on the diary form filled out by the patient. The total score is composed of symptoms from airways, sinus and ears together with malaise and antibiotic consumption. The idea is to monitor several aspects of an infectious episode and thus monitor the total infectious burden, rather than a specific symptom.

Evaluation of secondary endpoints (microbiological and biochemical endpoints, collected by the study personnel)

1. 25-OH vitamin D3 in serum
2. Microbiological findings and numbers of cultures taken. This information will be collected from patients' clinical records with a focus on samples taken from the respiratory tract.
3. Levels of antimicrobial peptides in nasal fluid. Every fifth patient (according to a special randomization list) will be asked to leave nasal fluid for analysis of antimicrobial peptides.
4. Antibiotic consumption collected from patient records. Information on how many prescriptions of antibiotics will be collected from patients' records.

Evaluation of clinical safety for participants

Patients will leave blood for analyses of plasma levels of creatinin, calcium, phosphate and albumin as well as serum levels of 25-OH vitamin D3 at times 0, 3, 6, 9 and 12 months. The information regarding all time-points except at inclusion will be sent to an unblinded senior physician who will serve as an external clinical safety monitor. He will contact the study physicians in case of clinically relevant abnormalities in the blood chemistry.

Samples and clinical chemistry

Serum and plasma from the first sampling will be sent to Dept of Clinical Chemistry, Karolinska University Hospital, Huddinge for routine analyses. These answers will be recorded in patients' records. For all other time points samples will be sent to Study Center Karolinska which will coordinate all samples for clinical chemistry and send answers to the unblinded clinical safety monitor. These answers will not appear in patients' records in order to keep the blinded design intact.

Adverse events (AE) and Severe adverse events (SAE)

All adverse events and severe adverse events will be recorded in special forms. They will further be classified for severity (mild, moderate and severe) and for connection with the study drug (probable, possible and unlikely). All SAE will be reported to the sponsor within 24 hours after it has been known by the investigator.

Statistics

Handling of data: All data will be registered in a database especially constructed for the study.

Analysis of excluded patients: Excluded patients will be recorded and followed for adverse events. After the study, special analyses will be performed to understand why these patients did not complete the study.

Statistical analysis and power calculation: The statistical calculation is based on the assumption that the infectious score is reduced with 30 % from 42 days (42x5=240 points) to 28 days (28x5=160 points) with full infectious score. If we include 60 patients per group a significance level of p=0.02 will be reached with a power of 90%. To compensate for expected exclusions, we will increase the number of patients per group to 70. Thus, the total number of patients in the study will be 140.

Quality control

Source data: The information that will be collected from each participant will be: study title, screening number, patient number, written informed consent, main and concomitant diagnoses, study treatment, medication, data on blood chemistry and other investigations carried out.

Monitoring: All study personnel have knowledge on clinical trials and ICH-GCP. The sponsor will sign a contract with Karolinska Trial Alliance for monitoring. The investigator will allocate time for monitoring and supply all available and relevant information to the monitors.

Ethics

The sponsor has applied for ethical approval from the regional Ethical Board. The study will be carried out according to ICH-GCP and the Helsinki-declaration.

Informed consent: The patient will have information sent home via regular mail. One week later the study nurse will call the patient and discuss the study. The first visit will involve the meeting with a physician and time is extended for questions before the written informed consent is signed. One copy will stay at the study site and one copy will go with the patient.

Handling of data

Case Report Forms (CRF) will be used. These will kept at the study site until the end of the study. After completion of the study, all material will be archived at least 10 years.

Insurance

All participants are insured via patient insurance and the Swedisg drug insurance.

Publication of the results

The study group wishes to publish the data in a refereed international scientific journal and to communicate the results at conferences and other venues.

The original protocol is written in Swedish and is available on request.