

Bergman et al, Vitamin D₃ 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 D₃ 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 D₃ can induce antimicrobial peptides both in macrophages and in epithelial cells¹, 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 *Enterobacteriaceae* 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, 2011².

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 in². The lyophilized polypeptide extract was reconstituted in 0.1% TFA to a concentration of 5 $\mu\text{g}/\mu\text{l}$ 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 in³. Antibodies used were a LL-37 monoclonal⁴ 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 D₃ in serum

Levels of 25-hydroxyvitamin D₃ 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 D₃ 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 D₃ supplementation. Genomic DNA was isolated from 200 µl peripheral blood leucocytes using the DNA Blood Mini kit (Qiagen, Hilden Germany). 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 used⁵. 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 D₃ 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 literature⁵⁻¹⁰.

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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