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Neme et al.: In vivo response of the human PBMC transcriptome to vitamin D

In vivo transcriptome changes of human white blood cells in response to vitamin D

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HIGHLIGHTS

- In human PBMCs 702 genes are significantly \((p < 0.05)\) affected by a vitamin \(D_3\) bolus.
- These genes are involved in general protein translation, monocyte differentiation and cellular growth control.
- The expression pattern of vitamin D target genes differed significantly between individuals.

ABSTRACT

In the vitamin D intervention study VitDbol (NCT02063334) blood samples were drawn directly before an oral bolus \((2000 \mu g \text{ vitamin } D_3)\) and 24 h later. The focus of phase II of VitDbol was the transcriptome-wide analysis of the effects of vitamin D gene expression in human peripheral blood mononuclear cells (PBMCs). All five participants responded in an individual fashion to the bolus by increases in serum levels of the vitamin D metabolites 25-hydroxyvitamin \(D_3\) \((25(OH)D_3)\) and \(1\alpha,25\)-dihydroxyvitamin \(D_3\) \((1,25(OH)_2D_3)\). RNA sequencing identified 15,040 commonly expressed genes in PBMCs, 702 \((4.7\%)\) of which were significantly \((p < 0.05)\) affected by the vitamin \(D_3\) bolus. KEGG pathway analysis suggested that these genes are involved in general protein translation, monocyte differentiation and cellular growth control. Previously published transcriptome-wide studies in comparable cell systems confirmed 234 of the 702 vitamin D target genes, leaving many genes, such as \(HLA\)-A and \(HLA\)-C, as novel discoveries. Interestingly, \textit{in vivo} stimulated PBMCs of this study showed a larger number of common vitamin D target genes with the monocytic cell line THP-1 than with \textit{in vitro} stimulated PBMCs. The expression pattern of vitamin D target genes differed significantly between individuals and the average expression change can serve as a marker for vitamin D responsiveness. In conclusion, this study demonstrates that under \textit{in vivo} conditions changes in 25(OH)D3 and 1,25(OH)_2D3 serum
concentrations alter the expression of more than 700 vitamin D target genes in human leukocytes.

**ABBREVIATIONS**

- $1,25(\text{OH})_2\text{D}_3$: 1α,25-dihydroxyvitamin D$_3$
- 25(OH)D$_3$: 25-hydroxyvitamin D$_3$
- BMI: body mass index
- FC: fold change
- FE: fold enrichment
- HLA: human leukocyte antigen
- KEGG: Kyoto Encyclopedia of Genes and Genomes
- MHC: major histocompatibility complex
- PBMC: peripheral blood mononuclear cell
- RNA-seq: RNA sequencing

**Keywords:** VitDbol; vitamin D$_3$; 25(OH)D$_3$; transcriptome; RNA-seq; vitamin D$_3$ bolus supplementation; PBMCs; vitamin D target genes.
INTRODUCTION

Vitamin D₃ is a biologically inert pre-hormone that mediates its physiological functions, such as maintaining the balance of calcium and phosphorus homeostasis, modulating innate and adaptive immunity as well as controlling cellular growth and differentiation, via its metabolite 1,25(OH)₂D₃ [1-3]. The latter molecule is a high affinity ligand to the transcription factor vitamin D receptor, i.e. vitamin D directly affects the expression of hundreds of genes [4]. Serum levels of the most stable vitamin D metabolite, 25(OH)D₃, serve as a biomarker of the vitamin D status of individuals [5]. A 25(OH)D₃ serum concentration below 50 nM can have musculoskeletal consequences, such as rickets in children and osteomalacia and fractures in adults [6]. Moreover, vitamin D insufficiency is linked to multiple sclerosis, diabetes, cardiovascular disease and cancers of the breast, prostate and colon [7-11].

The design of the vitamin D intervention trial VitDbol (NCT02063334, ClinicalTrials.gov) allowed to study in human vitamin D-triggered effects on gene expression under in vivo conditions. In phase I of VitDbol, 35 healthy young adults were exposed to a vitamin D₃ bolus (2000 µg) and PBMCs were isolated before as well as one and two days after the bolus. PBMCs are a mixture of the vitamin D-responsive cell types, such as monocytes, T and B lymphocytes, that can be obtained with minimal harm from human volunteers in less then an hour after drawing blood, i.e. their status is as close as possible to the in vivo situation [12]. Excluding any further in vitro culture, RNA and chromatin were isolated from PBMCs for testing expression changes in selected primary vitamin D target genes [13] and alterations in chromatin accessibility at a few genomic regions [14]. A ranking of the data collected for each VitDbol participant using the machine learning algorithm k-means suggested a classification of the individuals into high, mid and low vitamin D responders [15]. Since this so-called vitamin D response index classification was based only on a limited number of parameters, we started phase II of VitDbol, in which data were collected for epigenome- and transcriptome-wide assessment. In a proof-of-principle study one individual had been exposed three times in a row every 28 days to a vitamin D₃ bolus and epigenome-wide changes in chromatin accessibility were assessed at nine time points over 58 days [16]. This approach
allowed the identification of 70 genomic loci that showed already after one day and 361 sites after two days significant (p < 0.001) vitamin D-triggered chromatin opening or closing, i.e. a modulation of the epigenome.

In this study, we extended phase II of VitDbol by using RNA sequencing (RNA-seq) for the transcriptome-wide analysis of the effects of a vitamin D₃ bolus on human PBMCs. On the basis of five individuals we found 702 genes being significantly (p < 0.05) affected by vitamin D, 234 of which were confirmed by other transcriptome-wide studies using comparable conditions. The transcriptome-wide vitamin D target gene profile allowed the ranking of the study participants with higher accuracy than previous investigations. This study is the first report of short-term, transcriptome-wide effects of vitamin D in human under in vivo conditions.

MATERIAL AND METHODS

Sample collection

In the VitDbol (NCT02063334) study individuals were exposed once to a vitamin D₃ bolus (2000 µg). The bolus was provided in 25 tablets of 80 µg, the first 13 of which were drawn after blood sampling during an ad libitum breakfast and the remaining 12 some 3 h later during ad libitum lunch. Individuals 1, 2, 4 and 5 were 22 years old females that already participated in phase I of VitDbol [14], i.e. no new blood samples were drawn. In contrast, individual 3 was a 51 years old male, who was recruited exclusively for phase II of the study. The individuals were selected based on their basal 25(OH)D₃ levels, in order to represent a wide range (39.7 to 106.5 nM, Table 1). The study took place in January and February 2015, i.e. during a period of no UVB exposure from the sunlight in Finland. The research ethics committee of the Northern Savo Hospital District had approved the study protocol (#9/2014). All participants gave a written informed consent to participate in the study.

Blood samples for serum and PBMC isolation were collected after overnight (12 h) fasting of the study participants at days 0 and 1. Serum 25(OH)D₃ concentrations were measured using
a high performance liquid chromatography/coulometric electrode array as described previously [17]. The levels of 1,25(OH)₂D₃ were determined by applying a chemiluminescence immunoassay involving a paramagnetic microparticle solid phase (LIAISON® XL, DiaSorin, Saluggia, Italy).

**RNA-seq from PBMCs**

PBMCs were isolated within one hour after blood draw from 20 ml of peripheral blood in Vacutainer CPT Cell Preparation Tubes with sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions. Total RNA was extracted using the Direct-zol™ RNA MiniPrep kit (Zymo Research) including DNAse I digestion following the manufacturer’s protocol. RNA quality was assessed by using an Agilent Bioanalyzer and library preparation was performed after rRNA depletion.

**RNA-seq analysis**

RNA sequencing (RNA-seq) is a next generation sequencing method that has largely replaced microarray technology being based on hybridization with pre-selected oligonucleotide samples [18]. RNA-seq libraries were sequenced at 50 bp read length on a HiSeq2000 system using standard Illumina protocols at the Gene Core of the EMBL (Heidelberg, Germany). After quality control using afterQC [19], RNA-seq analysis was conducted by processing the high-quality reads by applying kallisto [20] with parameters –b 100 –single –I 180 –s 20. This software approximates abundance in a fast and efficient way via a pseudo-alignment stage to a reference genome (hg19). Differential gene expression was computed using DESeq2 [21], which implements a negative binomial test over the reads in the two conditions (treated/untreated), with standard parameters and a p-value cutoff of 0.05. DESeq2 is robust enough as to detect differential expression in relatively low numbers of datasets, like in this study, since the assumptions behind the negative binomial distribution are stringent and allow proper extrapolations. Equally relevant is that the low number of datasets can also be obtained
from notoriously distinct individuals, i.e., subjects of different ages or life styles, and the algorithm will be able to find differential expression.

RESULTS

Vitamin D metabolite changes after a vitamin D₃ bolus

For phase II of the VitDbol study four participants of phase I [14] and one new recruit were selected, their basal vitamin D status covering a rather wide range of 39.7 to 106.5 nM serum 25(OH)D₃ concentrations (Table 1). The individuals were exposed to an oral vitamin D₃ bolus of 2000 µg, which resulted within 24 h in 25(OH)D₃ serum level rises of 12.3 to 40.9 nM (14 to 62%) and an increase in 1,25(OH)₂D₃ concentrations of 2.6 to 58.8 pM (3 to 35%). These increases seem to occur in an individual fashion that neither depends on the basal vitamin D status nor on the 25(OH)D₃/1,25(OH)₂D₃ ratio of the study participants.

In summary, all participants of VitDbol’s phase II responded within 24 h after the vitamin D₃ bolus treatment by changes of the level of both vitamin D metabolites, 25(OH)D₃ and 1,25(OH)₂D₃.

The vitamin D-modulated transcriptome in PBMCs

PBMCs were isolated from blood samples drawn at days 0 and 1 and total RNA was extracted directly, i.e. there was no further in vitro culture of the cells. After rRNA depletion DNA libraries were prepared, which were exposed to massive parallel sequencing. Within the 5 x 2 PBMC samples 15,040 genes (64% of all) were commonly expressed, 702 of which were significantly (p < 0.05, for a negative binomial test computed with DESeq2) affected by the vitamin D₃ bolus (Table S1). For comparison, more strict statistical thresholds of p < 0.01 and p < 0.005 resulted in 146 and 82 target genes, respectively. From the pool of 702 vitamin D target genes 501 were up-regulated in a range of 1.09- to 5.48-fold and 201 were 1.22- to 3.29-fold down-regulated (Fig. 1). The list included many genes, such as HLA-
A, HLA-C, CEBPE, SPI1 (encoding for PU.1) and NR1H2 (encoding for LXRβ), which so far had not been reported as vitamin D targets. The 702 genes were found in all 22 autosomal chromosomes and the X chromosome, but highest density of vitamin D targets was observed on chromosomes 16, 19 and 22, while they were rarely found on chromosomes 4, 6, 12, 21 and X.

In order to understand how the vitamin D-modulated transcriptome translates into physiological functions, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed by using the webtool Enrichr [22]. Surprisingly, the top-scoring pathway was “Ribosome”, because many ribosomal genes lighted up as vitamin D targets (Table 2). This suggested a general increase of ribosomal protein translation activity after vitamin D stimulation. Second ranking was the pathway “Acute myeloid leukemia” prominently represented by genes encoding for the myeloid lineage determining transcription factors CEBPA and PU.1 (SPI1). Ranks 3 to 5 were the “p53 signaling pathway” containing vitamin D target genes, such as CDK6, CDKN2A and ATM, the “Non-alcoholic fatty liver disease” pathway with genes, like TGFB1 and PIK3CA, and the “Osteoclast differentiation” pathway being based on genes, such as JUN, JUND, JUNB and RELB, respectively.

Taken together, from 15,040 expressed genes in PBMCs, 702 (4.7%) were significantly (p < 0.05) affected within 24 h by a vitamin D₃ bolus. KEGG pathway analysis suggested that these target genes are involved in protein translation, monocyte differentiation and cellular growth control.

**Confirmation of vitamin D target genes**

The 702 vitamin D target genes that were identified by the human in vivo experiment reported here, were compared with genes reported by comparable studies (Fig. 2). RNA-seq analysis of THP-1 human monocytes, which were stimulated with 1,25(OH)₂D₃ in three biological repeats for 2.5, 4 and 24 h, found 3722 significantly (p < 0.05) regulated genes [23, 24], 181 of which overlapped with the 702 vitamin D targets in PBMCs (Fig. 2A). The well-known
vitamin D target genes $CD14$, $FBP1$, $LRRC25$, $DENND6B$ and $NINJ1$ were members of this list. In a study by Kariuki et al. [25] PBMCs from 85 individuals had been treated in vitro with 1,25(OH)$_2$D$_3$ for 24 h and were then analyzed by microarrays for transcriptome changes. We re-analyzed the raw data of this study by using the same settings for gene set analysis as in this study as well as in the THP-1 RNA-seq dataset [24]. This increased the number of significantly regulated genes from initially 720 to 1234 (Table S1). From these 1234 genes found in PBMCs stimulated in vitro with 1,25(OH)$_2$D$_3$, 78 overlapped with the 702 genes identified in in vivo stimulated PBMCs (Fig. 2B). The list of these overlapping vitamin D targets comprised genes, such as $CEBPB$, $CD14$, $CDK6$, $ESRRA$ and $SIRT6$. Finally, the study of Hossein et al. [26] used PBMCs from eight individuals, which had been supplemented daily with either 10 or 50 µg vitamin D$_3$ for two winter months. Gene expression was assessed by microarrays and 291 genes changed their expression by more than 1.5-fold during the supplementation period. From these 291 genes, 204 were found within the list of genes identified by RNA-seq (Table S1). However, only 13 of these genes, such as $JUND$, $JUNB$ and $JUN$, belonged to the list of 702 vitamin D target genes reported in this study (Fig. 2C).

In summary, 234 of the 702 identified vitamin D target genes had been observed in at least one previously published transcriptome study with comparable cell types. Interestingly, in vivo stimulated PBMCs showed a higher level of overlap with THP-1 monocytes than with PBMCs treated in vitro.

**Individuals’ responsiveness to vitamin D**

In order to evaluate the overall responsiveness of the five study participants to vitamin D based on their gene expression changes after the vitamin D$_3$ bolus, we plotted for each of them the FC of the 702 common vitamin D target genes (Fig. 3). The individuals were sorted by the FC of their 25(OH)D$_3$ serum concentrations caused by the bolus (Table 1). The range in gene expression changes and number of up-regulated genes varied significantly between the individuals (Table S1) suggesting the ranking individual 3 (621 genes), individual 4...
(551 genes), individual 2 (530 genes), individual 5 (499 genes) and individual 1 (304 genes). However, in the ranking of the average FC of the 702 genes, participant 4 came first with a value of 2.01-fold increase, then participant 3 with 1.53-fold increase, participant 5 with 1.09-fold increase, participant 1 with 1.02-fold decrease and finally participant 2 with 1.50-fold decrease (Fig. 3). Interestingly, the average FC of the 702 vitamin D target genes showed a negative correlation both with the change in 25(OH)D3 serum levels ($r^2 = 0.5098$, Fig. S1A) as well as in 1,25(OH)2D3 serum levels ($r^2 = 0.7198$, Fig. S1B).

Taken together, the five individuals can be distinguished and ranked by the average FC of their 702 vitamin D target genes. However, this ranking shows an unexpected negative correlation with the individuals’ changes in 25(OH)D3 and 1,25(OH)2D3 levels.

**DISCUSSION**

This study represents a safe human *in vivo* experiment, in which changes in the transcriptome of PBMCs of five individuals in response to a vitamin D3 bolus were determined. To our knowledge, this is the first study where under *in vivo* conditions the molecular response of a primary human tissue to vitamin D was tested in a rather short time frame, such as 24 h. Thus, in respect to timing the results of this study are directly comparable to *in vitro* cell culture experiments [4]. In fact, *in vitro* experiments use high 1,25(OH)2D3 concentrations, such as 10 to 100 nM, for stimulation and thus are *per se* bolus treatments. Moreover, in most cases vitamin D-depleted cells are the references for *in vitro* experiments, while *in vivo* experiments refer to the vitamin D status of individuals before vitamin D3 bolus supplementation. By purpose, the participants of this study were selected, in order to represent a rather wide range of 25(OH)D3 serum levels from 39 to 106 nM. Accordingly, the FC expression values of vitamin D target genes are *in vivo* studies far lower than *in vitro* experiments.

Based on five individuals, the vitamin D3 bolus treatment resulted in a statistically significant ($p < 0.05$) modulation of 702 genes. For an *in vivo* investigation this is a rather high number of genes and primarily relates to the experimental design of the study. However, even 2- to 5-
fold higher numbers of vitamin D target genes had been reported, when the same statistical threshold was applied to in vitro experiments in comparable cellular systems [24, 25]. The latter datasets confirmed a third of the 702 vitamin D target genes demonstrating that the regulation of a reasonable number of genes is stable enough to keep their responsiveness to vitamin D even when the cells are transferred from an in vivo to an in vitro environment. Although monocytes represent only some 10% of the cells in PBMCs, in vivo stimulated PBMCs show a higher percentage of overlap in vitamin D target genes with the monocytic cell line THP-1 than with in vitro cultured PBMCs. This parallels the results of other genome-wide dataset comparisons between in vivo stimulated PBMCs and THP-1 cells. For example, more than 85% of the prominent sites of chromatin accessibility in PBMCs are also found in THP-1 cells [23]. Accordingly, monocytes may be the most vitamin D-responsive cell type of PBMCs.

The study of Hossein et al. [26] represents another example of human in vivo PBMC investigations, but in this experiment eight individuals were treated for two months daily with either 10 or 50 µg vitamin D₃. This long-term experiment has the difficulty that most cellular components of PBMCs are rather short-lived, i.e. two largely different cell populations at begin of the study and at its completion two months later were compared. This may be an explanation for the very low number of overlapping vitamin D target genes reported in this study. Similar problems of a direct comparison apply to another PBMC study where 47 subjects were supplemented weekly with 500 µg vitamin D₃ even over 3 to 5 years [27].

In addition, there are a number of transcriptome-wide studies studying in vitro the effect of 1,25(OH)₂D₃ on other components of PBMCs, such B cells [28], or other immune cells that derive from monocytes, such as dendritic cells [29-31]. However, the raw data of some studies were not accessible for re-analysis or the cellular systems were too distant from components of PBMCs, so that were not considered for direct comparison with our data. However, prominent vitamin D target genes found here, such as FBP1, CAMP and CD14, have already been identified in these studies. Moreover, qPCR-based studies have already highlighted CCR10 as vitamin D target gene [32].
This study found some 450 genes that had not yet been reported as vitamin D targets in comparable cellular systems. Within the list of these genes there are very interesting candidates, such as *HLA-A* and *HLA-C*, which encode for class I major histocompatibility complex (MHC) proteins. These cell surface proteins are expressed on basically all tissues and cell types and are essential for T cells to recognize foreign molecules expressed in virus-infected and transformed cells [33]. On the level of protein expression the effects of 1,25(OH)_{2}D_{3} on MHC class I expression had already been reported long time ago [34]. The up-regulation of *HLA-A* and *HLA-C* suggests an increased responsiveness of the immune system and a better protection from infectious diseases and cancer. Interestingly, we recently identified in another VitDbol, phase II study [16] the *HLA* cluster in chromosome 6 as a “hotspot” of vitamin D-induced *in vivo* changes of the human epigenome. This provides an important hint for understanding the impact of vitamin D on the control of the immune system [35].

Interestingly, KEGG-based pathway analysis of the 702 vitamin D target highlighted genes encoding for ribosomal proteins as top-ranking. This suggests that vitamin D stimulation results in a general boost of ribosomal activity and protein translation, which is important for preparing the immune cells encountering challenges. The second ranking result is summarized under the keyword “acute myeloid leukemia”, a disease that is majorly represented by inefficient monocyte differentiation. Accordingly, the up-regulation of the myeloid lineage determining transcription factors CEBPA and PU.1 by vitamin D primarily induces the differentiation of monocytes. The keyword “p53 signaling pathway” represents vitamin D target genes encoding cell cycle proteins, such as CDK6, CDKN2A and ATM, suggesting that vitamin D is involved in cell cycle control of PBMCs. The term “non-alcoholic fatty liver disease” provides a link to metabolism, as reported previously in dendritic cells [30], while the term “osteoclast differentiation” is another hint towards the control of cellular differentiation.

The range of the responsiveness of the 702 vitamin D target genes and the resulting average FC differs between individuals. Accordingly, the transcriptome is suited to characterize
individuals and to rank them by their responsiveness to vitamin D. The number of individuals investigated in this study is too low, to provide a full scheme of high, mid and low vitamin D responders, as we developed it based on individual genes, genomic regions and biochemical parameters for the 71 participants of the VitDmet study [36, 37] and the 35 persons studied in phase I of VitDbol [13, 14]. However, it is obvious that participant 4 is a high responder, while participant 2 is a low responder. This classification fits with the categorization of both persons in phase 1 of VitDbol [14]. Individual 3 did not participate in the first VitDbol study but appears to be a mid to high responders. Future transcriptome-wide investigations with a higher number of individuals will allow a more accurate classification of the grade of vitamin D responsiveness.

The observed negative correlation between the average FC of the 702 vitamin D target genes and the changes in 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ serum levels parallels some of our observations with individual genes [13]. A higher number of individuals being studied transcriptome-wide may shed light into this puzzling result. However, so far the observation suggests that a strong metabolic response to a vitamin D$_3$ bolus on the level of changes in 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ serum concentrations does not predict any high vitamin D responsiveness on the level of gene expression.

In conclusion, this study is the first report of short-term effects of vitamin D on the human transcriptome under in vivo conditions. A number of known vitamin D target genes were confirmed but many new candidates await future detailed investigation. The use of a transcriptome-wide approach promises to provide a more accurate characterization of the vitamin D responsiveness of individuals than previous reports.

**ACKNOWLEDGEMENTS**

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REFERENCES

TABLES

Table 1: Changes in vitamin D metabolite levels in serum. Serum was isolated before (d0) and 24 h after (d1) five individuals were exposed to a vitamin D₃ bolus of 2000 µg. Gender, age and BMI are indicated as well as concentrations of 25(OH)D₃ and 1,25(OH)₂D₃, their absolute change and their percent increase between d1 and d0. Moreover, the ratio of the concentrations of 25(OH)D₃ and 1,25(OH)₂D₃ was calculated. Please note that individuals 1, 2, 4 and 5 participated in phase I of VitDbol [14], in which their vitamin D metabolite levels have already been reported.

<table>
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Table 2: Pathway analysis of vitamin D target genes. KEGG cell signaling pathway analysis was performed of the 702 target genes identified in PBMCs (Table S1) using the webtool Enrichr [22]. The five most significant biological pathways are listed.

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FIGURE LEGENDS

Fig. 1: Genome-wide view on vitamin D target genes in PBMCs. RNA-seq was performed with RNA isolated from PBMCs of five individuals, before and 24 h after a vitamin D₃ bolus. A Manhattan plot-like graph is used to display the fold changes (FC, in log scale) of 702 significantly (p < 0.05) regulated genes over their chromosomal position. For each chromosome the number of vitamin D target genes and their percentage of all genes is indicated; those with high (red) and low (blue) density of target genes are highlighted.
Fig. 2: *Vitamin D target genes confirmed by other studies.* Venn diagrams display the overlap between the 702 vitamin D target genes identified by this study (purple) with reports on 3722 genes in THP-1 treated for 24 h with 1,25(OH)₂D₃ (red) [23, 24] (A), 1234 genes in PBMCs from 85 individuals treated *in vitro* for 24 h with 1,25(OH)₂D₃ (blue) [25] (B) and 204 genes in PBMCs from eight individuals that were daily supplemented with vitamin D₃ for 2 winter months (green) [26] (C). Examples of overlapping target genes were listed below the respective diagrams.
Fig. 3: Personal ranges of transcriptome-wide responses to vitamin D. The full range of the fold changes (FC, in log scale) of the 702 vitamin D target genes is plotted for each of the five study participants. The average FC (red line) is indicated and the individuals are sorted by the FC of their 25(OH)D$_3$ levels between d1 and d0 (Table 1).