

Dissecting high from low responders in a vitamin D<sub>3</sub> intervention study

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## ABSTRACT

Vitamin D<sub>3</sub> is a pleiotropic signaling molecule that has via activation of the transcription factor vitamin D receptor (VDR) a direct effect on the expression of more than 100 genes. The aim of this study was to find transcriptomic and clinical biomarkers that are most suited to identify vitamin D<sub>3</sub> responders within 71 pre-diabetic subjects during a 5-month intervention study (VitDmet). In hematopoietic cells, the genes *ASAP2*, *CAMP*, *CD14*, *CD97*, *DUSP10*, *G0S2*, *IL8*, *LRRC8A*, *NINJ1*, *NRIP1*, *SLC37A2* and *THBD* are known as primary vitamin D targets. We demonstrate that each of these 12 genes carries a conserved VDR binding site within its genomic region and is expressed in human peripheral blood mononuclear cells (PBMCs). The changes in the expression of these genes in human PBMCs at the start and the end of the vitamin D-intervention were systematically correlated with the alteration in the circulating form of vitamin D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>). Only 39–44 (55–62%) of the study subjects showed a highly significant response to vitamin D<sub>3</sub>, i.e., we considered them as “responders”. In comparison, we found for 37–53 (52–75%) of the participants that only 12 biochemical and clinical parameters, such as concentrations of parathyroid hormone (PTH) and insulin, or computed values, such as homeostatic model assessment and insulin sensitivity index, show a correlation with serum 25(OH)D<sub>3</sub> levels that is as high as that of the selected VDR target genes. All 24 parameters together described the pleiotropic vitamin D response of the VitDmet study subjects. Interestingly, they demonstrated a number of additional correlations that define a network, in which PTH plays the central role. In conclusion, vitamin D<sub>3</sub>-induced changes in human PBMCs can be described by transcriptomic and serum biomarkers and allow a segregation into high and low responders.

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## 1. Introduction

The endocrine system of vitamin D involves (i) vitamin D<sub>3</sub> that is produced in the skin from 7-dehydrocholesterol with energy provided by the UV-B component of sun light [1], (ii) 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>), which is the most abundant

vitamin D metabolite and the widely accepted indicator of the vitamin D status within the human body [2], and (iii) 1, 25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), which is a specific high-affinity ligand of the transcription factor VDR [3]. The classical physiological function of vitamin D is the regulation of calcium and phosphate homeostasis and its impact on bone mineralization [4].

**Abbreviations:** 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; 25(OH)D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>; ADIPOQ, adiponectin; ASAP2, ArfGAP with SH3 domain, ankyrin repeat and PH domain 2; B2M, beta-2-microglobulin; CAMP, cathelicidin antimicrobial peptide; CD14, CD14 molecule; CD97, CD97 molecule; ChIP, chromatin immunoprecipitation; ChIP-seq, ChIP sequencing; DR3, direct repeat spaced by 3 nucleotides; DUSP10, dual specificity phosphatase 10; FFA, free fatty acid; G0S2, G0/G1switch 2; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; GEO, gene expression omnibus; GTP, glutamic-pyruvate transaminase (alanine aminotransferase); HOMA, homeostatic model assessment; HPRT1, hypoxanthine phosphoribosyltransferase 1; IGV, integrative genomics viewer; IL6, interleukin 6; IL8, interleukin 8; INS, insulin; IR, insulin resistance; LPS, lipopolysaccharide; LRRC8A, leucine rich repeat containing 8 family, member A; NINJ1, ninjurin 1; NRIP1, nuclear receptor interacting protein 1; PBMC, peripheral blood mononuclear cell; PTH, parathyroid hormone; qPCR, real-time quantitative polymerase chain reaction; RPLP0, ribosomal protein, large, P0; SLC37A2, solute carrier family 37, member 2; TNFRSF1B, tumor necrosis factor receptor superfamily, member 1B; TSS, transcription start site; THBD, thrombomodulin; VDR, vitamin D receptor.

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However, vitamin D<sub>3</sub> is a pleiotropic signaling molecule, which via 1,25(OH)<sub>2</sub>D<sub>3</sub> and VDR regulates numerous physiological processes ranging from the control of cellular growth, intracellular metabolism, and innate and adaptive immunity [5,6].

In order to achieve an optimal vitamin D status, sufficient exposure to natural UV-B radiation or, instead, adequate intake from diet or supplements is needed. However, serum 25(OH)D<sub>3</sub> levels show high inter-individual variations that are based on age, sun exposure, diet, level of obesity and (epi) genetic polymorphisms [7–9]. The US Institute of Medicine recommends a serum 25(OH)D<sub>3</sub> concentration of 50 nM [10], but other scientists suggest 75 nM or even more [11]. Based on these threshold definitions worldwide billions of people have to be considered as vitamin D deficient. As a consequence, in these deficient individuals not only the bone health is compromised but vitamin D insufficiency is also associated with a number of diseases, such as cancer, autoimmune disorders and all components of the metabolic syndrome [12]. In those cases where a sufficient vitamin D status cannot be achieved by appropriate sun exposure or dietary changes, a direct supplementation with vitamin D<sub>3</sub> seems to be the most straightforward intervention. However, the inter-individual variation suggests that a “one-size-fits-all” approach is not suited for vitamin D<sub>3</sub> supplementation [13].

VDR belongs to the nuclear receptor transcription factor superfamily, whose members are directly activated by small lipophilic compounds [14]. Insight into the genome- and transcriptome-wide actions of VDR and 1,25(OH)<sub>2</sub>D<sub>3</sub> can help in a more accurate evaluation of the human individual's responsiveness to, and needs for, vitamin D<sub>3</sub>. Transcriptome-wide analysis indicated that depending on cell type between 200 and 600 genes are primary targets of 1,25(OH)<sub>2</sub>D<sub>3</sub> [15–18]. The chromatin immunoprecipitation (ChIP) method coupled with massive parallel sequencing (ChIP-seq) allows the genome-wide mapping of nuclear proteins to their genomic locations [19]. VDR ChIP-seq data had been published for the human cellular models (i) GM10855 and GM10861 lymphoblastoid cells [15], (ii) THP-1 monocyte-like cells [16], (iii) lipopolysaccharide (LPS)-differentiated macrophage-like THP-1 cells [20], (iv) LS180 colorectal cancer cells [21] and (v) LX2 hepatic stellate cells [22]. A re-analysis of these data using identical peak calling settings identified genome-wide more than 23,000 individual VDR loci, of which more than 70% are specific for one cellular system [20]. This indicates that vitamin D signaling is largely cell-type specific.

In order to determine how the vitamin D responsiveness of human individuals could be determined most accurately, in this study we investigated changes in the expression of primary VDR target genes after vitamin D<sub>3</sub> supplementation. Since PBMCs are the easiest available vitamin D responsive human cell types to be achieved, we took advantage of already existing samples from 71 participants of the vitamin D<sub>3</sub> intervention trial VitDmet. We selected the primary VDR target genes ArfGAP with SH3 domain, ankyrin repeat and PH domain 2 (*ASAP2*), cathelicidin antimicrobial peptide (*CAMP*), CD14 molecule (*CD14*), CD97 molecule (*CD97*), dual specificity phosphatase 10 (*DUSP10*), G0/G1 switch 2 (*G0S2*), interleukin 8 (*IL8*), leucine rich repeat containing 8 family, member A (*LRRC8A*), ninjurin 1 (*NINJ1*), nuclear receptor interacting protein 1 (*NRIP1*), solute carrier family 37, member 2 (*SLC37A2*) and thrombomodulin (*THBD*) and investigated their expression on PBMCs obtained before and after the VitDmet trial in correlation to changes in serum 25(OH)D<sub>3</sub> concentrations. In parallel, we tested some 200 biochemical and clinical parameters from the same study participants for a possible correlation with alterations in 25(OH)D<sub>3</sub> levels, out of whom we found exactly 12 to be significantly correlated. All 24 parameters together described the pleiotropic actions of vitamin D in the VitDmet study subjects and allow their segregation into high and low responders.

## 2. Material and methods

### 2.1. ChIP-seq data visualization

The integrative genomics viewer (IGV) [23] was used to visualize the VDR ChIP-seq data, which had been summarized by Tuoresmäki et al. [20], which is accessible at Gene Expression Omnibus (GEO, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)) under the accession number GSE53041.

### 2.2. Samples of the VitDmet study

The inclusion criteria of the 73 participants of the VitDmet study (NCT01479933, ClinicalTrials.gov) were (i) to be ≥60 years of age, (ii) to show evidence of disturbed glucose homeostasis, such as impaired fasting glucose or impaired glucose tolerance, but not yet manifest type 2 diabetes, and (iii) to have a body mass index between 25 and 35. Out of these 73 subjects, we selected those 71, for whom PBMC isolates both from the start and the end of the trial were available. The research ethics committee of the Northern Savo Hospital District had approved the study protocol (#37/2011). All participants gave a written informed consent to participate in the study (for further details see [24]).

Serum concentrations for 25(OH)D<sub>3</sub> were measured from venous blood samples using a high performance liquid chromatography with coulometric electrode array as described previously [25]. Moreover, serum protein levels for the bone health marker parathyroid hormone (PTH), the soluble tumor necrosis factor receptor superfamily, member 1B (TNFRSF1B), the cytokine interleukin 6 (IL6), the adipocyte cytokine adiponectin (ADIPOQ) and the liver enzyme alanine aminotransferase (GTP) were determined by standard methods as described previously [25]. Other biochemical parameters were assayed at a local laboratory service provider (ISLAB, Kuopio, Finland).

At the start and the end of the 5-month intervention a 2 h oral glucose tolerance test was carried out with 75 g glucose; at time points 0, 30 and 120 min glucose, free fatty acids (FFA) and insulin (INS) were measured. Homeostatic modeling assessment (HOMA) indices were computed according to the nonlinear function using the approach of Wallace et al. [26], while the insulin sensitivity index was calculated as described by Matsuda and DeFronzo [27].

### 2.3. qPCR from PBMC samples

PBMCs were isolated, RNA was extracted and cDNA synthesized as described previously [24,28]. qPCR reactions were performed using 280 nM of reverse and forward primers (Table S1), 1/20 diluted cDNA template and LightCycler 480 SYBRGreen I Master mix (Roche). In the PCR reaction the hotstart Taq polymerase was activated for 10 min at 95 °C, followed by 43 amplification cycles of 20 s denaturation at 95 °C, 15 s annealing at primer-specific temperatures (Table S1) and 15 s elongation at 72 °C and a final elongation for 10 min at 72 °C. PCR product specificity was monitored using post-PCR melt curve analysis. Relative mRNA expression levels were determined using the formula  $2^{-(\Delta Ct)}$ , where  $\Delta Ct$  is  $Ct_{(targetgene)} - \text{mean of } Ct_{(referencegenes)}$ . The four internal reference genes beta-2-microglobulin (*B2M*), glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and ribosomal protein, large, P0 (*PRLP0*) were used as references. The stability of the expression of the reference genes was determined using the geNorm algorithm [29].

## 2.4. Correlation analysis

The correlations between the changes in mRNA expression, serum protein, metabolite levels or other clinical parameters in relation to serum 25(OH)<sub>2</sub>D<sub>3</sub> level alterations were assumed to be linear. The extent of the correlation was measured by the  $r^2$  value. Starting with all 71 study participants (for each of the 24 parameters individually) not more than 35 were removed and in a systematic way all possible correlations were computed. The largest participant subgroup that provided a correlation with an  $r^2$  value of 0.5 or higher was selected for further analysis.

## 2.5. Network construction

The Python-based software package NetworkX (<https://networkx.github.io>) was applied to represent the information obtained from correlation analysis by a network graph. The latter was further processed with the open software Gephi (<https://gephi.github.io>), which is an interactive visualization and exploration platform for all kinds of networks and complex systems, dynamic and hierarchical graphs.

## 3. Results

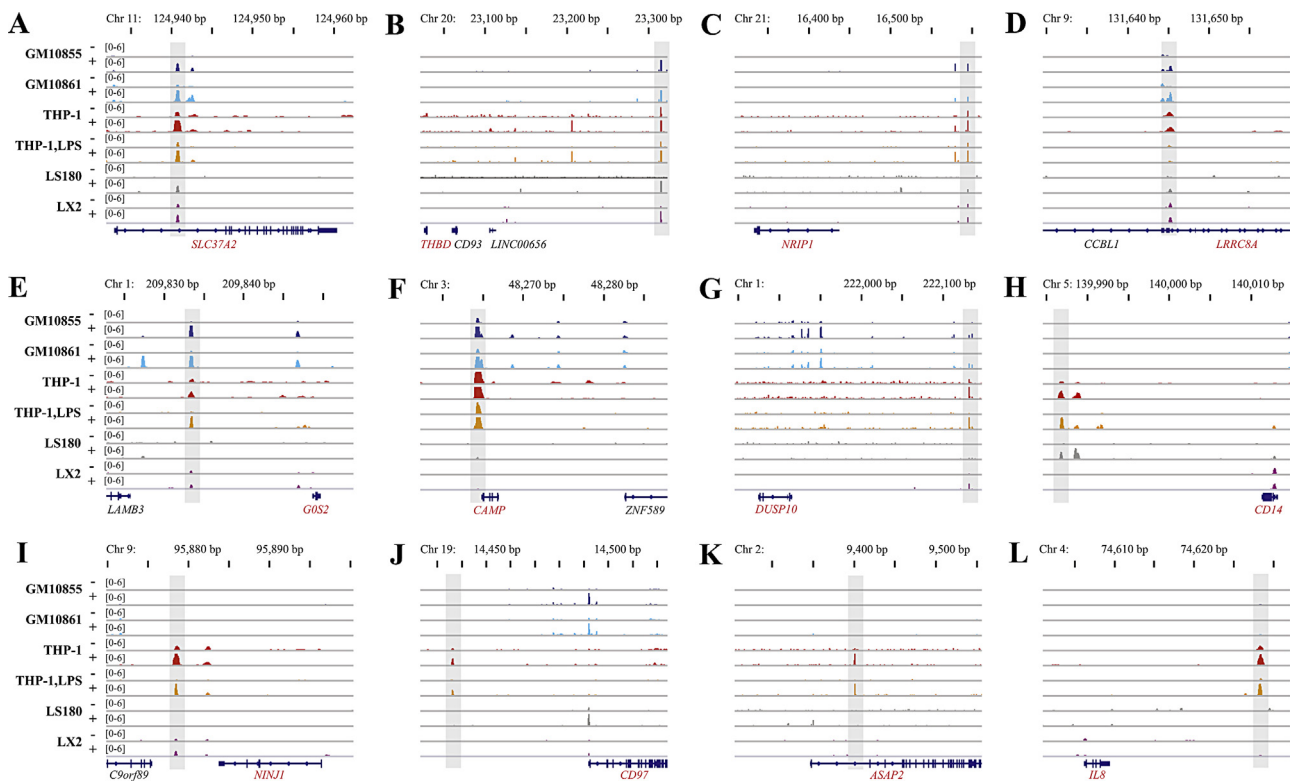
### 3.1. Primary 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes

For a selection of the most suited primary 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes for studies in human PMBCs, we selected the genes *SLC37A2* (Fig. 1A) [28], *THBD* (Fig. 1B) [30], *NR1P1* (Fig. 1C) [28], *LRRC8A* (Fig. 1D) [28], *GOS2* (Fig. 1E) [31], *CAMP* (Fig. 1F) [30], *DUSP10*

(Fig. 1G) [30], *CD14* (Fig. 1H) [32], *NINJ1* (Fig. 1I) [20], *CD97* (Fig. 1J) [28], *ASAP2* (Fig. 1K) [33] and *IL8* (Fig. 1L) [34]. The selection criteria for the genes were that they (i) represent the pleiotropic physiological actions of vitamin D<sub>3</sub>, (ii) were demonstrated to be primary 1,25(OH)<sub>2</sub>D<sub>3</sub> targets in a human hematopoietic cell line and (iii) have a conserved genomic VDR binding site in less than 300 kb distance from the gene's transcription start site (TSS).

The pleiotropy of the actions of the proteins encoded by the 12 genes was verified as the wide range of their preferential location being the nucleus (*NR1P1* and *DUSP10*), mitochondria (*GOS2*), Golgi (*ASAP2*), the plasma membrane (*SLC37A2*, *THBD*, *LRRC8A*, *CD14*, *NINJ1* and *CD97*) or even extracellular (*CAMP* and *IL8*). Moreover, the 12 vitamin D-regulated proteins are known to be involved in different physiological functions ranging from gene regulation (*NR1P1*), signal transduction (*DUSP10*), innate immunity (*CAMP* and *CD14*), inflammation (*IL8*), cell adhesion (*LRRC8A*, *NINJ1* and *CD97*), vesicle transport (*ASAP2*), apoptosis (*GOS2*), blood coagulation (*THBD*) to glucose transport (*SLC37A2*). Moreover, all 12 genes have been shown to be primary 1,25(OH)<sub>2</sub>D<sub>3</sub> targets in THP-1 human monocytic leukemia cells [16].

Using VDR ChIP-seq datasets from six different cellular models, we demonstrated that the genomic region of each of the 12 genes carries a prominent VDR binding site (Fig. 1). Each binding site was observed in THP-1 cells and in LPS-polarized macrophage like THP-1 cells. Interestingly, while the VDR peaks in vicinity of the genes *CD97*, *ASAP2* and *IL8* seem to be specific to these cell types, the VDR site of the *NINJ1* gene was also found in hepatic stellate cells, that of the *CD14* gene also in colon cells, the VDR loci of the genes *DUSP10* and *CAMP* also in B cells and that of *GOS2*, *LRRC8A* and *NR1P1* in all test cell types with the exception of colon cells (Fig. 1). Finally, the



**Fig. 1.** Different levels of cell type specificity of VDR peak locations. The IGV browser was used to display normalized VDR ChIP-seq signals from unstimulated (–) and ligand-stimulated (+) lymphoblastoid cell lines GM10855 ([15], dark blue) and GM10861 ([15], light blue), undifferentiated THP-1 cells ([16], red), LPS-differentiated THP-1 cells ([20], red), LS180 cells ([21], grey) and LX2 cells ([22], purple) for the loci of the genes *SLC37A2* (A), *THBD* (B), *NR1P1* (C), *LRRC8A* (D), *GOS2* (E), *CAMP* (F), *DUSP10* (G), *CD14* (H), *NINJ1* (I), *CD97* (J), *ASAP2* (K) and *IL8* (L). The genomic regions were sorted in a descending order by amount of inter-cell type overlap of the VDR peaks. Gene structures are indicated in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

VDR binding site of *THBD* and *SLC37A2* were observed in all tested cellular models.

In order to test, whether the 12 selected genes are also expressed in primary human cells, at the start of the VitDmet vitamin D<sub>3</sub> intervention trial RNA was isolated from PBMCs of 71 participants and qPCR was performed in relation to four reference genes (Fig. S1). All 12 genes were expressed but in a wide range that differed up to 327-fold between the most prominently (*CD14*) and the lowest expressed candidate (*CAMP*).

In summary, we selected with *ASAP2*, *CAMP*, *CD14*, *CD97*, *DUSP10*, *GOS2*, *IL8*, *LRRRC8A*, *NINJ1*, *NRIP1*, *SLC37A2* and *THBD* a divergent set of genes, which (i) represent the pleiotropic effects of vitamin D, (ii) have a prominent VDR binding site in vicinity of the TSS, (iii) are primary 1,25(OH)<sub>2</sub>D<sub>3</sub> target in monocytes and (iv) are also expressed in human PBMCs.

### 3.2. Vitamin D<sub>3</sub> responders concerning VDR target gene expression

The VitDmet trial had recruited elderly (>60 years) pre-diabetic human subjects from the region of Kuopio, Finland (63°N) [35], who showed at the begin of the study a serum 25(OH)D<sub>3</sub> level ranging from 35.9 to 73.6 nM (Table S2), i.e., below the exclusion criterium of above 75 nM. The trial was performed during 5 months of the winter season, where in the northern hemisphere at this latitude no vitamin D<sub>3</sub> could be synthesized in the skin of the participants. The study participants daily received either 40 µg or 80 µg of vitamin D<sub>3</sub> or placebo, respectively, which led, at the end of the study, to serum 25(OH)D<sub>3</sub> concentrations ranging between 27.5 and 155.7 nM (Table S2). We decided to express all changes observed during the 5-month intervention as ratios and not as differences, i.e., we interpreted the highest individual decrease of 30.2 nM in serum 25(OH)D<sub>3</sub> levels as a 2.1-fold reduction and the highest individual increase of 87.2 nM as a 2.8-fold induction (Table S2). Importantly, in the same period neither the body mass index nor serum calcium concentrations of the study participants changed significantly (for further details see [24,35]).

In order to evaluate possible changes in the expression of the 12 selected primary VDR target genes during the 5 months of the VitDmet trial, we used qPCR to measure their relative mRNA levels in PBMCs at the start and end of the intervention. After normalization with four reference genes this resulted in expression changes, which ranged from 0.50 (i.e. a 1.98-fold decrease) to 3.74 (i.e., a 3.74-fold increase) for *ASAP2*, 0.24–4.93 for *CAMP*, 0.42–2.23 for *CD14*, 0.40–2.68 for *CD97*, 0.60–1.51 for *DUSP10*, 0.35–2.68 for *GOS2*, 0.28–11.59 for *IL8*, 0.50–4.17 for *LRRRC8A*, 0.29–2.42 for *NINJ1*, 0.61–2.96 for *NRIP1*, 0.27–5.21 for *SLC37A2* and 0.23–2.34 for *THBD* (Table S2). Assuming a linear correlation between VDR target gene expression changes and 25(OH)D<sub>3</sub> level alterations, we performed a systematic correlation analysis of the data by exploring all possible lines through the correlation graphs (Table S3). Starting with the data of all 71 study participants we allowed the elimination of up to 35 subjects, in order to obtain a correlation with an  $r^2$  value of 0.5 or higher. This approach left 43 study participants for *ASAP2* (Fig. 2A), 39 for *CAMP* (Fig. 2B), 44 for *DUSP10* (Fig. 2C), 41 for *GOS2* (Fig. 2D), 39 for *IL8* (Fig. 2E) and 44 for *NINJ1* (Fig. 2F). We call these subgroups of subject “responders” for the respective gene. We had already investigated the remaining six genes with a similar method based on a ranking of the study participants concerning their response to vitamin D<sub>3</sub> [28]. Using that approach we kept 28 subjects in the correlation analysis for *CD14*, 35 for both *CD97* and *LRRRC8A*, 41 for *NRIP1*, 36 for *SLC37A2* and 34 for *THBD*. In the present study we observed that the systematic correlation analysis allowed including more study participants, which were 40 for *CD14* (Fig. S2A), 41 for *CD97* (Fig. S2B), 39 for *LRRRC8A* (Fig. S2C), 41 for *NRIP1* (Fig. S2D), 39 for *SLC37A2* (Fig. S2E) and 43 for *THBD* (Fig. S2F). However, the

systematic approach suggested for the expression changes of the genes *DUSP10* (Fig. 2C) and *THBD* (Fig. S2F) a negative correlation with changes in 25(OH)D<sub>3</sub> levels over the 5-month intervention period, i.e. the long-term effect of vitamin D<sub>3</sub> seems to be a down-regulation of these two genes.

Taking together, a systematic correlation analysis of the relation of expression changes of 12 primary VDR target genes in relation to alterations of 25(OH)D<sub>3</sub> levels of 71 VitDmet study participants indicated that 39–44 (55–62%) of the subjects showed a highly significant response to vitamin D<sub>3</sub> in PBMCs, i.e., they are vitamin D<sub>3</sub> responders.

### 3.3. Vitamin D<sub>3</sub> responders concerning clinical parameters

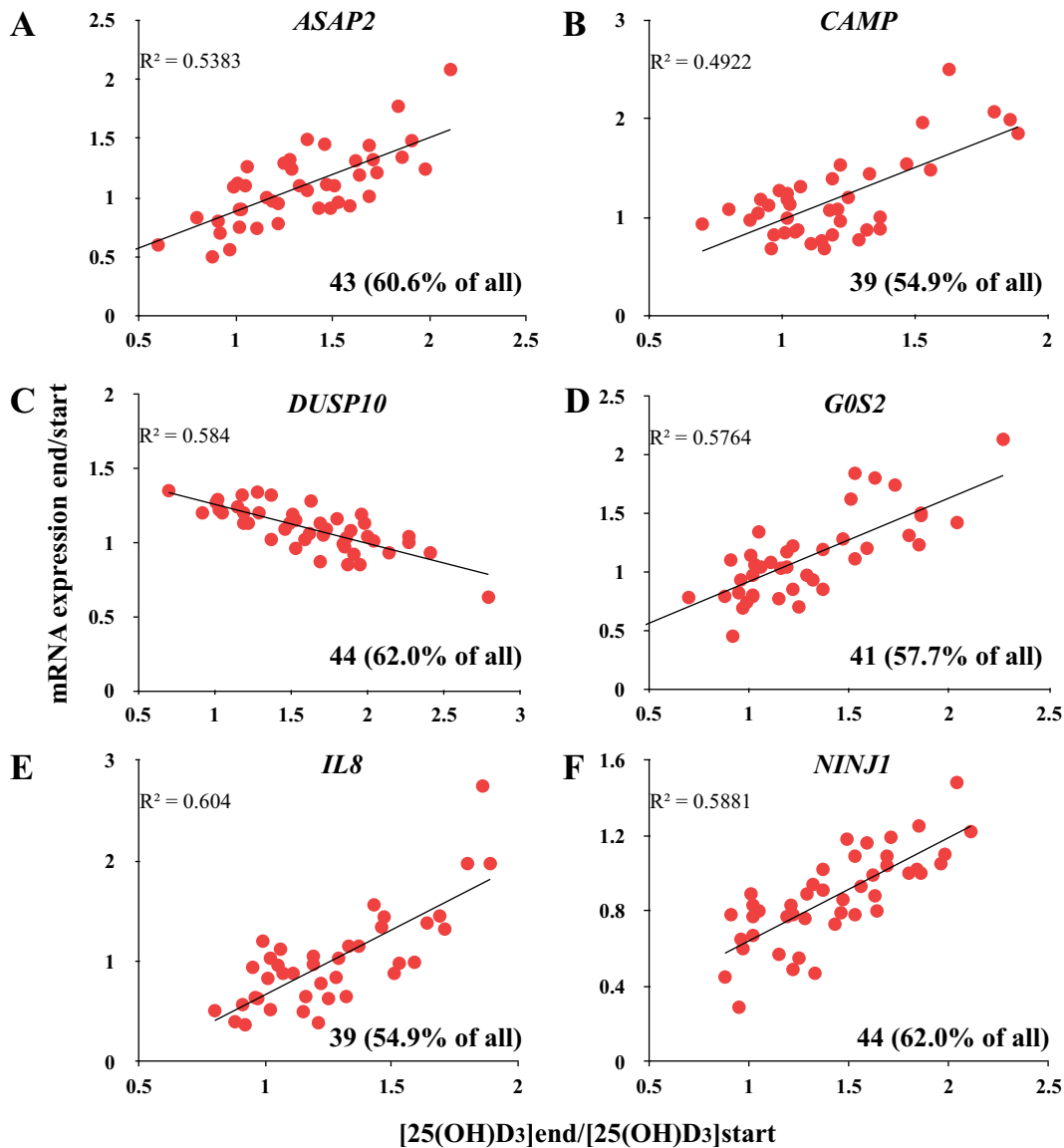
The 71 participants of the VitDmet study had been intensively investigated for their clinical parameters with focus on their serum protein and metabolite levels including a glucose tolerance test with measurements of glucose, FFA and INS at time points 0, 30 and 120 min [35]. In this way more than 200 biochemical and clinical parameters were determined, many of which were measured both at the start and at the end of the 5-months intervention. We focused on approximately 100 parameters for which at least 70 of the 71 study participants provided a value and performed the same type of systematic correlation analysis with serum 25(OH)D<sub>3</sub> concentrations as for VDR target gene expression. When we again restricted not to eliminate more than 35 participants, we obtained a highly significant ( $r^2 > 0.5$ ) correlation only for the changes of 12 parameters (Table S3). These were 53 subjects for serum PTH level (Fig. 3A), 43 for INS protein concentration at 0 min (Fig. 3A), 44 for both the indices HOMA-IR (Fig. 3C) and insulin sensitivity (Fig. 3D), 43 for heart rate (Fig. 3E), 39 for lymphocyte number (Fig. 3F), 37 for serum protein concentrations of TNFRSF1B (Fig. S3A), 40 for IL6 (Fig. S3B), 44 for ADIPOQ (Fig. S3C) and 41 for GTP (Fig. S3D) and 47 and 39 for FFA concentrations at time points 0 min (Fig. S3E) and 120 min (Fig. S3F), respectively. Interestingly, for the five parameters PTH, INS (0 min), HOMA-IR, heart rate and ADIPOQ we observed a negative correlation, i.e., the higher the 25(OH)D<sub>3</sub> levels the lower are these biochemical or clinical values.

In summary, we observed a high correlation for the changes in the 12 biochemical and clinical parameters of 37–53 (52–75%) of the VitDmet study participants with serum 25(OH)D<sub>3</sub> levels, i.e., they are responders concerning these parameters.

### 3.4. Network analysis of gene expression and clinical parameters

We identified 12 VDR target genes and the 12 biochemical/clinical parameters each of which presents a highly significantly correlation with serum 25(OH)D<sub>3</sub> levels (Table S3). The number of parameters, for which the 71 study participants showed responsiveness to vitamin D<sub>3</sub>, ranged from 21 to 6, i.e., there is a gradual decline of vitamin D<sub>3</sub> response within the investigated study group (Fig. S4). We have chosen the threshold of >50%, in order to segregate 49 high responders from 22 low responders.

In order to investigate, whether the investigated 24 parameters influence each other and form a correlation network, we calculated the fitness of correlation matrix (measured the square root of  $r^2$ , Table S3A). In addition, in order to determine, whether the correlation is positive or negative between a pair of parameters, we also calculated the Pearson correlation (Table S3B). Correlations with  $r$  values >0.3 are presented as a network (Fig. 4). A connection line between the parameters indicates that responders concerning the first parameter can be fitted with a relatively low error concerning the second parameter. The parameter PTH and the sensitivity-related variables, INS at fasting (0 min) and the calculated indices of



**Fig. 2.** VDR target gene expression changes of vitamin D<sub>3</sub> responsive study participants. RNA was isolated from PBMCs obtained from 71 participants before and after a 5-month vitamin D<sub>3</sub> intervention trial. qPCR was performed to determine the relative changes of the expression of the VDR target genes *ASAP2* (A), *CAMP* (B), *DUSP10* (C), *GOS2* (D), *IL8* (E) and *NINJI* (F) normalized by the reference genes *B2M*, *GAPDH*, *HPRT1* and *RPLP0*. The number of study participants selected for the correlation analysis (red data points) is indicated (see Table S2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

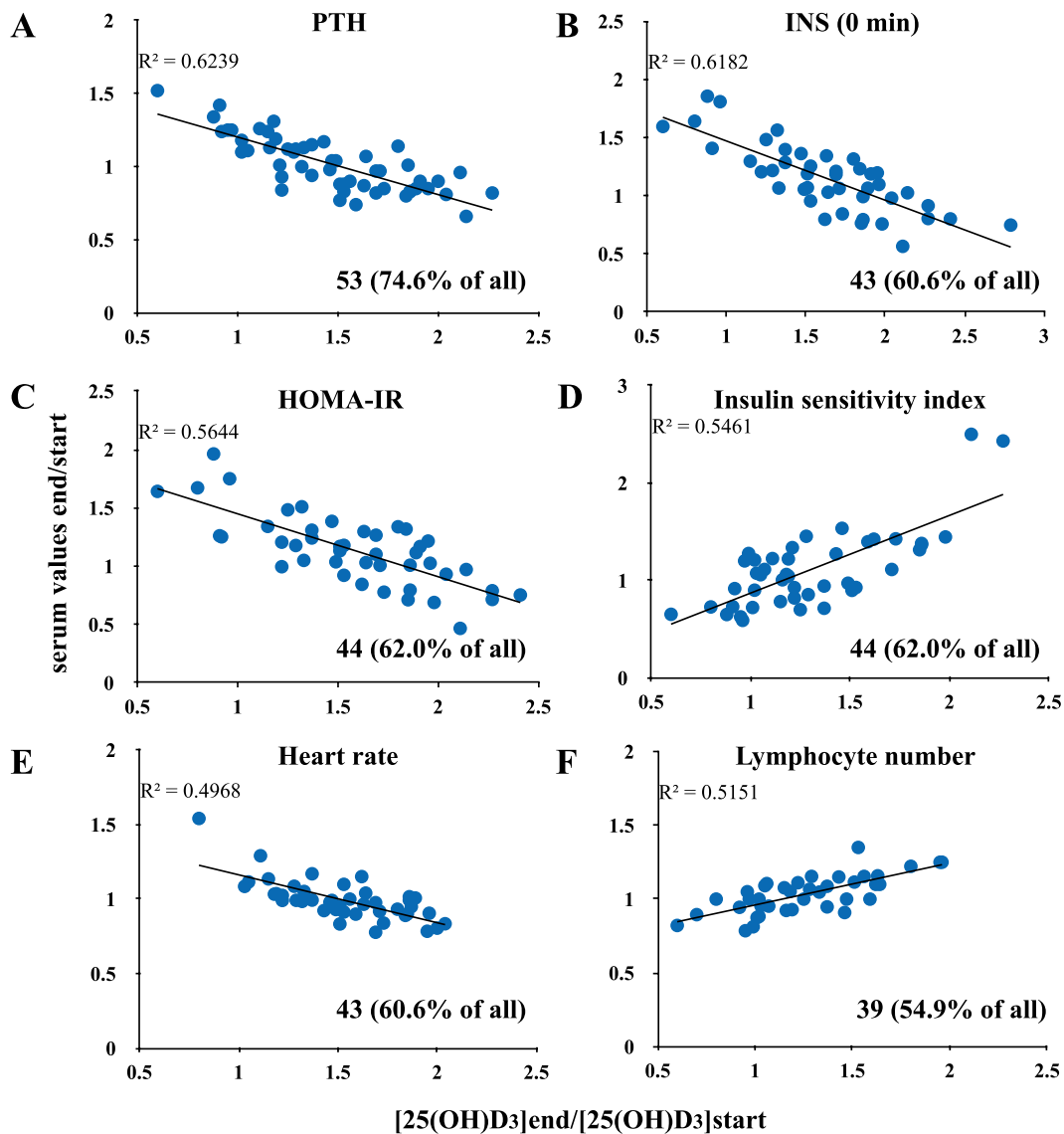
HOMA-IR and insulin sensitivity showed the highest correlation to each other and represent the core of the network. PTH constitutes a hub in the network with a degree of 21 (i.e., besides *CD14*, *CD97* and *LRRC8A* it correlated with all parameters), while *IL6* and *LRRC8A* are the parameters with the lowest number of correlations. However, we found also some correlations within the set of gene expression changes. For example, both *DUSP10* and *NRIP1* are connected each with four other genes. In addition, we observed a number of connections between genes and biochemical/clinical parameters; besides PTH levels also INS concentration, HOMA-IR index, insulin sensitivity index, heart rate, lymphocyte number and FFA (0 min) levels correlated with at least one gene.

Taken together, the 24 parameters of the VitDmet study that correlate with 25(OH)D<sub>3</sub> levels also show a number of additional correlations defining a network. The structure of this network describes an imbrication phenomenon, in which PTH seems to play a central role.

#### 4. Discussion

In this study, we presented a network of 12 primary VDR target genes and 12 biochemical/clinical parameters that describe the vitamin D responsiveness of human individuals. The changes of each of the 24 parameters demonstrated a high correlation with alterations of serum 25(OH)D<sub>3</sub> levels. In this context, we developed a connection network of these parameters on the basis of samples obtained from 71 elderly, pre-diabetic individuals, which participated in a 5-month vitamin D<sub>3</sub> intervention trial during Finnish winter [24].

The serum PTH level is an established biomarker and its down-regulation by increasing vitamin D levels is considered beneficial at least for bone health [36]. In this study, changes in serum PTH concentrations represent the most sensitive parameter that classified 75% of the study participants as vitamin D responders. In other words, 3 of 4 investigated individuals would benefit from vitamin D<sub>3</sub> supplementation, in order to optimize, for



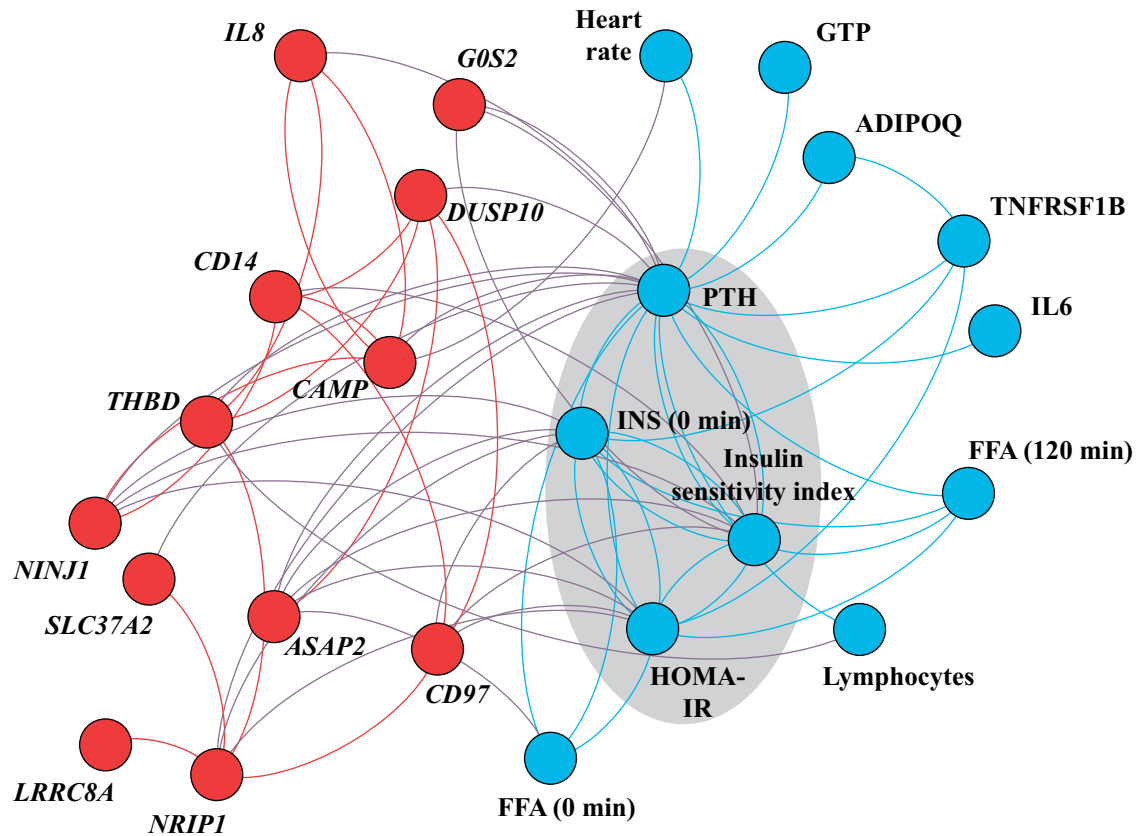
**Fig. 3.** Changes in serum parameters of vitamin D<sub>3</sub> responsive study participants. Blood was drawn in the context of an oral glucose tolerance test (0, 30 and 120 min) from 71 participants before and after a 5-month vitamin D<sub>3</sub> intervention trial. Plasma protein concentrations of PTH (A) and INS (B) were measured, HOMA insulin resistance (C) and insulin sensitivity index (D) were calculated and heart rate (E) and lymphocyte number (F) were determined. The number of study participants (blue data points) selected for the correlation analysis is indicated (see Table S2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

example, their bone mineralization. From the perspective of the 23 remaining parameters only between 37 and 47 (52–66%) of the investigated individuals are considered as vitamin D responders. This indicates that for the physiological functions, which are represented by these parameters, a smaller subgroup of the study participants seem to benefit from vitamin D<sub>3</sub> supplementation. Moreover, it implies that the pleiotropic actions of vitamin D show a differential sensitivity to serum 25(OH)D<sub>3</sub> levels, i.e., that different vitamin D levels are required, in order to obtain optimal effects.

For the pre-diabetic individuals studied here it is considered to be of benefit, when increased serum 25(OH)D<sub>3</sub> levels, i.e., an improved vitamin D status, lead to a decrease of (i) fasting serum INS concentrations, (ii) the HOMA insulin resistance index and (iii) the heart rate and an increase of the insulin sensitivity index. A general improve in insulin sensitivity was already observed in another recent vitamin D<sub>3</sub> intervention trial [37]. Taken together, this suggests that vitamin D<sub>3</sub> supplementation prevents

characteristics of the metabolic syndrome in a subsample of the pre-diabetic individuals. However, since a substantial proportion of the VitDmet participants were found low responsive at least for some of the investigated parameters, this conclusion should not be generalized. We assume that the majority of the low responsive subjects already had reached their individual optimal vitamin D status, so that any additional vitamin D<sub>3</sub> supplementation had no effect on the biochemical/clinical health characteristics, including insulin sensitivity. The finding of heart rate is an interesting observation and needs further studies.

On the level of the investigated primary VDR target genes expression changes of *DUSP10* and *NINJ1* appeared to be the most sensitive, since they represented the vitamin D responsiveness of 62% of the study participants. Importantly, the basal expression of *DUSP10* in PBMCs is relatively high, i.e., it may be measured more accurately than many other VDR target genes. Moreover, in our network model *DUSP10* showed the highest number of connections with other genes. This fits with our previous study on the



**Fig. 4.** Network analysis. The correlation analysis (Table S3) of the vitamin D<sub>3</sub>-dependent changes in the expression of 12 VDR target genes (red nodes) and of 12 serum protein and metabolite (blue nodes) suggested a network. Correlations with an  $r$  value higher than 0.3 between the parameters are indicated by a connection line, thicker lines represent more significant correlations. Red lines connect genes and blue lines clinical parameters, while purple lines demonstrate relations between the two parameter groups. A subgroup of four clinical parameters that display the closest correlation is shaded in grey. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

vitamin D response of adipose tissue samples from 47 VitDmet study participants [38], where we also identified *DUSP10* expression changes as the most representative marker. Interestingly, in that study we had applied a different approach for the identification of high vitamin D responsive individuals indicating that the result does not depend on the chosen method. However, the systematic correlation analysis that we applied in this study indicated a negative correlation between *DUSP10* expression changes and serum 25(OH)D<sub>3</sub> level alterations. In previous short-term monocyte cell culture experiments *DUSP10* was characterized as up-regulated primary 1,25(OH)<sub>2</sub>D<sub>3</sub> target gene [30]. In contrast, the apparent down-regulation of *DUSP10*, as detected in this study, was measured in primary PBMCs over a time period of 5 months, i.e. within a far longer time period, in which the chromatin on the genomic locus of the *DUSP10* gene could have been target of a number of epigenetic changes. For the *THBD* gene, which expression changes were in this study also negatively correlated with serum 25(OH)D<sub>3</sub> level alterations, was also reported as an rapidly up-regulated 1,25(OH)<sub>2</sub>D<sub>3</sub> target gene [24,30] and a similar explanation as for *DUSP10* may apply.

Since serum PTH concentration changes are the most sensitive marker and correlate with 20 of the 23 other parameters, it is located in the center of the interaction network. Moreover, the observation that the parameters INS concentration, HOMA-IR index and insulin sensitivity index form together with PTH the core of the network, is of special relevance for the studied group of pre-diabetic individuals. Therefore, the main conclusion from the network analysis is that optimal vitamin D levels are of

benefit for individuals with the metabolic syndrome and insulin resistance. In this direct comparison, the contribution of the 12 primary VDR target genes is not as prominent as that of the well-known biochemical and clinical parameters. Nevertheless, the network highlights the most relevant gene (*DUSP10*) and indicates a larger number of connections between gene expression changes and biochemical/clinical parameters. Moreover, it should be noted that we found for all 12 of the investigated primary VDR target genes a correlation with serum 25(OH)D<sub>3</sub> level changes, while most of the 200 tested clinical parameters did not show any correlation with vitamin D level alterations. In addition, the use of microarray or RNA-seq technology would allow a comprehensive measurement of all possible vitamin D target genes, while comparable methods for the assessment of all protein of metabolites are not equally well established. Therefore, the combination of genome- and transcriptome-wide data is more promising for future analyses than the measurement of additional individual biochemical/clinical parameters. Moreover, following this direction will increase the mechanistic understanding of the effects of vitamin D on the physiology of the investigated tissues or cell types.

In conclusion, in this study we present a set of 24 parameters, 12 VDR target genes and 12 biochemical/clinical parameters, which help to identify pre-diabetic individuals with indicators of the metabolic syndrome into high and low responders to vitamin D<sub>3</sub> supplementation. Using such a screening assessment will allow a better dissection of human individuals, whether they will benefit from vitamin D<sub>3</sub> supplementation or not.

## Contributors

N.S., A.N. and C.C. designed the current analysis, N.S. performed the experiments and A.N. the computational analysis, N.S., A.N. and C.C. analyzed the data, J.R. provided technical support, M.U., V.de M., S.V., T.N., J.V. and T.-P.T. designed and performed the VitDmet study and C.C. wrote the manuscript. All authors gave an intellectual contribution to the study and took part in editing the manuscript.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.jsbmb.2014.11.012>.

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