

Variable Genomic and Metabolomic Responses to Varying Doses of Vitamin D Supplementation

ARASH SHIRVANI¹, TYLER AREK KALAJIAN¹, ANJELI SONG¹, RACHEL ALLEN¹,
NIPITH CHAROENNGAM¹, RICHARD LEWANCZUK² and MICHAEL F. HOLICK¹

¹*Boston Medical Campus, Section Endocrinology, Diabetes, Nutrition and Weight Management, Department of Medicine, Vitamin D, Skin, and Bone Research Laboratory, Boston University School of Medicine, Boston, MA, U.S.A.;*

²*Division of Endocrinology and Metabolism, Department of Medicine, University of Alberta and Alberta Health Services, Edmonton, AB, Canada*

Abstract. *Background/Aim: To assess the impact of vitamin D supplementation on genomic and metabolomic profiles and relate them to the individual's responsiveness to varying doses of vitamin D₃. Patients and Methods: Healthy adults were given either 600, 4000 or 10,000 IUs vitamin D₃/day for 6 months. Circulating parathyroid hormone (PTH), 25-hydroxyvitamin D [25(OH)D], calcium, peripheral white blood cells broad gene expression and urine and serum metabolomic profiles were evaluated. Results: There was a dose-dependent effect of vitamin D supplementation on serum 25(OH)D, PTH and broad gene expression. Serum calcium levels remained normal for all study subjects and no untoward toxicity was observed. The metabolomic profiles were related to the genomic expression analysis. There were significant inter-individual effects on gene expression and metabolomic profile in response to the same dose of vitamin D₃ supplementation, despite similar changes in 25(OH)D and PTH concentrations. Conclusion: These results may help explain the variability observed in clinical trials regarding vitamin D's non-calceemic health benefits.*

It is generally accepted that vitamin D, the sunshine vitamin, is essential for the development and maintenance of bone health throughout life (1-4). The non-skeletal health benefits of vitamin D are supported by the observations that the vitamin D receptor is expressed in several types of tissues

Correspondence to: Michael F. Holick, Ph.D., MD, Boston University School of Medicine, Vitamin D, Skin, and Bone Research Laboratory, 85 E Newton St. M-1013, Boston, MA, 02118, U.S.A. Tel: +1 6173586139, e-mail: mfholick@bu.edu

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(1-4). A multitude of association studies and meta-analyses have demonstrated the potential benefits of vitamin D on longevity and reducing risk for cardio-metabolic disorders, several types of cancers, autoimmune disorders, all-cause mortality and other acute and chronic illnesses (1-3). However, the health benefit of vitamin D supplementation has been questioned based on recent clinical trials that not only challenged the concept of non-skeletal health benefits of vitamin D, but also suggested a potential detrimental effect of increased vitamin D supplementation on bone health (4). Furthermore, some studies have reported that improvement in vitamin D status can improve bone mineral density and reduce risk of fracture while others have not seen any benefit (1-3).

Different interpretation of results from observational studies with vitamin D led to some dispute in the field on the desired optimal vitamin D status *i.e.* serum 25-hydroxyvitamin D [25(OH)D] and also suggest that there may be individual sensitivity and responsiveness to vitamin D₃.

Two recent studies (VitDmet and VitDbol trials) have indicated that there were individual differences in response to supplementation with vitamin D₃ (5, 6). They concluded that the variable response to vitamin D₃ made be explained by epigenetic and genetic individual differences (5, 6). Other possibilities include an individual's ability to convert vitamin D to its active metabolites and the interaction of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] with its receptors and gene response elements (5-7).

The concept is based on the fact that 1 α ,25(OH)₂D₃, serving as the transcription factor for the vitamin D receptor and thus has a direct effect on the epigenome and transcriptome of many human tissues and cell types (5-7). Individuals can be categorized as robust, weak and low or non-responders to vitamin D by measuring vitamin D sensitive molecular parameters, such as changes in the epigenetic status and the respective transcription of genes of

blood immune cells. In addition, responsiveness can also be related to the changes and amounts of proteins or metabolites in the urine or serum (5).

Intracellularly, the interaction of 1,25(OH)₂D with its receptor is thought to result in some kind of physicochemical alterations, leading to its activation after complexing with retinoid acid X receptor. This heterodimer binds to the vitamin D response element of genes unlocking their genetic information (5-7). Our recent study has supported the concept of genomic responsiveness to vitamin D. We have shown that in subjects with a lower genomic response to vitamin D₃ supplementation, there was a response in ~2-5% of the genome while in more responsive subjects >5% of the genome responded to vitamin D₃ (8).

Some specific metabolites are known to be involved in this process of vitamin D dependent genomic activation. The experimental studies have shown that polyamines activate the vitamin D receptor *in vitro* and it is possible that they are related to the regulation of vitamin D action (9). The enhancement of spermidine N-acetyltransferase activity by 1,25-dihydroxyvitamin D is observed not only in the classical target tissues but also in the newly recognized target tissues of vitamin D (10). Some of these target tissues do not appear to be involved in the regulation of mineral metabolism. Indeed, metabolomic studies on blood and urine from subjects supplemented with calcium and vitamin D revealed different metabolic profiles (11). Thus, it seems that there is a close interaction between vitamin D status and metabolic profiles that may explain the individual's response to vitamin D. Less attention has been given to the more basic fundamental concepts related to inter-individual differences in vitamin D optimization (12).

The aim of this randomized controlled double-blind clinical trial was to assess the impact of vitamin D supplementation on metabolomic profiles by relating them to the individual's responsiveness to varying doses of vitamin D₃.

Patients and Methods

The protocol of this metabolomic study was approved by the Institutional Review Board (IRB) of Boston University Medical Campus (H-35506). This study is registered as a clinical trial at the ClinicalTrials.gov (NCT02856776; date of registration 05/08/2016) (8). All participants read and signed the written informed consent.

A deidentified bottle that contained 60 capsules of vitamin D₃ was given to each participant that was formulated with one of the following doses of vitamin D₃, 600 IU/day, 4,000 IU/day or 10,000 IU/day. All vitamin D₃ supplements were provided by Solgar Inc (Leonia, NJ, USA) and the contents evaluated by HPLC as previously described (7) and were found to contain concentrations within 10% of their specified content. The bottles were returned at each visit when the vitamin D₃ capsules were counted to track compliance.

The participants were randomized by a computer-generated randomization program into one of the three study groups.

Recruitment. Healthy young adult males and non-pregnant females were prescreened for serum concentrations of 25(OH)D deemed insufficient (below 30 ng/ml). Inclusion criteria included healthy young black and white adults with a BMI < 30 kg/m² without disorders or medications affecting vitamin D metabolism. To reduce the effect daylight on 25(OH)D level recruitment started in October and supplementation was completed by March. All subjects signed an informed consent that was accepted by the IRB of Boston University Medical Campus (8).

The exclusion criteria, consistent with our previous study (6), were: history of elevated serum calcium (>10.5 mg%); vitamin D supplementation with a dose of 600 IU/day or more; direct exposure to UV during the past month for greater than eight hours; any kind of malabsorption; history of chronic or acute renal or hepatic disease; current antiepileptic medications or glucocorticoids; pregnant/lactating women; and reluctance to consent to the study.

Study visits and sample collection. All participants visited in the GCRU (a unit for clinical research at Boston University Medical Campus). At baseline and every eight weeks, blood was drawn from each subject to determine serum calcium, albumin, creatinine, PTH and total 25(OH)D concentrations. At baseline and after 24 weeks, additional blood and urine were obtained for metabolomics analysis. Quest Diagnostics performed the 25(OH)D and PTH assays as previously described (7). The other blood biochemical tests were performed by Boston Medical Center Clinical Laboratory.

Expression analysis. Genetic expression analysis of circulating mononuclear leukocytes was performed before and after the treatment period to assess differentially expressed genes (DEGs) as previously described (7). RNA extraction was performed using Qiagen's RNeasy kit as described in the manual (Qiagen, Valencia, CA, USA). Isolated RNA was stored at -86°C until microarray analysis. Quality and quantity of the isolated RNA was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All RNA was sent to the Boston University Microarray Resource Facility for analysis. Procedures are described in the GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix, Santa Clara, CA, USA).

Metabolomics analysis. Blood and urine samples were stored at -80°C until processed. Metabolites identified & quantified in serum and urine samples by The Metabolomics Innovation Center (TMIC). Targeted analysis of serum sample was included 83 metabolites of biogenic amines, amino acids, acylcarnitines, phospholipids and sphingolipids. Metabolites identified and quantified in serum by Direct Flow Injection Mass Spectrometry (DI-LC/MS/MS). Targeted analysis of urine sample was mainly water-soluble metabolites, some lipids and organic acids. Metabolites identified & quantified in urine samples by GC-MS (TMIC00R4).

Safety and compliance. The amount of vitamin D₃ that given was within the guidelines recommended by the Institute of Medicine (now National Academy of Medicine) and the Endocrine Society Guidelines (13, 14). Serum calcium, albumin and creatinine was determined every 8 weeks to evaluate for potential toxicity. The participation of an individual who discovered to have serum calcium level above 10.5 mg/dl or serum creatinine increased by >20% was immediately discontinued and the primary care physician was informed (8).

Table I. Characteristics of the participants by dose group.

Characteristics	Vitamin D ₃ dose assignment (IU/day)		
	600 IU/day (n=9)	4,000 IU/day (n=13)	10,000 IU/day (n=8)
Gender (female)	6	8	5
Race (non-White)	6	5	4
Age (years)	26.3±2	25.3±2.1	26.1±2

A deidentified bottle containing 60 capsules of vitamin D₃ was given to each participant that had one of the doses of vitamin D₃ (600 IU/day, 4,000 IU/day or 10,000 IU/day). The bottles were returned at each visit when the vitamin D₃ capsules were counted to track compliance as previously described (7).

Statistical and functional analysis. The genomics and metabolomics data were normalized and the quality control and similarity were checked by using Principal Component Analysis (PCA) method as described previously. To compare the metabolomics alteration between groups, a 2-way ANOVA in the linear model was applied (8). The multivariate data matrix was analyzed and the univariate analysis was performed with SPSS software for Mac (SPSS v.25, US). The data were mean centered and Pareto-scaled prior to principal component analysis (PCA) and orthogonal projection to latent structures discriminate analysis (OPLS-DA). Potential markers of interest were extracted from the combining variable importance projection (VIP) plot that was constructed from the loading plots of OPLS-DA. VIP threshold of 1.5 was considered to select the metabolites. In the nonparametric univariate method, the Mann-Whitney-Wilcoxon test was applied to measure the significance of each metabolite, with results adjusted for multiple testing using false discovery rate (FDR) correction (8). The Pearson correlation was used to study the relations between biomarkers measured and the phenotypic characteristics. Statistical analyses were performed using SPSS software for Mac (SPSS v.25, USA). A *p*-value threshold of 0.05 and also a False Discovery Rate (FDR) of 0.1 considered as significant results. Data are presented as the mean and SD.

Pathways analysis. Pathway enrichment analysis was performed as previously described. DEGs with a significant fold change (>2.0-fold) were selected and reviewed for known associations to pathways involved in cell growth and proliferation using DAVID Bioinformatics 6.8 as previously described (7). Metabolite Set Enrichment Analysis and Pathway Analysis were carried out using the pathway analysis module (MetPA) (15, 16) of Metaboanalyst 3.0. Hypergeometric test and relative betweenness centrality (15, 16) were used for over-representation analysis and pathway topology analysis, respectively.

Results

A total of 33 adults who met the inclusion/exclusion criteria were enrolled and randomized into each study arm. Thirty participants (90%) completed the 6-month trial (600 IU/day, n=9; 4,000 IU/day, n=13; 10,000 IU/day, n=8). The characteristics of participants are shown in Table I.

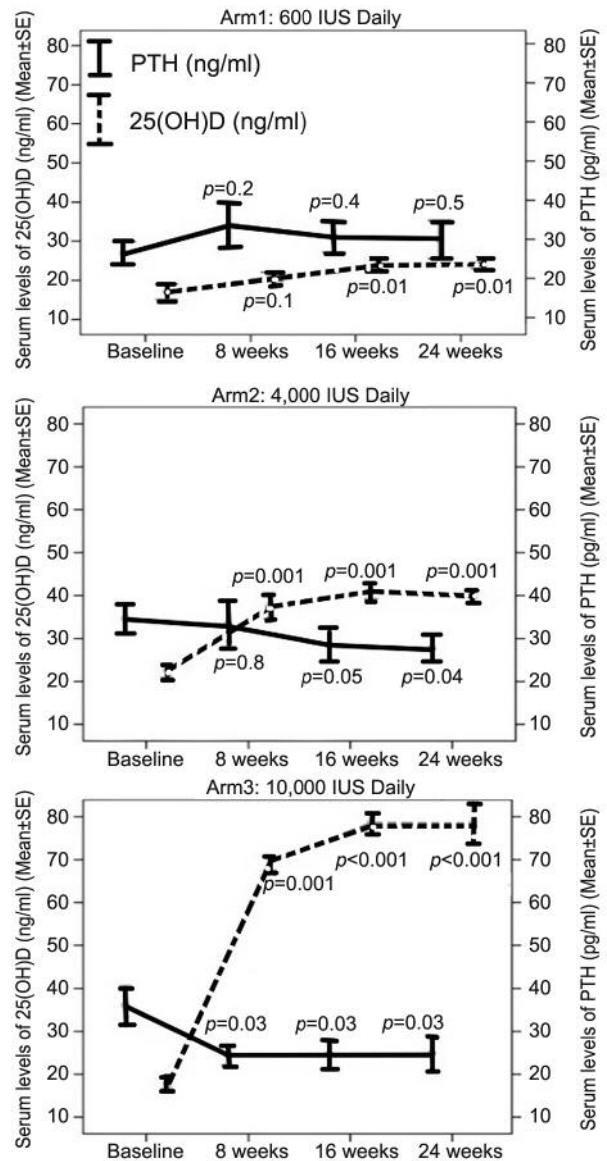


Figure 1. Total concentration of PTH and 25(OH)D during vitamin D₃ supplementation at baseline and every eight weeks for 24 weeks. This figure is reproduced with permission (8).

The average increase in 25(OH)D over 24 weeks was 7 ng/ml (18 nmol/l), 18 ng/ml (45 nmol/l) and 61 ng/ml (153 nmol/l) for 600 IU/day, 4000 IU/day and 10,000 IU/day, respectively. Figure 1 depicts serum concentrations of 25(OH)D and PTH by dose group. There was no significant change in serum concentrations of PTH or calcium in the 600 IU/day group ($p>0.05$). All participants in the 4000 and 10,000 IU/day groups achieved 25(OH)D levels >30 ng/ml (>75 nmol/l). There was no significant change in serum calcium for either group. Significant decreases in PTH

levels of 17.5% and 33.3% at 16 weeks were found for the 4000 and 10,000 IU/day group, respectively ($p=0.04$). PTH levels remained at that level for the remaining 8 weeks (Figure 1, reproduced with permission) (8). There were no significant differences between men and women with respect to changes in serum concentration of calcium, 25(OH)D or PTH in response to supplementation with vitamin D (8).

Differential expression analysis identified a dose-dependent 25(OH)D alteration in broad gene expression with 162 (86 up-regulated, 76 down-regulated), 320 (188 up-regulated, 132 down-regulated), and 1289 (800 up-regulated, 489 down-regulated) genes up- or down-regulated in subjects who received 600, 4,000, and 10,000 IU/day dose, respectively (8).

An evaluation of genome wide expression of genes influenced by vitamin D₃ revealed a greater change in genetic expression in subjects who received 10,000 IU/day/6 months compared to the other two groups. Visualizing of the gene expression alterations in the group of 10,000 IU/day is shown in Figure 2 reproduced with permission (8).

Variable pattern of broad gene expression in response to supplementation with Vitamin D₃. We compared gene expression between dose groups and related these data to changes in circulating levels of 25(OH)D and PTH to provide a clearer understanding of the biologic responsiveness to different doses of vitamin D. The pattern of gene expression in response to vitamin D supplementation showed an inter-individual variation. Approximately 30% of the adults who received different doses of vitamin D₃ supplement demonstrated a much smaller genomic response (8). The serum 25(OH)D levels of these subjects after 6 months of vitamin D₃ supplementation raised to the same degree as the other 70% (8). This variable pattern of expression is shown in Figure 2, which displays three subjects (ID: 34, 46 and 68) that had a very strong genomic response to vitamin D₃ supplementation with many genes being up or down regulated, in comparison with one subject (ID: 73) with a moderate response and two subjects (ID: 56 and 66) with a weak response (8).

Our broad gene expression analysis showed that in subjects with a lower genomic response to vitamin D₃ supplementation there was a response in ~2-5% of the genome while in more responsive subjects >5% of the genome responded to vitamin D₃ (8).

Pathway and functional analysis of the differentially expressed genes. We mapped upregulated genes to the STRING database (7) and selected high confidence for interactions score. The result was significant for protein-protein interaction (PPI) enrichment ($p\text{-value}=9.33e^{-14}$). The key genes in these clusters were *HIST1H2B*, *JUN*, *NFKB*, *TNF*, *IL8*, *HSPA8*, *EIF4A* and *PRS*. This network includes 4 clusters of which one is related to histone modification (7,

8). Epigenetic modifications such as histone modification and chromatin regulation are necessary mechanisms to control of gene expression (8). Gene Ontology showed that the genes in this network may regulate DNA accessibility and stability of chromosomes *via* histone modification as well as remodeling of the nucleosomes. This finding may explain the role of vitamin D supplementation on chromatin accessibility (8). Furthermore, the accessibility of chromatin and vitamin D supplementation might be considered as factors involved in the pattern of individual alterations in broad gene expression. The other clusters in this network are related to signaling pathways of *NF-kappa B*, *TNF*, NOD-like receptor, T cell receptor, *mTOR*, Chemokine, *MAPK*, Toll-like receptor and pathways in cancer. PPI network was also constructed from the differentially downregulated genes. The key genes in this network are *TLR1*, *CD180* and *LRRN3*.

Genes for known cancer-related pathways were significantly regulated in the 10,000 IU/day cohort, but not in the 600 or 4,000 IU/day cohorts. In the 10,000 IU/day cohort, 104 genes (8.1%; $p<0.001$) are involved in a cancer-related pathway, of which 41 genes (39.4%; $p<0.0001$; fold change >2.0; FDR<0.01) are related to B cell lymphomas. Vitamin D₃ significantly ($p<0.05$, FDR<0.01) down-regulated expression of the following genes (fold-change): *MYD88* (-4.7), *HCK* (-4.65), *SYK* (-4.98), *BCL2* (-3.99), *IKBKB* (-4.31), *IL6R* (-5.31), *TLR4* (-5.98), *NOTCH1* (-4.32), and *MYC* (-5.65). Vitamin D₃ also significantly up-regulated expression of the following genes (fold-change): *KDM6A* (+4.98), *NFKBIZ* (+8.97), and *NFKBIB* (+6.31).

In the 10,000 IU/day cohort, 65 genes are involved in the immune system. The 65 differentially expressed upregulated genes were mapped to the STRING database and screened for significant interactions of the highest confidence (Figure 3).

These biological connections compose 5 clusters and demonstrate that vitamin D₃ influences specific functions of the immune system. The key genes within the 5 clusters are phosphatidylinositol 3-kinase catalytic subunit alpha (*PIK3CA*), tumor necrosis factor (*TNF*), ubiquitin C (*UBC*), histone cluster 1 H2B (*HIST1H2B*), AP-1 transcription factor subunit (*JUN*), and NF-κB subunits (*NFKB* and *RELA*). Additionally, 5 genes were found to have no interactions (*PMAIP1*, *POLB*, *PPP3CC*, *SNWI*, *CREB3*). All clusters have biological functions relating to the regulation of immune system processes, response to stress, response to cytokine, cytokine-mediated signaling, cellular response to cytokine stimulus, and response to organic substance, among other immune functions. The significant pathways involved include NF-κB (light blue cluster) and TNF-signaling (upper right-hand dark blue cluster).

The light and dark blue clusters are related to rheumatoid arthritis, atherosclerosis, giant cell arteritis, sarcoidosis, graves' disease, celiac disease, inflammatory bowel disease,

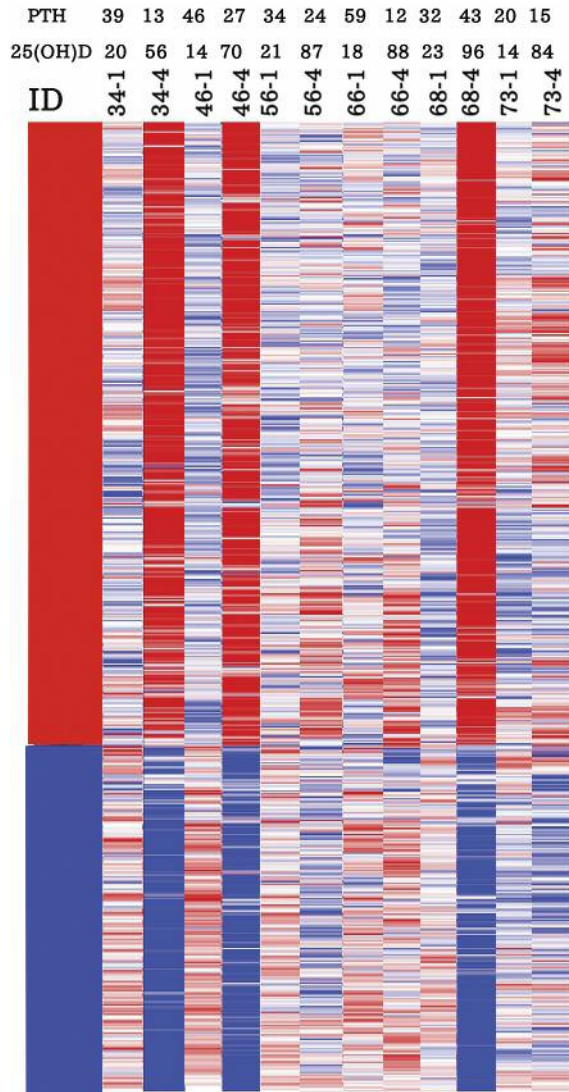


Figure 2. Visualization the gene expression alterations in response to 10,000 IU/day vitamin D₃ supplementation. The gene-expression alterations are demonstrated by different colors. Upregulation and downregulation of gene expression after 6-months vitamin D₃ supplementation showed by red and blue, respectively. Trends of gene expression are seen by range of colors from light blue to dark red. The red, white and blue represented of high, average and low gene expression, respectively. This figure is reproduced with permission (8).

and psoriasis. The dark blue cluster alone is related to graves’ ophthalmopathy. The red cluster is related to atherosclerosis, sarcoidosis, graves’ disease, celiac disease, and psoriasis. The green cluster is related to rheumatoid arthritis, atherosclerosis, celiac disease, and psoriasis. Additionally, the region circled in red corresponds to histone cluster 1 H2A, H2B, H3, and H4 family members which are involved in epigenetic modification and systemic lupus erythematosus.

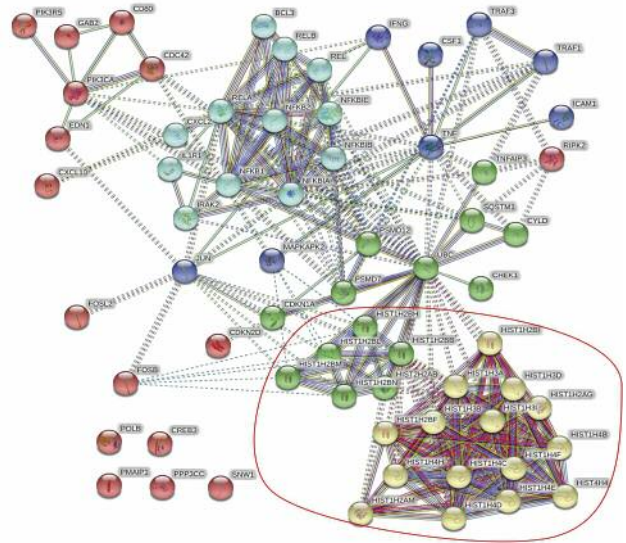


Figure 3. Protein-Protein Interaction (PPI) Network was constructed from differentially upregulated genes contributing to immune system-related pathways after 6-month supplementation with vitamin D₃.

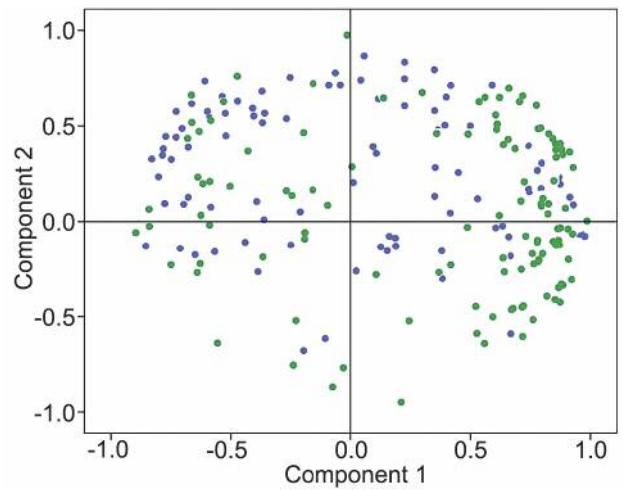


Figure 4. The PCA score plot of the study groups. There was not any pattern of separation in the differential metabolites between 600 IU/day (blue dots) and 10,000 IU/day groups (green dots).

Vitamin D₃ supplementation and metabolomic profile. Targeted analysis of serum samples was included 83 metabolites of biogenic amines, amino acids, acylcarnitines, phospholipids and sphingolipids. Metabolites were identified and quantified in serum by Direct Flow Injection Mass Spectrometry (DI-LC/MS/MS). Targeted analysis of urine samples was included 36 metabolites of mainly water-soluble metabolites, some lipids and organic acids. Metabolites were identified and quantified in urine samples by GC-MS.

Table II. *The most differentially regulated metabolites by vitamin D₃ after 6 months supplementation.*

Metabolites	Source	Fold change	Standard error	p-Value
Methionine sulfoxide	Urine	3.7	1.0	0.001
3 Hydroxyisovaleric acid	Urine	1.6	0.5	0.02
3 Methylglutaconic acid	Urine	1.7	0.3	0.001
2 Furoylglycine	Urine	3.2	0.8	0.05
PC aa C32:2 (Phospholipids)	Serum	1.5	0.1	0.005
C0 (Carnitine)	Serum	1.5	0.1	0.001
Uracil	Serum	1.5	0.2	0.001
C3 (Propionylcarnitine)	Serum	1.5	0.1	0.02
C4 (Butyrylcarnitine)	Serum	1.5	0.1	0.001
C5 (Valerylcarnitine)	Serum	1.5	0.1	0.006
Spermidine	Serum	1.5	0.03	0.001

To individually evaluate each metabolite, we calculated the statistically significant ($p < 0.05$ and fold change > 1.5) changes of 11 metabolites (7 from serum and 4 from urine) after 6 months vitamin D₃ supplementation, as shown in Table II.

The enrichment pathway analysis showed that these metabolites mostly involved in the oxidation of branched chain fatty acids. In the principal component analysis (PCA) score plot, the samples of the baseline were only partially separated from the samples of 6 months supplementation with vitamin D₃. PCA was first performed to discover intrinsic supplementation-related clusters within the datasets. Following this, partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were used to improve separation among the groups and screen differential metabolites. We also didn't observe any reasonable separation of the study groups in the PCA score plot (Figure 4).

There were no significant differences between the 3 study groups (600, 4000 and 10,000 IU/day) with respect to changes in metabolomic profile in response to supplementation with vitamin D₃ as shown by ANOVA.

Following this, partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were also used to improve separation among the vitamin D more responsive and less responsive groups and screen differential metabolites. The score plot resulted in unambiguous inter-group separation (Figure 5).

We observed a clear separation between the vitamin D₃ more responsive and less responsive groups, as shown in the score plot (Figure 5). Differential metabolites were selected based on the separation through the score plot (circle in Figure 5). These metabolites included 44 metabolites from serum and 10 metabolites from urine.

The metabolites with the highest VIP-scores and loading factors are shown in Table III.

Metabolomic pathway analysis. The underlying signaling pathways and molecular networks influenced by vitamin D₃ supplementation were explored by MetPA. Identified metabolites contributing to the separation of vitamin D more responsive and less responsive groups were imported into MetPA. This analysis of the relationship between the identified metabolites and diseases such as seizure, hartnup, diabetes mellitus, coronary, schizophrenia and stroke, among others, are mainly predicted (Table IV).

Discussion

There is an ongoing debate on recommendations of daily allowance for vitamin D as well as the optimal serum concentration to achieve efficacy (17). Our results demonstrated that PTH plateaued when 25(OH)D levels were ≥ 30 ng/ml (75 nmol/l) and confirmed previous observations that serum concentrations of PTH continued to decrease and reached a plateau when circulating levels of 25(OH)D were > 30 ng/ml (18-20). The increase in vitamin D₃ dose from 4000 IU/day to 10,000 IU/day had no significant additional effect on PTH levels (Figure 1). However, the gene expression analysis demonstrated a dose-dependent effect. Even for subjects who took 600 IU/day of vitamin D₃ for 24 weeks, a dose that had little effect on PTH levels, the expression of more than 100 genes was significantly affected. These results indicated that even a small increase in vitamin D₃ intake of 600 IU/day for 24 weeks exerted significant genomic effects.

These results may help explain the disparity of conclusions regarding the studies that have evaluated the impact of supplementation with vitamin D₃ on serum 25(OH)D improvement and clinical outcomes. The individual's response to vitamin D is explained by the individual's ability to convert vitamin D to its active metabolites and their interaction with their receptors and response elements (5-7).

Similar to our findings, two recent studies (VitDmet and VitDbol trials) have indicated that there are individual differences in response to supplementation with vitamin D (5, 6). Based on their conclusion, this response to vitamin D is explain by epigenetic and genetic individual differences (5, 6). In agreement, our results indicated that in subjects with a lower genomic response to vitamin D₃ supplementation there was a response in $\sim 2-5\%$ of the genome while in more responsive subjects $> 5\%$ of the genome responded to vitamin D₃. The most pronounced vitamin D response occurred in genes involved in genetic regulation. The major upregulated genes were *HIST1H2B*, *JUN*, *NFKB*, *TNF*, *IL8*, *HSPA8*, *EIF4A* and *PR3* and the major down regulated genes were *TLR1*, *CD180* and *LRRN3*. The upregulated genes were involved in 4 main clusters, one of which is related to histone modification. Epigenetic modifications, such as histone modifications and chromatin

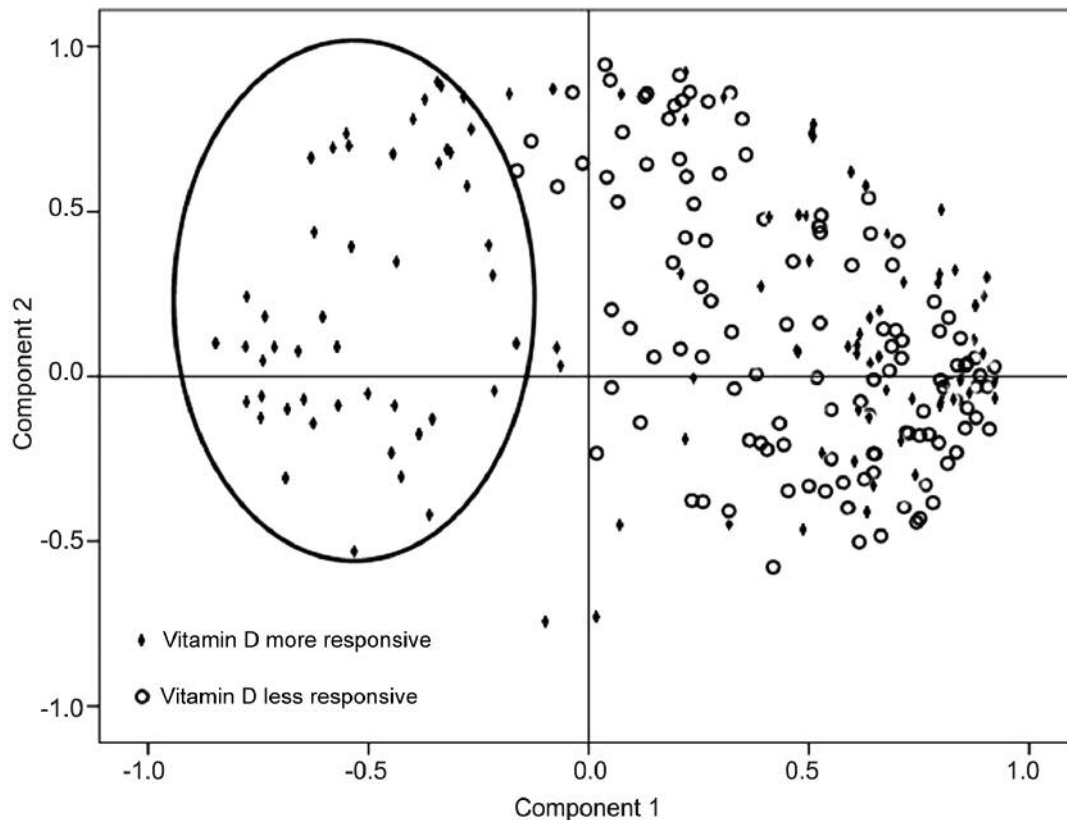


Figure 5. The PCA score plot of the vitamin D in the more responsive and less responsive groups. The score plot resulted in unambiguous inter-group separation. Differential metabolites in the vitamin D more responsive group are shown by diamond dots and in the less responsive by circle dots. The large circle points to the metabolites with the most discriminating power between vitamin D more responsive and less responsive groups.

regulation, are essential parts of gene regulation (21, 22). Current findings are consistent with our previous study (7) and several studies (23, 24) that showed that vitamin D supplementation resulted in alterations in genes related to epigenetic modifications.

We demonstrated that vitamin D supplementation altered the metabolomic profile by analyzing the serum and urinary metabolomics profile of subjects before and after 6 months supplementation with varying doses of vitamin D₃. Targeted analysis included 83 metabolites from serum and 36 metabolites from urine. To individually evaluate each metabolite, we calculated the statistically significant ($p < 0.05$ and fold change > 1.5) changes of 11 metabolites (7 from serum and 4 from urine) after 6 months vitamin D₃ supplementation, as shown in Table II. These findings are consistent with previous studies implicating vitamin D in metabolomic alteration. The relationship between some of these differentially affected metabolites like spermidine have been previously reported. The previous studies have indicated that spermidine N1-acetyltransferase activity by 1 $\alpha,25(\text{OH})_2\text{D}_3$ is earliest induced metabolite reported to

date and that the 1 $\alpha,25(\text{OH})_2\text{D}_3$ -induced duodenal synthesis of putrescine occurs by the pathways from both ornithine and spermidine (10). Furthermore, some studies have suggested that both spermidine and spermine are involved in the activation of vitamin D receptor (9, 10). Our results showed the ability of metabolomics to identify alterations in the metabolome of subjects more responsive to Vitamin D₃ compared with the less responsive subjects. We observed a clear separation in the targeted metabolites between the vitamin D₃ more responsive and less responsive groups, as shown in the score plot (Figure 5). Differential metabolites were selected based on the separation through the score plot (red circle in Figure 5). These metabolites included 44 metabolites from serum and 10 metabolites from urine.

Understanding the inter-relationships among genes, gene products and dietary habits is fundamental to identifying those who will benefit most from intervention strategies. Unravelling the multitude of nutrigenomic and metabolomic patterns that arise from the ingestion of foods or their bioactive components provides insights into a tailored approach to diet and supplementation (25). Hence,

Table III. The list of metabolites that were differentially regulated in vitamin D more responsive and vitamin D less responsive groups.

Metabolites	HMDB	PubChem	KEGG
Azelaic acid	HMDB0000784	2266	C08261
Suberic acid	HMDB0000893	10457	C08278
Glutamine	HMDB0000641	5961	C00064
Kynurenine	HMDB0000684	161166	C00328
Valine	HMDB0000883	6287	C00183
Pimelic acid	HMDB0000857	385	C02656
Leucine	HMDB0000687	6106	C00123
Tryptophan	HMDB0000929	6305	C00078
Arginine	HMDB0000517	6322	C00062
Histidine	HMDB0000177	6274	C00135
Isoleucine	HMDB0000172	6306	C00407
Serotonin	HMDB0000259	5202	C00780
Tiglylglycine	HMDB0000959	6441567	-
4-Deoxythreonic acid	HMDB0002453	10964471	-
Methylsuccinic acid	HMDB0001844	10349	C08645
Ethylmalonic acid	HMDB0000622	11756	-
Hydroxypropionic acid	HMDB0000700	68152	C01013
trans-Aconitic acid	HMDB0000958	444212	C02341
Adipic acid	HMDB0000448	196	C06104
p-Hydroxyphenylacetic acid	HMDB0000020	127	C00642
3-keto-2-Methylbutyrate	HMDB00029172	11966223	-
Vanillylmandelic acid	HMDB0000291	736172	C05584
3-Hydroxyphenylacetic acid	HMDB0000440	12122	C05593
Uracil	HMDB0000300	1174	C00106
Octadecenoylcarnitine	HMDB0013340	53481699	-
Glycolic acid	HMDB0000115	757	C00160
5-Hydroxyindoleacetic acid	HMDB0000763	1826	C05635
L-Acetylcarnitine	HMDB0000201	1	C02571
Quinolinic acid	HMDB0000232	1066	C03722
Pimelylcarnitine	HMDB0013328	53481675	-
Tetradecenoylcarnitine	HMDB0013330	53481679	-
Alpha-Hydroxyisobutyric acid	HMDB0000729	11671	-
3-Hydroxy-2-methylglutarate	HMDB00029169	20070700	-
Dodecanoylcarnitine	HMDB0002250	168381	-
3-Hydroxymandelic acid	HMDB0000750	86957	-
Pyroglutamic acid	HMDB0000267	7405	C01879
Palmitoylcarnitine	HMDB0000222	11953816	C02990

nutrigenomics is destined within the next decade to identify complex associations between nutrients and the expression of thousands of genes with simultaneous changes in an equivalent number of metabolites (26).

This research program emphasizes the importance of personalized medicine. Vitamin D supplementation at 10,000 IU/day for 6 months was safe, optimally regulated PTH levels and had a pronounced effect on genetic expression of 1200 genes as well as on metabolomic profile. Furthermore, broad gene expression and metabolomic

Table IV. Metabolite set enrichment analysis for the prediction of disease associated metabolites that were differentially regulated between vitamin D₃ more responsive and less responsive groups.

Predicted disease	p-Value	FDR
Acute seizures	0.00027	0.0738
Different seizure disorders	0.00122	0.166
Hartnup disease	0.00582	0.529
Diabetes mellitus (MODY), non-insulin-dependent	0.011	0.655
Pyruvate dehydrogenase deficiency (E3)	0.012	0.655
Continuous ambulatory peritoneal dialysis (CAPD)	0.0223	0.954
Maple syrup urine disease	0.0258	0.954
Short bowel syndrome (under arginine-free diet)	0.0316	0.954
Ornithine transcarbamylase deficiency (OTC)	0.0349	0.954
Refractory localization-related epilepsy	0.0349	0.954
Schizophrenia	0.0414	1.0
Spastic ataxia	0.0501	1.0
Stroke	0.0501	1.0

profile may predict individual response to vitamin D₃ supplementation.

Conflicts of Interest

The A.S., T.K., A.S., R.A., N.C., and R.L. declare no competing interests. M.H. is a consultant for Quest Diagnostics Inc., and was on the speaker's Bureau for Abbott Inc. and Hayat Pharmaceutical Industries Company PLC.

Author's Contributions

Study design: A.S., R.L. and M.H. Data acquisition: A.S., T.K., A.S., R.A., N.C., R.L. and M.H. Analysis: A.S. and M.H. All authors, A.S., T.K., A.S., R.A., N.C., R.L. and M.H., reviewed and edited the manuscript. M.H. is the guarantor of this work, and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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