



# Molecular Approaches for Optimizing Vitamin D Supplementation

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## Abstract

Vitamin D can be synthesized endogenously within UV-B exposed human skin. However, avoidance of sufficient sun exposure via predominant indoor activities, textile coverage, dark skin at higher latitude, and seasonal variations makes the intake of vitamin D fortified food or direct vitamin D supplementation necessary. Vitamin D has via its biologically most active metabolite  $1\alpha,25$ -dihydroxyvitamin D and the transcription factor vitamin D receptor a direct effect on the epigenome and transcriptome of many human tissues and cell types. Different interpretation of results from observational studies with vitamin D led to some dispute in the field on the desired optimal vitamin D level and the recommended daily supplementation. This chapter will provide background on the epigenome- and transcriptome-wide functions of vitamin D and will outline how this insight may be used for determining of the optimal vitamin D status of human individuals. These reflections will lead to the concept of a personal vitamin D index that may be a better guideline for an optimized vitamin D supplementation than population-based recommendations.

## ABBREVIATIONS

**1,25(OH)<sub>2</sub>D** 1 $\alpha$ ,25-dihydroxyvitamin D

**25(OH)D** 25-hydroxyvitamin D

**CYP** cytochrome P450

**FAIRE-seq** formaldehyde-assisted isolation of regulatory elements sequencing

**OGTT** oral glucose tolerance test

**PBMC** peripheral blood mononuclear cell

**PTH** parathyroid hormone

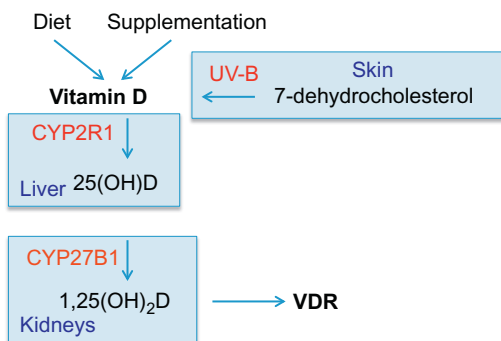
**SNP** single nucleotide polymorphism

**VDR** vitamin D receptor



## 1. INTRODUCTION

The natural way of obtaining vitamin D is its endogenous synthesis from the cholesterol precursor 7-dehydrocholesterol (Fig. 1). Thus the term “vitamin” is not used in its original sense. However, the essential need of UV-B for endogenous vitamin D synthesis restricts this pathway to sun exposed skin. Lifestyle changes during the last hundreds to thousand years, such as predominant indoor activities and textile coverage outdoors, as well as seasonal and climatic changes, often result in insufficient UV-B exposure and thus low endogenous vitamin D production. This can cause dependence on external vitamin D supply, i.e., under these conditions the compound is correctly termed a vitamin. Average human diet is rather low in vitamin D,



**Figure 1** Vitamin D synthesis pathway. Vitamin D is taken up by diet or supplementation or synthesized endogenously in the skin under essential catalysis by UV-B. In the liver, vitamin D is converted by the enzyme CYP2R1 into the circulating form 25(OH)D and then in the kidneys by the enzyme CYP27B1 into the high-affinity VDR ligand 1,25(OH)<sub>2</sub>D.

since only fatty fish and some mushrooms contain reasonable amounts of the vitamin D isomers vitamin D<sub>3</sub> and vitamin D<sub>2</sub>, respectively. Therefore, in some countries dietary products, such as milk, margarine, and juices, are fortified with vitamin D<sub>3</sub> or direct supplementation with the compound is recommended. This chapter discusses how for each human individual an optimal vitamin D level can be determined.

Vitamin D itself is an biologically inert molecule and has to be activated by hydroxylation first at position 25, leading to the prehormone 25-hydroxyvitamin D (25(OH)D), and then at position 1, creating the nuclear hormone 1 $\alpha$ ,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) (Norman, 2008). The latter molecule is the only natural high-affinity ligand to the transcription factor vitamin D receptor (VDR) (Haussler et al., 1997). The first step of vitamin D synthesis is catalyzed in the liver by the cytochrome P450 (CYP) enzyme CYP2R1 and the second step by CYP27B1 in the kidneys (Fig. 1). The individual's vitamin D status is assessed best via the serum 25(OH)D concentration, since this vitamin D metabolite has a half-life of 15 days, while the half-life of 1,25(OH)<sub>2</sub>D is with approximately 15 h far shorter. For both bone and overall health, the Institute of Medicine recommends 25(OH)D serum levels of  $\geq 50$  nM (Institute-of-Medicine, 2011). This translates to a daily vitamin D supplementation of 10–15  $\mu$ g (400–600 IU) for children and 15–20  $\mu$ g (600–800 IU) for adults. In contrast, the US Endocrine Society suggests a 25(OH)D serum concentration of 75 nM and daily vitamin D supplementations with 25  $\mu$ g (1000 IU) or more (Holick et al., 2011).

The classical action of 1,25(OH)<sub>2</sub>D is the promotion of dietary calcium and phosphorus absorption in the intestine, the facilitation of calcium reabsorption in the renal tubules, and the control of remodeling in the bones. Since CYP27B1 is not only expressed in the kidneys, there is also extra-renal production of 1,25(OH)<sub>2</sub>D in tissues, such as in macrophages, the gastrointestinal tract, skin, vasculature, and placenta, in which the nuclear hormone has paracrine and autocrine functions. Furthermore, the rather ubiquitous expression of VDR supports the view that vitamin D and its receptor have far more functions than only the control of calcium homeostasis and bone remodeling. Accordingly, vitamin D deficiency does not only result in rickets in children and in a higher incidence of osteoporotic fractures in adults (Carlberg, 2014b), but it may also compromise the protective roles of vitamin D against cancer, cardiovascular diseases, diabetes, infections, and neuropsychiatric disorders (Holick, 2007), since vitamin D is involved in the regulation of cellular growth and differentiation (Feldman, Krishnan,

Swami, Giovannucci, & Feldman, 2014) as well as innate and adaptive immunity (Chun, Liu, Modlin, Adams, & Hewison, 2014).

In summary, it is undisputed that a sufficient vitamin D status is essential for bone health, such as the prevention of osteoporosis (Institute-of-Medicine, 2011). Tissue calcification is the main possible side effect that might be caused by overdosing with natural and synthetic vitamin D analogs (Cheskis, Freedman, & Nagpal, 2006). Therefore, there is some hesitation to recommend higher vitamin D doses for reaching nonskeletal effects of vitamin D. The latter are mainly based on observational studies and most of them lack proof of causal relations from randomized controlled trials.



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## 2. A VIEW FROM EVOLUTION

Cholesterol synthesis is an evolutionary very old pathway, so that 7-dehydrocholesterol was already available in early marine organisms, such as phyto- and zooplankton (Holick, 2011). These species use the photochemical reaction resulting in vitamin D as a protection against UV-B-induced DNA damage, i.e., the historically first role of vitamin D was that of an inert molecule acting as a sunscreen. Since plankton is a major component in the marine food chain, vitamin D accumulates in the liver of many deep-water fish, such as cod. Vitamin D obtained endocrine functions when animals moved out of the water and needed to develop a stable skeleton based on calcium (Bouillon & Suda, 2014). Therefore, only vertebrates have a full vitamin D endocrine system, composed of plasma transport proteins, such as the vitamin D binding protein, metabolizing enzymes, such as CYP27B1 and CYP24A1, and a specific high-affinity nuclear receptor, such as VDR.

The evolutionary precursors of nuclear receptors were ligand-independent transcription factors being primarily involved in the control of cellular metabolism (Escriva, Bertrand, & Laudet, 2004). These ancestral nuclear receptors acquired in a multistep process the ability to bind and to be activated by metabolic compounds, such as bile acids in the case of the VDR precursor (Makishima et al., 2002). Further evolution of the ligand-binding domain created then a ligand-binding pocket that can harbor with high specificity and affinity 1,25(OH)<sub>2</sub>D (Carlberg & Molnár, 2012). After the move from the calcium-rich ocean to the calcium-poor terrestrial environment, the control of calcium homeostasis became a speciality of the vitamin D endocrine system (Bouillon & Suda, 2014). However, the

evolutionary background of vitamin D and the VDR implies that both are involved in a wider set of functions than only providing bones with calcium.

Anatomically modern humans developed some 200,000 years ago in East Africa. Already their ancestors had obtained dark skin following the loss of body hair more than 1 million years ago, in order to prevent UV-mediated degradation of the essential circulating methyl-group donor folate. The intensive sun exposure at the equator allowed, despite their dark skin, sufficient vitamin D<sub>3</sub> synthesis. In fact, the average circulating levels of 25(OH)D in members of the traditionally living Maasai tribe in East Africa is 119 nM (Luxwolda, Kuipers, Kema, Dijck-Brouwer, & Muskiet, 2012). Since modern humans lived some 150,000 years a similar lifestyle than the Maasai, at least concerning sun exposure, it can be assumed that human physiology and biochemistry has adapted well to this rather high vitamin D status. When some 50,000 years ago some modern humans started to move north toward Asia and Europe, the essential need of endogenous vitamin D production in less sunny regions at higher latitude caused an evolutionary pressure for skin lightening (Hochberg & Templeton, 2010). The gradual skin lightening process took 10,000–30,000 years and reflects the pace of human migration to nearly all regions of the planet. In an evolutionary scale, this is very fast, i.e., most other population-wide adaptations of the human genome took far more time. Nevertheless, the immigration of light skin Europeans to the Americas and Australia and the involuntary transfer of dark skin Africans to the Americas within the last 500 years were far too quick for any genetic adaptation. In net effect, nowadays many humans live at a latitude, i.e., at an average yearly UV-B exposure, for which their skin color is not adapted. For example, dark skin persons living in Canada or in the UK do not have the chance of sufficient endogenous production of vitamin D. In addition, colder climate as well as cultural and religious traditions imposed to most humans a textile coverage of nearly their entire body being another reason for a low endogenous vitamin D synthesis in most human populations. Furthermore, genetic variations between human individuals are primarily found on the level of single nucleotide polymorphisms (SNPs). Some of these SNPs have been shown to be related to serum 25(OH)D levels, i.e., to the vitamin D status of the individuals (Wang et al., 2010). Thus, based on their genetics some persons are exposed throughout their entire life to either a higher or a lower serum 25(OH)D level than the average population.

The agricultural revolution that started some 10,000 years ago and in particular the industrial revolution that took place during the last

100–200 years drastically changed the lifestyle of nearly all human populations concerning the composition of diet, physical activity, and the intestinal microbiome. These changes are closely related and result in homeostatic imbalances that are the basis of many common non-communicative disorders, such as cardiovascular disease, diabetes, autoimmune disease, and cancer. Thus, today's rather low rate of endogenous vitamin D production parallels with many other potentially disease-promoting lifestyle changes of contemporary humans that are genetically still adapted to a large extent to the environmental conditions and lifestyle of their ancestors in the savannahs of East Africa. However, we should bear in mind that evolution selects for benefits that result in higher number of offspring reaching a reproductive age, i.e., getting and raising children, but not for aging-related diseases. Therefore, the vitamin D status was probably high, in order to protect against infectious diseases, such as tuberculosis, but has not been adapted for the protection against disorders that normally occur at higher age, such as cancer and cardiovascular disease.

Taken together, the genetic origin of all contemporary humans is an equatorial region (East Africa), where there are no seasonal changes in UV-B exposure. This means that the human genome is rather adapted to a constant vitamin D status than to level changes between summer and winter. The very high average 25(OH)D serum concentration in Maasai may represent an optimal vitamin D status but it could also reflect the maximal levels that evolution allowed in the presence at excessive sun exposure. Interestingly, the 25(OH)D serum levels of Maasai individuals ranged from 58 to 167 nM (Luxwolda et al., 2012). This may suggest that there is a wide personal range in the optimal or maximal vitamin D status that could be either genetically or epigenetically programmed.



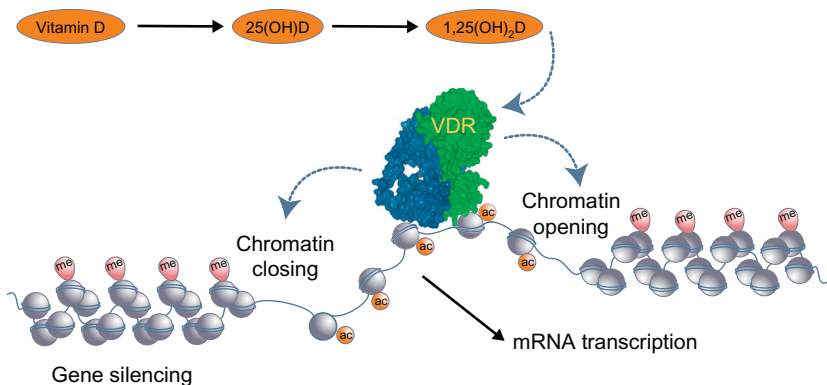
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### 3. VITAMIN D AND THE EPIGENOME

As a high-affinity ligand of the transcription factor VDR, 1,25(OH)<sub>2</sub>D and its precursor vitamin D belong to a small group of natural compounds that have a direct effect on gene regulation (Carlberg & Dunlop, 2006). VDR is a member of the nuclear receptor superfamily (Evans & Mangelsdorf, 2014) that contains other endocrine receptors specifically binding lipophilic molecules in the size of cholesterol, such as estrogen, testosterone, and cortisol (Carlberg & Molnár, 2012). The nuclear receptor is essential for all molecular actions of 1,25(OH)<sub>2</sub>D, but in addition VDR bears also ligand-independent functions (Polly et al., 2000), i.e., the functional

profile of the receptor exceeds that of its ligand. VDR acts as the mechanistic core of vitamin D signaling. It recognizes via its DNA-binding domain genomic target sequences, it uses its complete surface for the interaction with other nuclear proteins, and its inner surface, the ligand-binding pocket, serves as a cave specifically interacting with  $1,25(\text{OH})_2\text{D}$  and its synthetic analogs (Carlberg, Molnár, & Mourino, 2012). The protein–protein interaction partners of VDR are either other transcription factors, such as the retinoid X receptor (blue in Fig. 2) that help the receptor to stabilize its contact with genomic DNA (Carlberg & Polly, 1998), or nuclear adaptor proteins, such as corepressors, coactivators, and the Mediator complex.

In all stages of a cell, genomic DNA forms a complex with nucleosomes (gray balls in Fig. 2), referred to as chromatin. As densely packed heterochromatin, it prevents the access to genomic DNA (Beisel & Paro, 2011). Chromatin has this intrinsic repressive potential in order to conserve the epigenetic landscape of a differentiated cell, i.e., by default it largely restricts the access of transcription factors to promoter and enhancer regions leaving only in the order of 50–100,000 accessible chromatin regions per cell type (ENCODE-Project-Consortium et al., 2012). The accessibility of chromatin is modulated by methylation of genomic DNA and by posttranslational modifications, such as acetylations and methylations, of nucleosome-forming histone proteins (Fig. 2) (Bell, Tiwari, Thoma, & Schubeler,



**Figure 2** Chromatin model of vitamin D signaling. Gene regulation by VDR requires accessible genomic DNA, i.e., open chromatin. In turn, ligand-dependent actions of VDR result either in further opening of chromatin (i.e., in most cases, in upregulation of the transcription of the respective gene) or in closing of chromatin (and respective downregulation of transcription).

2011; Zhou, Goren, & Bernstein, 2011). These epigenomic modifications are controlled by chromatin modifying enzymes that read, write, or erase posttranslational marks on the histones.

Some of the protein–protein interaction partners of VDR attract chromatin remodeling complexes that rearrange the positioning of nucleosomes as well as chromatin modifying enzymes that locally open and close chromatin (Fig. 2). For example, VDR-mediated histone modifications can change the pattern of accessible chromatin regions. The interactions of VDR with nuclear proteins are modulated by the absence or presence of  $1,25(\text{OH})_2\text{D}$  within the receptor's ligand-binding domain, i.e., the most of them are ligand-dependent (Molnár, 2014). Experimentally, this can be monitored genome-wide by using the method formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq) (Giresi, Kim, McDaniell, Iyer, & Lieb, 2007). A detailed FAIRE-seq time course in a  $1,25(\text{OH})_2\text{D}$ -stimulated human monocytic cell line (THP-1) could demonstrate that some 87% of VDR binding sites colocalize with open chromatin (Seuter, Pehkonen, Heikkinen, & Carlberg, 2013). At approximately 20% of these loci, a stimulation with  $1,25(\text{OH})_2\text{D}$  leads to a significant increase in chromatin accessibility (Seuter et al., 2013). In this way, vitamin D has a direct effect on changes of the epigenome.

A gene can only be transcribed when the genomic regions of both its transcription start site and the binding sites of the transcription factors controlling the activity of RNA polymerase II, referred to as enhancers, are located within accessible chromatin (Carlberg & Campbell, 2013). Vitamin D-triggered epigenome changes are therefore the first step in the modulation of the transcriptome of a cell. Thus, these  $1,25(\text{OH})_2\text{D}$ -dependent epigenome modulations are the most direct functional readout of the molecular actions of vitamin D (Carlberg, 2014a). However, it should be kept in mind that not all changes within the epigenome result in a modulation of the transcriptome. Therefore, in most cases, changes in mRNA expression, such as measured by quantitative PCR or RNA-seq, act as molecular markers of the action of VDR and its ligand.

In summary, it can be assumed that the vitamin D status measured on the level of serum  $25(\text{OH})\text{D}$  concentrations is proportional to the availability of  $1,25(\text{OH})_2\text{D}$  in the nuclei of VDR expressing tissues and cell types. Since  $1,25(\text{OH})_2\text{D}$  has via the VDR a direct effect on chromatin accessibility, the vitamin D status of a human individual should have an impact on his/her epigenome and subsequently on the transcriptome.





#### 4. MOLECULAR INSIGHT FROM VITAMIN D INTERVENTION TRIALS

Most vitamin D intervention studies have so far focused on the evaluation of the health status of the study participants via questionnaires, medical examination, or serum biochemistry. The 5-month vitamin D<sub>3</sub> intervention study VitDmet (NCT01479933) that investigated 71 elderly prediabetic persons, measured in addition at start and end of the trial the mRNA expression in peripheral blood mononuclear cells (PBMCs) and adipose tissue biopsies (Carlberg et al., 2013). This allowed the evaluation of vitamin D target genes as biomarkers for functional consequences of changes in the vitamin D status. The analysis of the VitDmet samples had been performed in an unconventional way, since the samples of this three-arm intervention (daily either 0, 1600, or 3200 IU vitamin D) were pooled, and the ratios (and not the differences) of the investigated parameters at end and start of the study were correlated with the respective ratios of the 25(OH)D levels (Carlberg et al., 2013). Nevertheless, this analysis approach was successful and demonstrated for all 24 investigated primary vitamin D target genes a significant correlation between mRNA expression changes and variations in the vitamin D status (Table 1) (Vukic et al., 2015). From the more than 100 clinical and biochemical parameters that had been determined in the VitDmet trial, only 12 displayed correlations with vitamin D status changes that were as significant as that of the 24 vitamin D target genes (Table 1) (Saksa et al., 2015). These 36 parameters were linked in a correlation network, the center of which was the serum parathyroid hormone (PTH) concentration change (Saksa et al., 2015; Vukic et al., 2015). PTH is a well-established marker of the vitamin D status (Bouillon et al., 2013) and an indication of the validity of the analysis. Accordingly, PTH is leading the relevance ranking of the 36 vitamin D-triggered parameters (Table 1). Interestingly, the following 12 parameters in the ranking are all expression changes of vitamin D target genes (*STS*, *BCL6*, *ITGAM*, *LRRC25*, *LPGAT1*, *TREM1*, *DUSP10*, *CD14*, *CD97*, *CD274*, *FUCA1*, and *NEF2*), which is technically easier to determine than most clinical and biochemical parameters. Interestingly, a recent meta-analysis (Standahl Olsen, Rylander, Brustad, Aksnes, & Lund, 2013) had already identified *CD14* as the most suited gene for describing the vitamin D status of primary blood samples.

**Table 1** Parameters Relevant for Monitoring the Vitamin D Status of Human Individuals

| <b>Parameter</b>           | <b>Significant Correlation with</b>   |
|----------------------------|---|
| PTH                        | 25(OH)D, <i>STS, BCL6, ITGAM, LRRC25, LPGAT1, TREM1, CD274, FUCA1, CD38, FBP1, DUSP10, NRIP1, THBD, ASAP2, NINJ1, IL8, G0S2, CAMP, SLC37A2</i> , insulin resistance, HOMA-IR, fasting insulin, fasting FFAs TNFRSF1B, lymphocyte number, FFAs (120 min), IL6, ALAT, heart rate, adiponectin |
| <i>STS</i>                 | 25(OH)D, <i>BCL6, ITGAM, LRRC25, LPGAT1, TREM1, CD274, NFE2, CD38, DUSP10, CD14, CD97</i>   |
| <i>BCL6</i>                | 25(OH)D, <i>STS, ITGAM, LRRC25, LPGAT1, TREM1, FUCA1, NFE2, CD38, DUSP10, CD14, CD97</i>  |
| <i>ITGAM</i>               | 25(OH)D, <i>STS, BCL6, LRRC25, LPGAT1, TREM1, FUCA1, NFE2, DUSP10, CD14, CD97, NRIP1, SLC37A2</i>   |
| <i>LRRC25</i>              | 25(OH)D, <i>STS, BCL6, ITGAM, LPGAT1, TREM1, FUCA1, NFE2, FBP1, TMEM37, DUSP10, CD14</i> , lymphocyte number  |
| <i>LPGAT1</i>              | 25(OH)D, <i>STS, BCL6, ITGAM, LRRC25, CD38, DUSP10, CD97</i>  |
| <i>TREM1</i>               | 25(OH)D, <i>STS, BCL6, ITGAM, LRRC25, LPGAT1, FUCA1, NFE2, FBP1, DUSP10, CD14</i>   |
| <i>DUSP10</i>              | 25(OH)D, <i>STS, BCL6, ITGAM, LRRC25, LPGAT1, CD274, NFE2</i>   |
| <i>CD14</i>                | 25(OH)D, <i>STS, BCL6, ITGAM, LRRC25, LPGAT1, TREM1, FUCA1, NFE2, DUSP10</i>  |
| <i>CD97</i>                | 25(OH)D, <i>STS, BCL6, ITGAM, LRRC25, LPGAT1, TREM1, NFE2, DUSP10, CD14</i>   |
| <i>CD274</i>               | <i>STS, BCL6, LPGAT1, CD38, DUSP10, CD14, CD97, ASAP2, LRRC8A</i> , insulin resistance  |
| <i>FUCA1</i>               | 25(OH)D, <i>STS, BCL6, LRRC25, LPGAT1, TREM1, FBP1</i>  |
| <i>NEF2</i>                | 25(OH)D, <i>STS, BCL6, ITGAM, TREM1, FUCA1, CD14</i>  |
| Insulin sensitivity (OGTT) | 25(OH)D, <i>CD38, TMEM37, CD14, CD97, ASAP2, NINJ1, G0S2, PTH, HOMA-IR</i> , fasting insulin, lymphocyte number   |
| <i>CD38</i>                | 25(OH)D, <i>STS, ITGAM, CD274, DUSP10, NRIP1, LRRC8A</i>  |

**Table 1** Parameters Relevant for Monitoring the Vitamin D Status of Human Individuals—cont'd

| Parameter                 | Significant Correlation with   |
|---------------------------|--|
| <i>NR1P1</i>              | 25(OH)D, <i>STS</i> , <i>BCL6</i> , <i>ITGAM</i> , <i>LPGAT1</i> , <i>FUCA1</i> , <i>NFE2</i> , <i>CD97</i> , <i>THBD</i> , <i>LRRC8A</i> , <i>SLC37A2</i> |
| <i>FBP1</i>               | 25(OH)D, <i>STS</i> , <i>BCL6</i> , <i>ITGAM</i> , <i>LRRC25</i> , <i>LPGAT1</i> , <i>TREM1</i> , <i>FUCA1</i> , <i>NFE2</i> , lymphocyte number           |
| HOMA-IR (OGTT)            | 25(OH)D, <i>CD97</i> , <i>NR1P1</i> , <i>ASAP2</i> , <i>NINJ1</i> , insulin resistance, fasting insulin, TNFRSF1B, lymphocyte number                       |
| Fasting insulin (OGTT)    | 25(OH)D, <i>CD97</i> , <i>NR1P1</i> , <i>ASAP2</i> , <i>NINJ1</i> , insulin resistance, HOMA-IR, TNFRSF1B, FFAs (120 min)                                  |
| <i>THBD</i>               | 25(OH)D, <i>STS</i> , <i>LRRC8A</i> , <i>LPGAT1</i> , <i>TREM1</i> , <i>FUCA1</i> , <i>FBP1</i> , <i>DUSP10</i> , <i>IL8</i> , lymphocyte number           |
| <i>ASAP2</i>              | 25(OH)D, <i>STS</i> , <i>BCL6</i> , <i>ITGAM</i> , <i>LPGAT1</i> , <i>NFE2</i>   |
| <i>NINJ1</i>              | 25(OH)D, <i>BCL6</i> , <i>LRRC25</i> , <i>TREM1</i> , <i>FUCA1</i> , <i>CD14</i>   |
| Fasting FFAs (OGTT)       | 25(OH)D, <i>CD97</i> , <i>NR1P1</i> , <i>ASAP2</i> , <i>NINJ1</i> , insulin resistance, HOMA-IR, TNFRSF1B, FFAs (120 min)                                  |
| <i>IL8</i>                | 25(OH)D, <i>CD274</i> , <i>FUCA1</i> , <i>CD38</i> , <i>CAMP</i>   |
| <i>TMEM37</i>             | 25(OH)D, <i>G0S2</i>   |
| <i>LRRC8A</i>             | 25(OH)D, <i>ITGAM</i> , <i>CD38</i> , <i>NR1P1</i>   |
| <i>G0S2</i>               | 25(OH)D, <i>TMEM37</i> , insulin resistance  |
| <i>CAMP</i>               | 25(OH)D, <i>CD14</i> , <i>NINJ1</i> , <i>IL8</i> , heart rate  |
| TNFRSF1B                  | 25(OH)D, <i>STS</i> , <i>BCL6</i> , <i>LPGAT1</i> , <i>ALAT</i>  |
| Lymphocyte number         | 25(OH)D, <i>FBP1</i>   |
| <i>SLC37A2</i>            | 25(OH)D  |
| FFAs after 120 min (OGTT) | 25(OH)D  |
| <i>IL6</i>                | 25(OH)D, <i>TREM1</i>  |
| <i>ALAT</i>               | 25(OH)D  |
| Heart rate                | 25(OH)D  |
| Adiponectin               | 25(OH)D  |

With serum and PBMC samples of the 71 VitDmet study participants, 24 vitamin D target genes (italic) and 12 biochemical/clinical parameters were found to be suitable for monitoring the vitamin D status of the investigated individuals. Some of the biochemical parameters were measured in the context of an oral glucose tolerance test (OGTT). For further details, please refer to the original publication (Vukic et al., 2015).

None of the VitDmet study participants was responsive to all 36 measured parameters (Vukic et al., 2015). However, the study subjects differed significantly by responding only to 10 (27.8%) up to 30 (83.3%) of all. This allowed a segregation of the study subjects into 26 high and 45 low responders and is an important demonstration how individual the response to vitamin D can be (Vukic et al., 2015). Comparable segregations had not yet been performed for other studies. To a minor extent, the observed differences may be based on genetic variations, such as SNPs affecting the vitamin D status (Wang et al., 2010). However, in analogy to most other traits underlying common aging-related disorders, such as type 2 diabetes, osteoporosis, or cardiovascular disease, a molecular explanation is found on the level of the epigenome rather than on the genome. Since the epigenome can respond to environmental changes, such as those imposed by the individual's lifestyle, it is far more dynamic than the genome. This implies that this responsiveness to vitamin D can also change during the life span of the human individual.

The time span of 5 months between the two measurements of the VitDmet participants suggests that the observed changes on the level of gene expression cannot be a direct consequence of transcriptional regulation but must be the result of vitamin D-triggered epigenomic changes. In order to observe direct transcriptional effects, a different type of intervention had to be performed. In the VitDbol (NCT02063334) intervention study, healthy human adults were treated once with high dose of vitamin D (2000 µg) and samples were taken already 1 and 2 days after onset of the intervention (Vukic et al., 2015). This study demonstrated that some (for example, *CAMP*, *TREM1*, *CD14*, and *ITGAM*) but not all of vitamin D target genes used in the VitDmet study can be used as markers of the rapid transcriptional response of human individuals to vitamin D.

Taken together, the observations made in the VitDmet and VitDbol trials suggest that the response to vitamin D is biphasic being composed of a fast direct transcriptional response and a more long-lasting epigenomic response. Epigenetic changes underlie also the fast transcriptional effects, but these may be only transient and different to the longer lasting effects of vitamin D on the epigenome.



## 5. CONSEQUENCES FOR VITAMIN D SUPPLEMENTATION

The results of molecular analysis of vitamin D supplementation studies, such as VitDmet (Carlberg et al., 2013; Ryyänänen et al., 2014; Saksa

et al., 2015; Wilfinger et al., 2014) and VitDbol (Vukic et al., 2015), indicate that each human individual displays a personal response to vitamin D. Importantly, this dynamic response to vitamin D, i.e., a comparison of vitamin D-triggered parameters at two or more time points, does not correlate with the static description of the vitamin D status based on one single measurement as performed in most observational studies. This finding implies that it is advisable to measure at least twice a person's vitamin D status together with a number of molecular and clinical parameters. These measurements can be done within the period of a few months, such as at begin and end of the winter season, or even at two consequent days. The first type of measurement describes the epigenomic response and is independent of any vitamin D supplementation protocol, while the second test monitors the transcriptional response and requires a vitamin D bolus, in order to observe significant effects. Obviously, the latter test provides quicker results that may be immediately implemented in an appropriate vitamin D supplementation protocol. However, the transcriptional and the epigenetic response are not necessarily identical. Therefore, a combination of both approaches, such as obtained by measurements at days 0, 1, and 30, may be most suited for computing a vitamin D index describing the personalized response of human individuals to vitamin D.

The vitamin D index analysis can indicate for each human individual a vitamin D supplementation protocol that will direct to a personal optimal vitamin D status. This concept may dissolve the scientific dispute about recommended 25(OH)D serum levels and vitamin D amounts of daily supplementation. The fact that in the United States, sales of vitamin D supplements increased by a factor of nearly 15 within the last decade indicates that vitamin D supplementation became very popular in the general population (Kupferschmidt, 2012). For these people, a smartphone app integrating the results of a vitamin D index measurement with their dietary vitamin D intake (fatty fish or fortified food), outdoor physical activity (correlating with sun exposure), and adiposity (decreasing 25(OH)D bioavailability) will rather accurately recommend a personalized vitamin D intake (which may change from day to day). Follow-up studies of individuals with a stable optimized vitamin D status will more likely prove or disprove claims about the impact of vitamin D in a variety of symptoms and disorders than traditional observational studies.

In summary, the inclusion of vitamin D index measurements in the stratification of study cohorts may be most appropriate, in order to challenge observational studies suggesting that high serum concentrations

of vitamin D protect against cardiovascular disease, diabetes, colorectal cancer, and all-cause mortality.



## 6. CONCLUSION AND FUTURE DIRECTIONS

In the past, vitamin D and its metabolite 1,25(OH)<sub>2</sub>D were known best for their role in calcium homeostasis and bone formation, but to date most genome-wide data are available for the actions vitamin D in cells of the hematopoietic system (Carlberg, 2014a). This emphasizes the impact of vitamin D and VDR for innate and adaptive immunity. The response to vitamin D supplementation varies considerably from individual to individual and depends on many factors, such as baseline level, adiposity, and genotype, i.e., a “one dose fits all” approach is not anymore appropriate. Thus, the dynamic vitamin D status of human individuals, which is introduced here as the vitamin D index, can be considered as a biomarker of the lifestyle of the person. This index is obtained from PBMCs that represent cells of both the adaptive and the innate immune system being in contact with most tissues of the human body. PBMCs are not only the most representative cell types for what is happening in the human body, but they are also the primary tissue that is easily obtained by a simple draw of blood. In future, the vitamin D index may be used in two rather different ways. On one hand, the test may be reduced to a routine measurement of a few biomarker-type genes, such as *CD14*, *ITGAM*, and *CAMP*, while on the other hand, next-generation sequencing type measurements may be applied that will allow an assessment far beyond the role of vitamin D. Since the vitamin D index has a large epigenetic component, measurements via epigenome- and/or transcriptome-wide methods, such as RNA-seq and FAIRE-seq, can integrate the environmental influences to the individual, such as physical activity, diet, and sun exposure. In this context, the vitamin D index exceeds its original scope describing the response to vitamin D but can monitor the health status of a human individual as a whole. Moreover, this will also better integrate confounding factors affecting the function of vitamin D, such as adiposity. This approach also implies that the status of a person in health as well as in disease cannot be reliably deduced from a single genotyping experiment, such as suggested by classical pharmacogenetics, but needs to be profiled on the level of the epigenome and transcriptome in a time series experiment (Carlberg & Raunio, 2014).

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