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Molecular evaluation of vitamin D responsiveness of healthy young adults

Sabine Seuter[a], Jyrki K. Virtanen[b], Tarja Nurmi[b], Jussi Pihlajamäki[b], Jaakko Mursu[b], Sari Voutilainen[b], Tomi-Pekka Tuomainen[b], Antonio Neme[a], Carsten Carlberg[a,*]

[a] School of Medicine, Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland
[b] Institute of Public Health and Clinical Nutrition, University of Eastern Finland, Kuopio, Finland

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ABSTRACT

Vitamin D_3 has via its metabolites 25-hydroxyvitamin D_3 (25(OH)D_3) and 1α,25-dihydroxyvitamin D_3 (1,25(OH)$_2D_3$) direct effects on the transcriptome and the epigenome of most human cells. In the VitDbol study we exposed 35 healthy young adults to an oral vitamin D_3 dose (2000 μg) or placebo and took blood samples directly before the supplementation as well as at days 1, 2 and 30. Within 24 h the vitamin D_3 intake raised the average serum levels of both 25(OH)D_3 and 1,25(OH)$_2D_3$ by approximately 20%. However, we observed large inter-individual differences in these serum levels, reflected by the average ratios between 25(OH)D_3 and 1,25(OH)$_2D_3$ concentrations ranging from 277 to 1365. Interestingly, average serum parathyroid hormone (PTH) levels increased at day 1 by some 10% but then decreased within the following four weeks to levels 5% below baseline. In peripheral blood mononuclear cells (PBMCs) that were isolated at the same time points we determined vitamin D-modulated chromatin accessibility by FAIRE-qPCR at selected genomic loci. This method is well suited to evaluate both short-term and long-term *in vivo* effects of vitamin D on the epigenome of human subjects. The differential vitamin D responsiveness of the VitDbol study participants was determined via individual changes in their PTH levels or chromatin accessibility in relation to alterations in 25(OH)D_3 concentrations. This led to the segregation of the subjects into 14 high, 11 mid and 10 low responders. In summary, the vitamin D responsiveness classification provides additional information compared to a vitamin D status assessment based on single 25(OH)D_3 serum measurements. The study was registered at Clinicaltrials.gov (NCT02063334).

1. Introduction

The pre-hormone vitamin D_3 has direct effects on both the epigenome and the transcriptome of most human cells via its metabolites 25(OH)D_3 and 1,25(OH)$_2D_3$ [1,2]. The natural way of vitamin D_3 production is its synthesis from 7-dehydrocholesterol in UV-B exposed human skin [3]. Since today's lifestyle, being represented by primary indoor activity, textile coverage and life at latitudes above 40°, often leads to insufficient sun exposure, vitamin D has to be taken up by diet or supplementation [4]. In general, serum 25(OH)D_3 levels are used as a biomarker of the vitamin D status for the human body [5] and a concentration below 50 nM (20 ng/ml) is considered as vitamin D deficiency [6]. Clinically low vitamin D serum concentrations can have musculoskeletal consequences, such as rickets in children and osteomalacia and fractures in adults [7]. In addition, vitamin D insufficiency is linked to a number of other diseases, such as type 1 and type 2 diabetes, cardiovascular disease and cancers of the breast, prostate and colon [8–12].

The biologically most active vitamin D_3 metabolite, 1,25 (OH)$_2D_3$, is a high affinity ligand for the transcription factor vitamin D receptor (VDR) [13,14]. The first effect of activating VDR by its ligand is a transient opening or closing of chromatin at specific enhancer and transcription start site (TSS) regions that

Abbreviations: 1,25(OH)$_2D_3$ or 1,25D, 1α,25-dihydroxyvitamin D_3; 25(OH)D_3, 25-hydroxyvitamin D_3; BMI, body mass index; CAMP, cathelicidin antimicrobial peptide; CLIA, chemiluminescence immunoassay; ELL, elongation factor RNA polymerase II; FAIRE, formaldehyde-assisted isolation of regulatory elements; GAPDH, glycerinaldehyde-3-phosphate-dehydrogenase; MB, myoglobin; PBMC, peripheral blood mononuclear cell; PTH, parathyroid hormone; qPCR, quantitative polymerase chain reaction; RAP2B, RAP2B member of RAS oncogene family; TSS, transcription start site; VDR, vitamin D receptor; WBP1, WW domain binding protein 1.

* Corresponding author at: School of Medicine, Institute of Biomedicine, University of Eastern Finland, POB 1627, FI-70211 Kuopio, Finland.
E-mail address: carsten.carlberg@uef.fi (C. Carlberg).

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results in a second step in the activation or repression of vitamin D target genes, such as cathelicidin antimicrobial peptide (*CAMP*) or *PTH*, respectively [15]. In THP-1 human monocytic leukemia cells we have demonstrated that the method formaldehyde-assisted isolation of regulatory elements (FAIRE) [16] is well suited to monitor vitamin D-modulated changes in chromatin accessibility [17,18]. This means that vitamin D stimulation of cultured cells, and probably also of human tissues and cell types *in vivo*, results in changes of the epigenome. In this study, we challenged the latter assumption by the use of PBMCs, which i) are a mixture of the vitamin D-responsive cell types, i.e. monocytes, T and B lymphocytes, ii) can be obtained with minimal harm from human volunteers and iii) are isolated within less then an hour after drawing blood, so that their status is as close as possible to the *in vivo* situation.

The approach to use a threshold of serum $25(OH)D_3$ concentration, such as 50 nM, as an indicator of vitamin D sufficiency is easy and straightforward, but it ignores the possibility that human subjects display an individual response to vitamin D. Therefore, we suggest to test the personal vitamin D responsiveness [19]. When we investigated samples from the VitDmet study (Clinicaltrials.gov: NCT01479933), in which elderly, pre-diabetic persons were during Finnish winter daily supplemented with vitamin D_3 (0, 40 and 80 μg), we found that changes in serum $25(OH)D_3$ concentrations do not completely correlate with alterations in the expression of vitamin D target genes in PBMCs and adipose tissue [20–24]. We used these vitamin D status-triggered gene expression changes to classify the pre-diabetic individuals into high and low vitamin D responders.

In this study, we describe the VitDbol study, in which 35 healthy young adults were exposed to an oral dose of 2000 μg vitamin D_3 or placebo. Serum biochemistry, such as $25(OH)D_3$, $1,25(OH)_2D_3$ and PTH levels, at days 0, 1, 2 and 30 were compared with effects of vitamin D on chromatin accessibility in PBMCs as measured by FAIRE-qPCR. We demonstrate that both types of molecular evaluations of the *in vivo* effects of vitamin D_3 can be combined for a multiple parameter determination of the vitamin D responsiveness of the VitDbol study participants. This allows a classification of healthy, young individuals into high, mid and low vitamin D responders.

2. Material and methods

2.1. VitDbol study

The participants of the VitDbol study (NCT02063334, ClinicalTrials.gov) were males and females around 25 years of age having a body mass index (BMI) between 20 and 25 kg/m^2 (Table S1). Subjects were given a total of 2000 μg of vitamin D_3 in two doses. The study supplement was provided in 25 tablets, each containing 80 μg of vitamin D_3 or placebo. The first 13 tablets were taken during the visit at the University of Eastern Finland, Kuopio campus, for blood sampling, followed by an *ad libitum* breakfast. The remaining 12 tablets were taken approximately 3 h later during lunch. Blood samples were taken directly before the dose (day 0) and at days 1, 2 and 30. The study took place between March and May in 2014 and February and March in 2015, i.e. during a period of little or no UVB exposure from the sun in Finland. The research ethics committee of the Northern Savo Hospital District had approved the study protocol (#9/2014). All participants gave a written informed consent to participate in the study.

2.2. Serum measurements

Blood samples for serum and PBMC isolation were collected after overnight (12 h) fasting of the study participants. Serum 25$(OH)D_3$ concentrations were measured using a high performance liquid chromatography/coulometric electrode array as described previously [25] and $1,25(OH)_2D_3$ levels were determined by applying a chemiluminescence immunoassay (CLIA) involving a paramagnetic microparticle solid phase (LIAISON® XL, DiaSorin, Saluggia, Italy). Serum concentrations of PTH were also assayed by CLIA (LIAISON®, DiaSorin). Serum calcium was determined with colorimetric Arsenazo III test using a Konelab 20XT clinical chemistry analyzer (Thermo Fisher Scientific).

2.3. FAIRE-qPCR from PBMCs

PBMCs were isolated within one hour after blood draw from 8 ml of peripheral blood in a Vacutainer CPT Cell Preparation Tube with sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. FAIRE analysis was performed according to the protocol published by Giresi et al. [26] with some modifications. In short, $5–7.5 \times 10^6$ PBMCs were cross-linked for 10 min and stopped with glycine. The washed cell pellets were resuspended and incubated sequentially in 500–750 μl of buffer L1, 500–750 μl of buffer L2 and 300 μl of buffer L3. The lysates were sonicated in a Bioruptor Plus (Diagenode) to result in DNA fragments of 200–500 bp and cellular debris was removed by centrifugation. Aliquots (45 μl) of the sonicated lysates were reverse cross-linked and proteinase K-digested overnight at 65 °C to prepare control (input) samples. The FAIRE samples and reverse cross-linked reference samples were subjected to two sequential phenol/chloroform/isoamyl alcohol (25/24/1) extractions, resuspended in 10 mM Tris-HCl (pH 7.4) and treated with 1 μl of RNase A (10 mg/ml) for 1 h at 37 °C. FAIRE samples were then reverse cross-linked and proteinase K-digested for 2 h at 65 °C. The genomic DNA was purified from control and FAIRE samples using the ChIP DNA Clean&Concentrator Kit (Zymo Research).

Quantitative polymerase chain reactions (qPCRs) to detect the TSS regions of the genes elongation factor RNA polymerase II (*ELL*), RAP2B, member of RAS oncogene family (*RAP2B*) and WW domain binding protein 1 (*WBP1*), were performed using 250 nM of reverse and forward primers (Table S2), 2 μl diluted FAIRE template (0.5 ng/μl) and the Roche LightCycler 480 SYBRGreen I Master mix in a total volume of 8 μl. In the PCR reaction the hotstart Taq polymerase was activated for 10 min at 95 °C, followed by 42 amplification cycles of 20 s denaturation at 95 °C, 15 s annealing at primer-specific temperatures (Table S2) 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 expression levels were determined with the comparative delta threshold cycle (ΔCt) method. For normalization, the relative quantities were divided by the normalization factor being the geometric mean of the FAIRE-qPCR signal at the reference regions of the genes glycerinaldehyde-3-phosphate-dehydrogenase (*GAPDH*) and myoglobin (*MB*).

The Integrative Genomics Viewer [27] was used to visualize regions of genome-wide FAIRE-seq data from VitDbol study participant #7 (A.N., S.S., C.C., unpublished data).

2.4. Correlation analysis and statistics

The assumed linear correlations between the changes in PTH levels or chromatin accessibility in relation to serum $25(OH)D_3$ or $1,25(OH)_2D_3$ concentration alterations were evaluated by the r^2 value. All possible correlations were computed by testing the line that fits best the maximum number of participants following the concepts of Dolia et al. [28]. We simplified the latter method by substituting ellipsoidal kernels by lines. The largest participant subgroup that provided a correlation with an r^2 value above 0.4 was selected for further analysis. Participants were excluded under

biologically plausible assumptions, such as a preponderant direction of changes in parameters as a function of 25(OH)D_3 level variations.

The statistical significance of the findings was evaluated using the nonparametric Wilcoxon-Mann-Whitney test, which does not require the assumption of normal distributions. The k-means algorithm finds a pre-determined number of centroids in data based on the density, that is, more centroids are placed in regions with higher density, whereas sparse regions contain less centroids or means. K-means generates a partition based on the actual distribution of data instead of a biased partition assuming data are identically distributed. This creates categories with individual ranges that reflect the actual distribution.

3. Results

3.1. Characteristics of the VitDbol cohort

Thirty-five healthy, normal weight (BMI $22.4 \pm 1.5\ kg/m^2$), young adults (16 females, 19 males, age 25.8 ± 5.2 years) were recruited for the VitDbol study (Table S1). Twenty-seven of these individuals consumed once 2000 μg vitamin D_3 in two doses, while eight study participants were only exposed to placebo. Blood samples were taken directly before the dose (day 0) and at days 1, 2 and 30. Serum was preserved for the determination of 25(OH)D_3, 1,25$(OH)_2D_3$, PTH and calcium levels and PBMCs were isolated for FAIRE assays. Within 30 days after the vitamin D_3 dose none of the study participants showed a significant rise in serum calcium levels compared to basal levels (Table S1).

The baseline serum 25(OH)D_3 concentrations of the 35 individuals ranged between 37.4 and 106.5 nM with an average of 65.2 nM (Table S1). Compared to day 0, the 25(OH)D_3 levels at days 1, 2 and 30 rose in average by 17.4, 21.1 and 19.7 nM, respectively, for the 27 supplemented participants and remained unchanged for the eight placebo controls (Fig. S1A). A Wilcoxon-Mann-Whitney test showed a significant difference between days 1, 2 and 30 when compared to day 0 (U(day 1, day 0) = 2.87, 0.004, U (day 2, day 0) = 3.53, 0.004, U(day 30, day 0) = 3.29, 0.009). For the placebo group, no significant difference was found. Similarly, the average 1,25$(OH)_2D_3$ concentration of 104.4 pM at day 0 rose by 21.1, 28.8 and 6.7 pM at days 1, 2 and 30, respectively, for the supplemented individuals, while in average it stayed the same for the placebo controls (Fig. S1B). A Wilcoxon-Mann-Whitney test showed for the supplemented group a significant difference for days 1 and 2 when compared to day 0 (U(day 1, day 0) = 2.77, 0.022 and U(day 2, day 0) = 2.24, 0.024). No significant differences were found when comparing days 30 and 0. For the placebo group, as expected, no significant difference was found at any time point. The average PTH serum level of 39.8 pg/ml at day 0 initially increased for supplemented study participants by 3.7 pg/ml at day 1, lowered by 3.1 pg/ml within the following 24 h until day 2 and further decreased by 1.8 pg/ml within the 4 weeks until day 30 (Fig. S1C). In contrast, in the placebo control group the average PTH serum level rose by 1.1, 1.0 and 2.0 pg/ml at days 1, 2 and 30 compared to day 0. No statistically significant difference was found for neither of the two groups.

Although the changes in average serum levels of 25(OH)D_3, 1,25$(OH)_2D_3$ and PTH showed the expected tendencies, there was large inter-individual variation of these values (Fig. S1). For example, a study with postmenopausal women showed that in a concentration range of 40–100 nM the serum levels of 25(OH)D_3 and 1,25$(OH)_2D_3$ are positively correlated and the ratios between the vitamin D metabolite concentrations ranged between 400 and 750 [29]. In fact, the median of the averages of the four 25(OH)D_3/1,25$(OH)_2D_3$ ratios that was measured for each of the 35 study participants was 714 (Fig. 1). However, compared to the median subjects No. 4, 10, 18, 20, 21 and 25 showed a significantly ($p < 0.05$) higher ratio between their 25(OH)D_3 and 1,25$(OH)_2D_3$ values, while for participants No. 9, 13, 15, 28, 29, 31, 32 and 34 the ratio was clearly lower.

Taken together, the VitDbol study demonstrates that in healthy young adults an oral vitamin D_3 dose of 2000 μg is able to increase within 24 h the average serum levels of both 25(OH)D_3 and 1,25$(OH)_2D_3$ by approximately 20%. Interestingly, average serum PTH levels also initially increased by some 10% but then decreased in the following four weeks to levels 5% below baseline. There were large inter-individual differences in these serum levels, leading to average ratios between 25(OH)D_3 and 1,25$(OH)_2D_3$ levels ranging from 277 to 1365.

3.2. Correlation of PTH levels with 25(OH)D_3 and 1,25$(OH)_2D_3$ concentration changes

In order to better monitor the individual responses of the VitDbol study participants concerning baseline serum levels of 25$(OH)D_3$, 1,25$(OH)_2D_3$ and PTH and their changes after a vitamin D_3

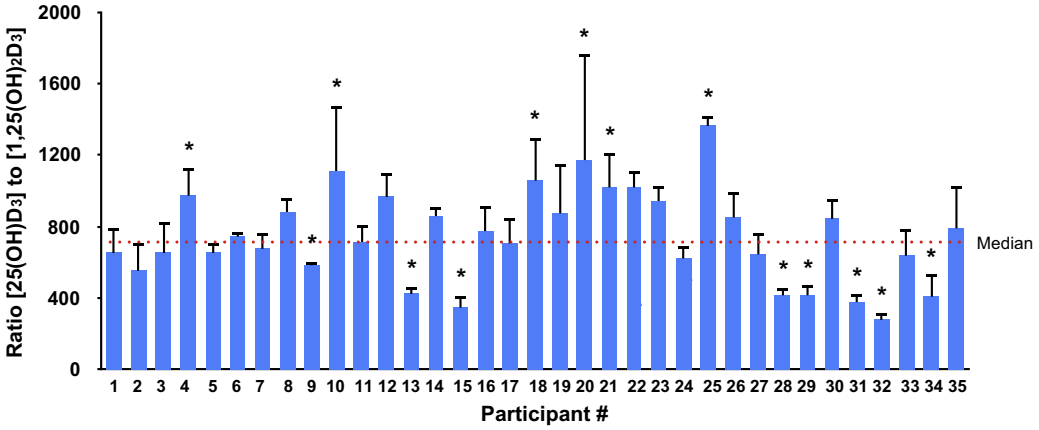

Fig. 1. Personal 25(OH)D_3 to 1,25$(OH)_2D_3$ serum levels ratios. Columns indicate the mean ratio of 25(OH)D_3 to 1,25$(OH)_2D_3$ serum levels at days 0, 1, 2 and 30 for the 35 participants of the VitDbol study and bars represent the standard deviation. A Wilcoxon-Mann-Whitney test was applied, in order to determine significant deviation from the median (* $p < 0.05$).

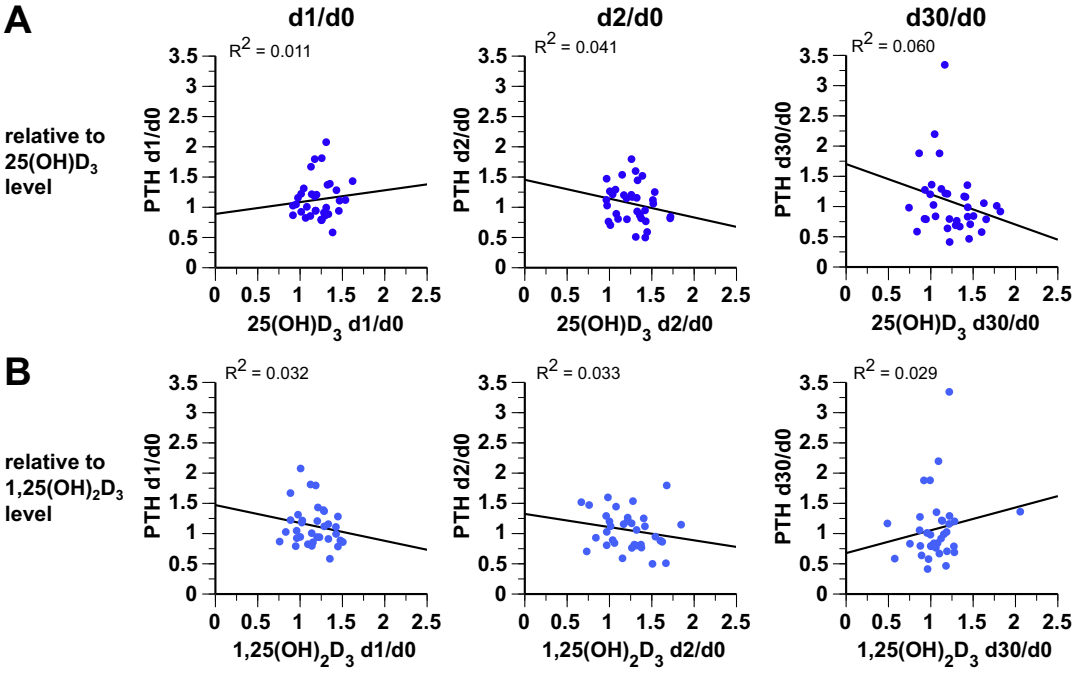

Fig. 2. Correlation of PTH status with $25(OH)D_3$ and $1,25(OH)_2D_3$ serum levels. Linear regression analysis was used, in order to display the correlation of the changes of the PTH status of the 35 VitDbol participants in relation to alterations in $25(OH)D_3$ (A) and $1,25(OH)_2D_3$ (B) serum levels at days 1, 2 and 30 compared to day 0.

dose, we displayed the data as ratios between serum levels at days 1, 2 and 30 compared to day 0, each (Fig. 2). Linear regression analysis of the changes of PTH levels in relation to alterations of $25(OH)D_3$ concentrations indicated a positive correlation after the first day (d1/d0) and a negative correlation after two and 30 days (d2/d0 and d30/d0, Fig. 2A). In contrast, a linear regression analysis of PTH level changes in relation to alterations of $1,25(OH)_2D_3$ concentrations provided a negative correlation after days 1 and 2 but a positive correlation after day 30 (Fig. 2B). This means that at days 1 and 30 the correlation analyses diverge between changes in $25(OH)D_3$ and $1,25(OH)_2D_3$ levels. The difference at day 30 could be explained by the far shorter half-life of $1,25(OH)_2D_3$ than that of $25(OH)D_3$ (15 h versus 15 d). Moreover, the $25(OH)D_3$-based correlation analyses (Fig. 2A) but not the $1,25(OH)D_3$-based correlation analyses (Fig. 2B) confirmed the observations based on average absolute serum concentrations (Fig. S1C) that PTH levels increase within the first day after vitamin D_3 application, but then decrease within the following 29 days. Therefore, further analyses will only be related to changes in $25(OH)D_3$ levels.

As a next step, we followed the approach of Dolia et al. [28] and applied a systematic correlation analysis, in which we explored all possible lines through the PTH/$25(OH)D_3$ correlation graphs. This method involved the step-wise exclusion of study participants resulting in higher correlation coefficients. In addition, we included the constraint to preserve the orientation of the correlation line. In this way, we obtained highly significant correlations with r^2 values between 0.41 and 0.60 (Fig. S2). This implies that those individuals that were not eliminated from the correlation graphs have a higher correlation between their PTH and $25(OH)D_3$ serum concentrations, i.e. in respect to PTH levels these individuals can be considered as vitamin D responders.

In summary, a ratio-based display of the relation between PTH levels and $25(OH)D_3$ concentrations confirmed the up-regulation of PTH at day 1 after a high dose vitamin D_3 supplementation and a down-regulation at days 2 and 30. Systematic correlation analysis could identify those study participants that in respect to their PTH levels responded well to vitamin D_3.

3.3. *Evaluation of vitamin D responsiveness via* in vivo *chromatin accessibility*

In search for an independent method to monitor the vitamin D response characteristics of human subjects, we tested the quantification of chromatin accessibility via the FAIRE-qPCR assay. Based on genome-wide FAIRE-seq data of one VitDbol participant (A.N., S.S., C.C., unpublished data) we selected three prominent vitamin D-responsive sites at the TSS regions of the genes *ELL*, *RAP2B* and *WBP1* in reference to the negative control regions at the genes *MB* and *GAPDH* (Fig. S3). The chromatin cross-linking for the FAIRE assay was performed immediately after isolation of PBMCs from freshly drawn blood, i.e. the chromatin accessibility of the cells was preserved as close to the *in vivo* status as possible. After the FAIRE procedure qPCR determined the relative chromatin accessibility at the five selected genomic regions. We used again the display of ratios, in order to monitor the effect of changes in 25 $(OH)D_3$ serum levels on alterations in chromatin accessibility at days 1, 2 and 30 in comparison to day 0 (Fig. S4). Linear regression analysis indicated at days 1 and 2 an increase of open chromatin at the TSS regions of the genes *ELL*, *RAP2B* and *WBP1*, while at day 30 there was hardly any change detectable. At the negative control regions in the *MB* and *GAPDH* gene loci at all three time points a horizontal regression line was obtained, i.e. at these genomic sites vitamin D did not affect the chromatin status. Normalization of the FAIRE data of *ELL*, *RAP2B* and *WBP1* with that of the control regions slightly improved the linear regression values, but the overall result stayed the same, i.e. there was prominent vitamin D-induced chromatin opening at days 1 and 2 but far weaker effects at day 30, when all participants were included (Fig. 3). However, when we applied a systematic correlation analysis also at day 30 clear effects were observed (Fig. S5). At the loci of the genes *ELL* and *WBP1* vitamin D increased chromatin accessibility, while at the *RAP2B* TSS chromatin closing was observed.

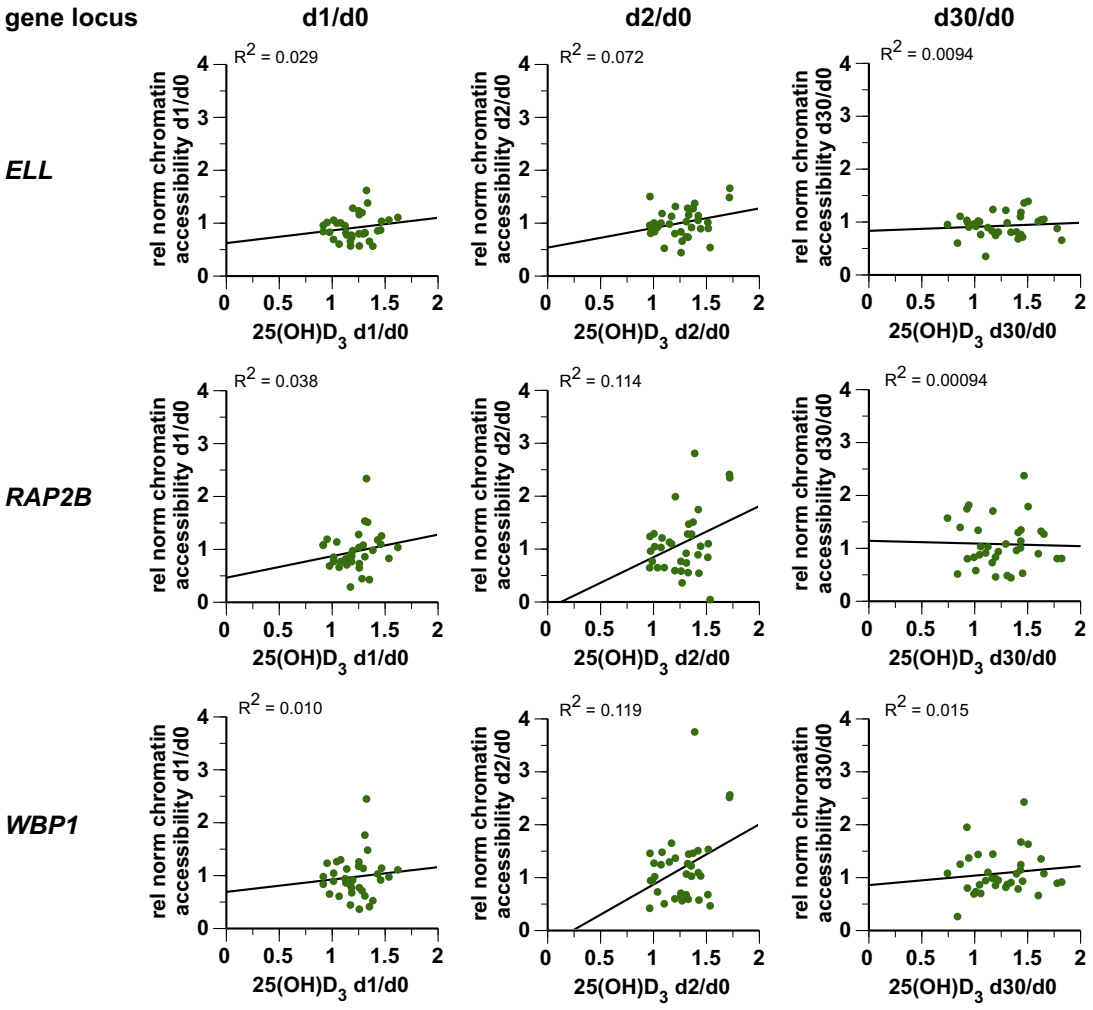

Fig. 3. Correlation of chromatin accessibility with 25(OH)D_3 serum levels. FAIRE-qPCR was used to determine the change in chromatin accessibility at the TSS regions of the genes *ELL*, *RAP2B* and *WBP1* (normalized to control regions at the genes *MB* and *GAPDH*) in relation to alterations in 25(OH)D_3 serum levels at days 1, 2 and 30 compared to day 0. Linear regression analysis for data from all 35 VitDbol participants is shown.

Taken together, vitamin D-modulated chromatin accessibility of freshly isolated PBMCs at selected genomic loci, as measured by FAIRE-qPCR, can be used both for a short-term (days 1 and 2) and a long-term (day 30) assessment of the vitamin D responsiveness of human subjects.

3.4. High, mid and low responders to vitamin D

The evaluation of vitamin D-dependent changes in PTH serum levels (Fig. 2) and in opening (or closing) of chromatin within PBMCs at three different time points (days 1, 2 and 30, Fig. 3) provides for each of the 35 VitDbol participants up to 12 parameters to evaluate their vitamin D responsiveness. In fact, the most responsive study participant (No. 23) was retained in 11 of these 12 correlation analyses, while the least responsive subjects (No. 11 and 12) were kept in only 3 analyses (Fig. 4). Please note that the latter two participants were older than the average and had a BMI close to 25. We applied the k-means algorithm, in order to segregate high from mid and low responders. The k-means algorithm offers a way to discretize continuous data or to partition discrete ranges. In this study, three discrete states were obtained from a discrete variable in the range determined by the number of parameters (n = 12). This identified 14 study participants as high responders, 11 as mid responders and 10 as low responders. It should be noted that this vitamin D responsiveness classification does not reflect the actual 25(OH)D_3 serum levels but the inducibility by the hormone. For example, at the beginning of the VitDbol study, participants No. 3 and 29 had a 25(OH)D_3 level below 50 nM, i.e. a low vitamin D status, but are classified as high responders, while individual No. 27 started with a 25(OH)D_3 concentration of more than 100 nM, i.e. a high vitamin D status, but was found to be a low responder.

In summary, the correlation analyses based on changes in PTH serum concentrations or chromatin accessibility within PBMCs allowed the classification of the 35 VitDbol study participants into high (n = 14), mid (n = 11) and low (n = 10) responders. This vitamin D responsiveness classification gives additional information to a vitamin D status determination based on single 25(OH)D_3 measurements.

4. Discussion

We described the vitamin D_3 intervention study VitDbol, where healthy, young human adults were supplemented with a high dose of vitamin D_3 (2000 μg) and serum and PBMCs were isolated at days 0, 1, 2 and 30. We had a special focus on what happens within the first 24 h after the vitamin D_3 dose, since this time frame we already studied extensively in our THP-1 human monocyte cell culture model [18]. As expected, the vitamin D_3 dose increased the vitamin D status of the study participants, as measured by 25(OH)

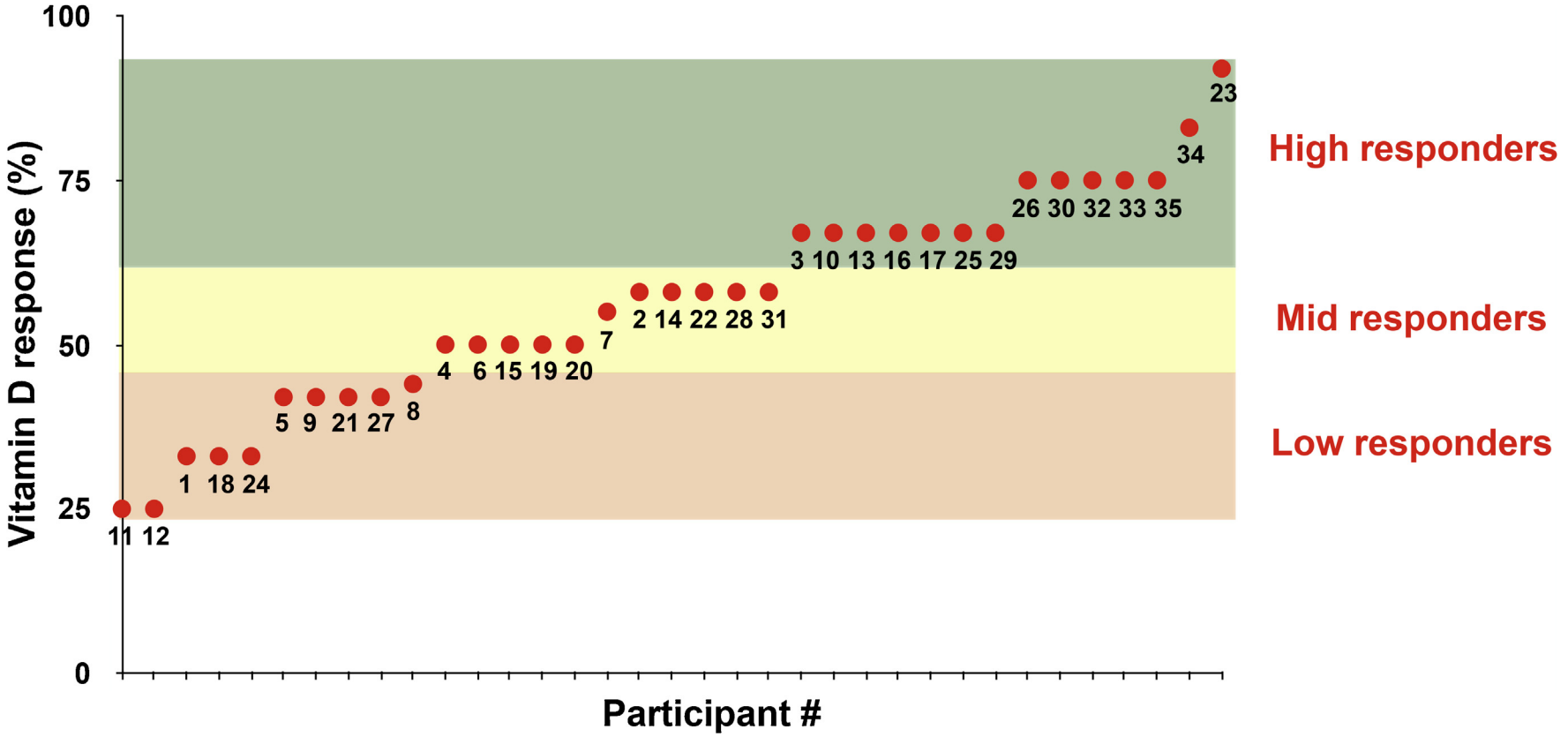

Fig. 4. Evaluation of vitamin D responsiveness. The vitamin D_3 response of the 35 VitDbol study participants is expressed by the percentage of the vitamin D_3-triggered parameters, to which they show response (red data points). A k-mean approach was used to distinguish 14 high responders (green), from 11 mid responders (yellow) and 10 low responders (orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

D_3 serum levels, within one day by some 20%. In this way, the vitamin D deficient subject went into the sufficiency range, i.e. the supplementation goal was reached immediately. More important we consider the observation that a period of 24 h was also sufficient to raise $1,25(OH)_2D_3$ serum levels by some 20%. Moreover, we observed the functional consequences of this VDR ligand concentration boost on the level of chromatin opening at three representative TSS regions. This demonstrates that a vitamin D_3 intake has a direct effect on the epigenome and that 24 h are sufficient time not only to convert vitamin D_3 to $25(OH)D_3$ but also to produce enough new $1,25(OH)_2D_3$ molecules, in order to induce the VDR signaling cascade. In a previous study [24], we already showed that 24 h are long enough to induce some prominent VDR target genes, such as *CAMP*. With the VitDbol study we confirmed that also the first step in the gene activation process, the epigenetic process of opening of chromatin, can be monitored under *in vivo* conditions within this time frame.

In contrast to changes in mRNA or protein levels that represent an accumulative signal, monitoring the effects of vitamin D via changes of the epigenome has the advantage that the latter are on/off markers on either genomic DNA (cytosine methylation) or histone proteins (lysine acetylation or methylation), mediated by fast enzyme reactions [30]. In principle, epigenome changes could be monitored at the level of DNA methylation or specific histone modifications. However, the measurement of the resulting increases or decreases of chromatin accessibility, by methods such as FAIRE, is technically easier than the determination of histone modifications or DNA methylation and integrates the effects of the latter specific epigenome markers. Therefore, for the evaluation of vitamin D responsiveness of human individual subjects, in particular after a high vitamin D_3 dose, we consider epigenome changes better suited than transcriptome alterations.

In general, PTH serum levels are negatively correlated with the vitamin D status of human subjects [31,32]. Therefore, on the first view it is puzzling that a vitamin D_3 intake results in a short-term increase of PTH protein concentrations. However, the inverse relation between PTH and $25(OH)D_3$ serum levels had been established based on long-term measurements, such as weeks or even months. After 30 days also the VitDbol study monitored the expected decrease of PTH levels. In addition, despite the identification of VDR binding sites within the promoter region of the *PTH* gene in several species in the pre-genomic era [33–35], a formal confirmation that the *PTH* gene is a primary down-regulated VDR target in humans is still missing. Therefore, it can be speculated that the primary effect of VDR binding to the *PTH* promoter results in an up-regulation that after more than 24 h turns via secondary effects into a down-regulation. Since the *PTH* gene is only expressed in parathyroid glands, an experimental validation of this hypothesis in a human *in vivo* system is very difficult. Nevertheless, changes of PTH serum levels already one day after the vitamin D_3 intake seem to be a good indicator of the vitamin D responsiveness of human subjects.

This study confirms the value of $25(OH)D_3$ serum concentrations as a biomarker for the vitamin D status of human subjects. The far longer half-life of $25(OH)D_3$ (approximately 15 days) compared to $1,25(OH)_2D_3$ (less than 24 h) provides more stable measurements. Therefore, we decided to base the vitamin D responsiveness evaluations on changes of $25(OH)D_3$ levels and not on alterations of $1,25(OH)_2D_3$ concentrations. Moreover, in this study we demonstrated that the $25(OH)D_3/1,25(OH)_2D_3$ ratio can vary a lot between human subjects. The molecular basis of this observation may primarily be different activities of the CYP27B1 enzyme (or its encoding gene) that catalyzes the conversion of $25(OH)D_3$ into $1,25(OH)_2D_3$. In any case, this example of an inter-individual difference seems to be one component of the different vitamin D responsiveness of humans.

In our previous VitDmet study we evaluated on the basis of VDR target gene expression and biochemical parameters the vitamin D responsiveness of 71 elderly, pre-diabetic persons in response to a daily vitamin D_3 supplementation over 5 months and found the majority of them (n = 46) to be low responders [24]. This could potentially explain the lack of effect of vitamin D_3 supplementation on most measured parameters in the VitDmet study [36]. In the VitDbol study with healthy young persons, we found more high responders (n = 14) than low responders (n = 10) and introduced the extra class of normal (or mid) responders (n = 11). The higher percentage of high responders in the VitDbol cohort may be related to the fact that these individuals were young and disease-free. This could mean that during aging and/or the onset of disease the vitamin D responsiveness decreases. Alternatively, this observation could indicate that low vitamin D responsiveness may pre-dispose to diseases, such as impaired glucose tolerance as the VitDmet study subjects were not only older but also were pre-diabetic. Investigations with larger cohorts are necessary, in order to provide a better answer to this question.

In conclusion, in this study we demonstrated that the analysis of venous blood samples after a high dose vitamin D_3 supplementation is well suited for the evaluation of the vitamin D responsiveness of human subjects. Moreover, we showed that epigenome changes, such as alterations in chromatin accessibility, are valuable parameters for these assessments.

Author contribution

S.S. and C.C. designed the current analysis, S.S. and T.N. performed the experiments and A.N. the computational analysis, S.S., A.N. and C.C. analyzed the data, J.V., T.N. J.P., S.V., J.M., T.-P.T. and C.C. designed and performed the VitDbol 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.2016.06.003.

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