RESEARCH ARTICLE

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Biomarkers of Vitamin D Sufficiency: Vitamin D Metabolite Levels do not depend on 25-Hydroxyvitamin D2 levels in Healthy Turkish Individuals

ABSTRACT

Objective: Patient-specific factors may influence the adequate supplemental vitamin D dose. In this study, we evaluated the relationship between 25(OH)D_2 and free vitamin D levels and vitamin D deficiency in a healthy Turkish population.

Method: Blood samples were obtained from 92 healthy adults aged ≥18 years. Total 25(OH)D was determined by CMIA. Serum 25(OH)D_3 and D_2 levels were measured by LC-MS. Free 25(OH)D was calculated according to the Bikle method.

Results: In 54% of the participants, 25(OH)D_3 levels were below 20 ng/mL. Those with 20 ng/mL or higher had higher mean serum 25(OH)D_3 and free vitamin D levels (P<0.001). Mean 25(OH)D_2 concentration did not differ according to deficiency criteria. Serum 25(OH)D_2 levels were consistent regardless of free vitamin D concentrations.

Conclusions: Serum 25(OH)D_3 and free vitamin D concentrations measured by LC-MS indicate deficiency by influencing the total 25(OH)D_3 concentration. However, serum 25(OH)D_2 concentration did not differ between individuals and does not directly indicate deficiency.

Keywords: Vitamin D, 25-Hydroxyvitamin D_2, 25-Hydroxyvitamin D_3, free vitamin D.

D Vitamini Yetersizliğinin Biyobelirteçleri: Sağlıklı Türk bireylerde D Vitamini Metabolit Düzeyleri 25-Hidroksivitamin D2 Düzeylerine Bağlı Değildir

ÖZET

Amaç: Hastaya özgü faktörler yeterli ek D vitamini dozunu etkileyebilir. Bu çalışmada, sağlıklı bir Türk popülasyonunda 25(OH)D_2 ve serbest D vitamini düzeyleri ile D vitamini eksikliği arasındaki ilişki değerlendirilmiştir.

Yöntem: 18 yaş üstü 92 sağlıklı yetişkinden kan örnekleri alınmıştır. Toplam 25(OH)D CMIA ile belirlenmiştir. Serum 25(OH)D_3 ve D_2 seviyeleri LC-MS ile ölçülmüştür. Serbest 25(OH)D hesaplaması Bikle yöntemine göre yapılmıştır.

Bulgular: Katılımcıların %54'ünde 25(OH)D_3 seviyesi 20 ng/mL'nin altındaydı. 20 ng/mL veya daha yüksek olanlarda, ortalama serum 25(OH)D_3 ve serbest D vitamini seviyeleri daha yüksekti (P<0.001). Ortalama 25(OH)D_2 konsantrasyonu eksiklik kriterlerine göre fark göstermemiştir. Serum 25(OH)D_2 seviyeleri, serbest D vitamini konsantrasyonlarından bağımsız olarak tutarlıydı.

Sonuç: LC-MS ile ölçülen serum 25(OH)D_3 ve serbest D vitamini konsantrasyonları, toplam 25(OH)D_3 konsantrasyonunu etkileyerek eksikliği göstermektedir. Ancak, serum 25(OH)D_2 konsantrasyonu bireyler arasında fark göstermemiştir ve eksikliği doğrudan göstermez.

Anahtar Kelimeler: D vitamini, 25-Hidroksivitamin D_2, 25-Hidroksivitamin D_3, serbest D vitamini

INTRODUCTION

Vitamin D occurs predominantly in two forms: ergocalciferol (D_2) and cholecalciferol (D_3). Most vertebrates' skin produces around 80% of their D_3 by UVB irradiation of 7-dehydrocholesterol. In contrast, UVB radiation produces vitamin D_2 in plants and fungus (1). Vitamin D is essential for bone metabolism, among other metabolic and catabolic pathways, and functions in the congenital and adaptive immune systems that influence the cure, severity and mortality of various acute and chronic diseases and bacterial and viral illnesses (2-4). Multiple forms of vitamin D can play a key role in modulating immunity, including ergocalciferol (D_2), cholecalciferol (D_3), 25-hydroxyvitamin D_2 ($25(OH)D_2$), 25-hydroxyvitamin D_3 ($25(OH)D_3$), and 1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)2D_3$) (5). The primary metabolic pathway for vitamin D physiology involves the formation of $25(OH)D_2$ and $25(OH)D_3$ from 25(OH)D (6). Through the bloodstream, vitamin D and its metabolites are transported predominantly attached to vitamin D binding protein (VDBP) (about 85%) and albumin (about 15%) (7). Upon reaching target cells, the vitamin D complex dissociates from either VDBP or albumin, allowing vitamin D to enter the cells and engage with nuclear vitamin D receptors (VDRn), which are present in various tissues and act as transcriptional factors (8).

Vitamin D insufficiency is commonly defined as serum $25(OH)D_3$ concentrations less than 20 nmol/L, while other recommendations and published research have varying cut-off values (9). It is often necessary to augment low serum $25(OH)D_3$ levels with ergocalciferol (D_2) or cholecalciferol (D_3), although their therapeutic equivalentity is debatable (10-12). Research indicates that taking D_3 orally increases levels of both free and total 25(OH)D more than using D_2 supplements (10, 13, 14). Factors unique to each patient may also influence how much extra vitamin D is needed (15) and for this reason, it's critical to validate vitamin D metabolite thresholds as well as which metabolite is clinically meaningful in order to recommend vitamin D supplementation. This study provides a comprehensive analysis by including both $25(OH)D_3$ and $25(OH)D_2$ levels in a larger cohort, allowing for a more detailed evaluation of vitamin D_2 status in healthy individuals. We present new findings on the potential role of $25(OH)D_2$ in vitamin D metabolism, which has been largely overlooked in previous studies. Owing to variations in patient reactions to vitamin D therapy and demographic variables, our goal was to look at the relationship between $25(OH)D_2$ and free vitamin D levels as well as vitamin D insufficiency in a cohort of Turkish adults who were otherwise in good health.

MATERIAL AND METHODS

Study Design: Ninety-two healthy adults, aged 18 or older, of both sexes (38 men and 54 women), who applied for a normal yearly check-up at the Biruni University Hospital's Check-up Unit, satisfied the research's requirements, and consented to take part in the investigation were included in the study. The following three requirements were met in order to be eligible for this prospective study: 1) no health condition, such as obesity, that might influence vitamin D concentrations; 2) no vitamin D supplementation during the previous two years; and 3) at least two generations of Turkish ancestry. The exclusion criteria included the following: 1) refusing to participate in the study; 2) not being of Turkish ethnic descent; 3) taking any kind of vitamin D supplement in the previous two years; 4) having a diagnosis of an infectious disease that is actively active (such as acute hepatitis, AIDS, or tuberculosis); 5) using steroids or their derivatives in the previous two years; 7) being under the age of eighteen, pregnant, or nursing.

Ethics committee permission for the study was obtained from Biruni University Ethics Committee (Ethical apporaval number: 2020/43-20). This study was conducted in full compliance with the Helsinki Declaration. After notifying all subjects about the study, formal permission forms were obtained from each.

In our previous study, we genotyped vitamin D binding protein in 51 patients (16). In this study, we added 41 more patients to the patients and performed a different statistical study and biochemical analysis of vitamin D_2 from a different perspective. In addition, the measurement differences between vitamin D_2 and D_3 levels were also revealed.

Collection of Blood Samples: Venous blood samples were taken and divided into two tubes: one for serum with a gel and the other for EDTA (Nest- UK). Blood samples collected in serum tubes at 4100 rpm and centrifuged (NF 800, Nuve, Turkey), the blood was split into two aliquots and stored at -80°C.

Metabolic Measurements of Vitamin D: 25(OH)D concentrations were evaluated using chemiluminescence microparticle immunoassay (CMIA). The Architect 25-OH Vitamin D kit (5P02, Abbott Diagnosis, USA) and i1000SR analyzer (Abbott Laboratories, USA) were used in the study. The 2011 IOM report on dietary reference intakes of 20 ng/ml was taken as the cut-off for vitamin D deficiency (17). To measure the albumin concentration of the samples, a Roche/Hitachi cobas C instrument was used in accordance with the manufacturer's instructions, which uses a colorimetric assay technique. Serum VDBP concentration was determined in accordance with the literature (18) and Quantikine kit for

monoclonal immunoassay measurement of human vitamin VDBP was performed using the manufacturer's instructions (R&D Systems, Cat No: DVDBP0, USA).

The analysis of 25(OH) D_3 and vitamin D_2 was conducted using liquid chromatography–mass spectrometry (LC-MS), employing an Agilent Infinity 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA). This system featured a binary pump (G4220A), column compartment (G1316C), and autosampler (G7167B), which were coupled to a 6470 triple quadrupole mass spectrometer (6470A, Agilent Technologies, Santa Clara, CA, USA). The process utilized a CE-in vitro diagnostic certified Jasem vitamin D LC-MS/MS analysis kit (Sem Laboratuar Cihazları Pazarlama Inc., Turkey).

For sample preparation, patient samples and serum-based calibrants/quality control materials were handled according to the kit's protocol. This procedure included a protein precipitation phase before injection into the system. The HPLC system was operated using the chromatographic parameters specified in the kit, and detection was performed with MS/MS using positive electronic spray ionization in multiple-reaction monitoring mode. For accurate measurement of analyte concentrations, the ratio of the peak area of vitamin D_2 (25(OH) D_3) to the internal standard (labeled stable isotope-d6 25(OH) D_3) was calculated. The Bikle Method, generally known as the following equation, was utilized to determine free 25(OH)D (19):

Free 25(OH)D = Total 25(OH)D/ (1 + (KALBx[ALB]) + (KDBPx[VDBP]))

Where [VDBP] is the concentration of vitamin D binding protein, [KALB] is the affinity constant for 25(OH)D with albumin, and [ALB] is the albumin concentration. Similarly, KDBP is the affinity constant for 25(OH)D_3 with vitamin D binding protein.

The authors' previously published work (16) examined VDBP gene polymorphisms using data on blood 25(OH) D_3, albumin, and VDBP concentrations of 51 people.

Statistical Analysis: A priori power analysis was conducted using G*Power 3.1.9.7 to determine the appropriate sample size for this study. The analysis was based on an expected effect size of 0.5, an alpha level of 0.05, and a statistical power of 95%. The results indicated that a minimum of 34 participants was required to achieve sufficient power. Since our study included 92 participants, we ensured adequate statistical power to detect significant effects.

The presentation of all the data was as mean ± standard deviation. Kolmogorov-Smirnov distance test was used to test the normality of all in-group variables. The numerical variables were compared to a normal distribution using an unpaired t-test with Welch correction. The correlation between 25-OH-Vit D_2 and 25-OH-Vit D_3 levels was assessed using Spearman's rank correlation coefficient due to the non-parametric nature of the data distribution. Statistical significance was defined as $P<0.05$. For all statistical analyses, GraphPad InStat ver. 3.06 (USA) was used.

RESULTS

Patient Characteristics: Ninety-two people had a mean age of 37.28 ± 15.23. Among the patients, 58.7% were female and 41.3% were male. Although there was a substantially greater 25(OH) D_3 in females compared to men, the mean concentration of total 25(OH) D_3, 25(OH)D_2, and 25(OH) D_3 as determined by LC-MS and free vitamin D did not differ significantly across genders (Table 1). Compared to males (17 ng/mL), women had mean 25(OH) D_3 concentrations of 22 ng/mL, which suggests that women had greater amounts of vitamin D_3. Men and women have similar free vitamin D levels (pg/mL), indicating that while women have greater total 25(OH) D_3, there is no discernible difference in free vitamin D levels between the sexes. Females show significantly higher levels of both 25(OH) D_3 and free vitamin D compared to males. This suggests that women in the sample have greater amounts of bioavailable (free) vitamin D in addition to having higher total vitamin D_3 levels.

Table 1. Vitamin D metabolite levels of healthy individuals according to the gender

	Total (n = 92)	Male (n = 38)	Female (54)	P value
Total 25(OH)D_3 (ng/ml)	19.45 ± 13.08	16.78 ± 8.19	21.02 ± 15.83	*0.413*
25(OH)D_2 (ng/ml)	2.95 ± 0.46	3.00 ± 0.50	2.90 ± 0.42	*0.375*
25(OH)D_3 (ng/ml)*	18.74 ± 11.85	15.98 ± 8.77	20.85 ± 13.54	*0.054*
Free vitamin D (pg/ml)	3.89 ± 2.45	3.24 ± 1.83	4.17 ± 2.56	*0.130*

*measured by LC-MS/MS

Comparison of Vitamin D Metabolites: Among the total participants, 54% had blood levels of total 25(OH) D_3 below 20 ng/mL, which led to a diagnosis of vitamin D insufficiency. Subjects with total 25(OH) D_3 levels equal to or greater than 20 ng/mL had significantly higher mean concentrations of 25(OH) D_3 (measured by LC-MS) and free vitamin D compared to those with levels

below 20 ng/mL (P < 0.001). However, there was no significant difference in the mean serum concentration of 25(OH) D_2 between the two groups, based on the criteria for vitamin D deficiency (Table 2).

Table 2. Vitamin D metabolite levels of healthy individuals compared with the total 25- Hydroxyvitamin D_3 levels

	Total 25(OH)D_3 <20ng/ml (n = 50)	Total 25(OH)D_3≥ 20ng/ml (n = 42)	P value
25(OH)D_2 (ng/ml)	2.91 ± 0.48	2.99 ± 0.44	*0.266*
25(OH)D_3 (ng/ml)*	10.63 ± 5.36	28.40 ± 10.07	***<0.001***
Free vitamin D (pg/ml)	2.29 ± 1.31	5.82 ± 2.08	***<0.001***

*measured by LC-MS/MS

Additionally, 30.4% of all participants had free vitamin D levels under 2 pg/mL. Individuals with serum free vitamin D levels of 2 pg/mL or higher showed significantly higher mean concentrations of both total 25(OH) D_3 and 25(OH) D_3, compared to those with free vitamin D levels below 2 pg/mL (P < 0.001), as determined by LC-MS. Nevertheless, no significant differences were observed in the mean serum 25(OH)D_2 levels across subjects when categorized by free vitamin D levels (Table 3).

Table 3. Vitamin D metabolite levels of healthy individuals compared with the free vitamin D levels

	Free vitamin D < 2 pg/ml (n = 28)	Free vitamin D ≥ 2 pg/ml (n = 64)	P value
Total 25(OH)D_3 (ng/ml)	7.50 ± 2.59	23.75 ± 13.0	***<0.001***
25(OH)D_2 (ng/ml)	2.93 ± 0.41	2.94 ± 0.40	*0.902*
25(OH)D_3 (ng/ml)*	6.18 ± 2.88	23.41 ± 10.80	***<0.001***

*measured by LC-MS/MS

The scatter plot analysis demonstrates the relationship between serum 25-OH-Vit D_2 and 25-OH-Vit D_3 levels. The Spearman correlation coefficient (r= 0,03062) was calculated as (p= 0,7695), indicating a weak correlation between the two variables (Figure 1). The trendline with confidence intervals suggests that higher levels of 25-OH-Vit D_3 are not strongly associated with variations in 25-OH-Vit D_2 levels.

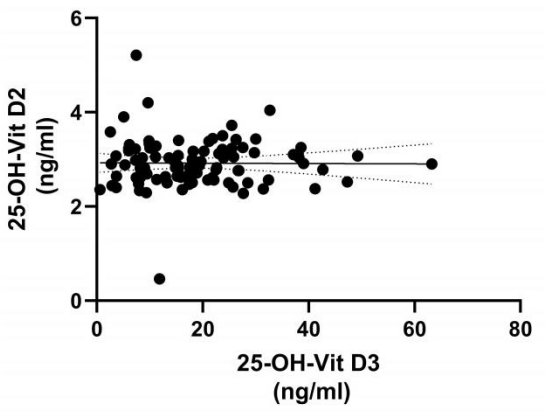

Figure 1. Scatter plot showing the correlation between serum 25-hydroxyvitamin D_2 (25-OH-Vit D_2) and 25-hydroxyvitamin D3 (25-OH-Vit D_3) levels. Each dot represents an individual patient sample. The correlation was assessed using Spearman's rank correlation coefficient (r = 0,03062, p = 0,7695). The dotted lines indicate the confidence interval of the regression trendline.

DISCUSSION

Serum total 25(OH)D is a widely used biomarker to assess vitamin D reserves and estimate human vitamin D status as it detects both forms of 25(OH)D_2 and 25(OH) D_3 (16). We looked into the relationship between the levels of 25(OH)D_2 and of free vitamin D and vitamin D insufficiency in a healthy Turkish population since the type of vitamin D (D_2 or D_3) might impact the dosage response of 25(OH) D_3 to vitamin D_3. The mean concentration of 25(OH) D_2 in serum did not differ among individuals according to Vitamin D deficiency criteria and to concentration of free vitamin D, and therefore does not directly indicate vitamin D deficiency. However, the mean concentration of 25(OH) D_3 measured by LC-MS and free vitamin D affects the total 25(OH) D_3 concentration measured by automated systems and may indicate vitamin D deficiency. These findings may point to gender-specific variations in vitamin

D exposure or metabolism, such as variations in vitamin D binding proteins or increased sun exposure (Table 1).

Over the previous 20 years, a number of sensitive and focused commercial tests have been created (16). The variations in the cross-reactivity of antibodies with epimers and/or metabolites, as well as the processes of vitamin D extraction, deproteinization, and purification, account for the variations among these tests. Even with these technological advancements, measuring 25(OH) D_3 concentration precisely and accurately remains difficult due to the presence of various hydrophobic vitamin D metabolites and fluctuating ratios of 25(OH) D_2 to 25(OH) D_3 in the bloodstream. These metabolites also have low free quantities in serum due to their ability to bind to lipids, albumins, and vitamin D binding protein (VDBP). Therefore, the challenges are attributable to the accuracy and sensitivity of assays which might result in discrepancies across different testing methodologies. Of these methods, automated immunoassays account for 90% of routine 25(OH)D testing because of their low manual labor requirements, high throughput, and automated sample handling (20). All immunoassays should assess D_2 and D_3 metabolites similarly (with equimolar reactivity), although detection of 25(OH) D_2 and 25(OH) D_3 largely depends on the antibody specificity. Immunoassays that are capable of detecting 25(OH) D_2 are unable to distinguish it from 25(OH) D_3 (21). Because it is significantly more sensitive than automated methods but also more costly, isotope-dilution LC-MS/MS is now the gold standard for 25(OH) D_3 testing (22). In the current investigation, we additionally assessed 25(OH) D_3 using LC-MS/MS and compared the results with the overall levels of 25(OH) D_3 determined by automated CMIA. The results demonstrate that there was consistency in the 25(OH) D_3 levels across the two techniques of assessment.

Several studies have demonstrated that vitamin D_2 in equimolar doses is less effective at increasing blood 25(OH) D_3 levels compared to vitamin D_3 (23-25). Additionally, research suggests that vitamin D_3 metabolites exhibit a stronger affinity for VDBP and interact differently with the vitamin D receptor compared to vitamin D_2 metabolites. Furthermore, the 25-hydroxylation rate of vitamin D_3 is higher than that of vitamin D_2 (26). Although the majority of commercially available vitamin D supplements are in the D_3 form, vitamin D_2 is still present in certain dietary sources and fortified foods. As a result, individuals may have varying contributions of 25(OH)D_2 to their total vitamin D levels, depending on their dietary intake and metabolism. Furthermore, evaluating both 25(OH)D_3 and 25(OH)D_2 provides a more comprehensive understanding of vitamin D metabolism. Previous studies have primarily focused on total 25(OH)D_3 levels, overlooking the potential role of 25(OH)D_2 in maintaining overall vitamin D status (27-29). Given these considerations, this study aims to explore the presence and significance of 25(OH)D_2 in healthy individuals, contributing to a more detailed assessment of vitamin D homeostasis across different populations. One possible reason for the higher production of 25(OH)D_3 over 25(OH)D_2 could be the increased hydroxylation efficiency of vitamin D_3. In our current study, 25(OH)D_3, rather than 25(OH) D_2, seems to be the primary contributor to overall 25(OH)D_3 levels. A possible explanation for the difference between 25(OH)D_2 and 25(OH)D_3 may be the lower affinity of vitamin D_2 metabolites for VDBP, resulting in a shorter half-life and quicker clearance from the bloodstream (30). Additionally, it has been shown that 1,25$(OH)_2D_2$ undergoes a more rapid inactivation phase during 24-hydroxylation, whereas 1,25,24$(OH)_3D_3$ requires further steps for deactivation. These findings suggest that 25(OH)D_3 remains physiologically active for a longer period, thereby playing a direct role in sustaining vitamin D levels (3). Research by Shieh et al. comparing high doses of vitamin D_2 and D_3 found that vitamin D_3 increased both total and free 25(OH)D levels more effectively than vitamin D_2 (13). Our results, based on 25(OH)D_3 levels measured by LC-MS and free vitamin D levels calculated using the Bikle method, align with the literature and point to vitamin D deficiency, rather than low 25(OH)D_2 levels, as the likely issue. Moreover, 25(OH)D_3 and free vitamin D appear to be better markers of vitamin D bioactivity than 25(OH)D_2, making them the more reliable indicators of physiological vitamin D sufficiency. In our Turkish research population, we also discovered that females had greater levels of 25(OH)D_3 than males. Similar results have also been noted in Indian (31), and Norwegian (32) sample populations, but not in Saudi (33) populations, despite the anthropometric, ethnic, and geographic disparities in the populations. These findings may reflect physiological differences between males and females, such as variations in PTH levels, or cultural factors, like differences in sun exposure, or possibly a combination of both. Previous studies have indicated that variations in total 25(OH)D levels and VDBP gene polymorphisms may account for the differences in free and bioavailable 25(OH)D levels among healthy Turkish individuals. This points to the significant role of genetic factors in influencing vitamin D metabolite levels (16). 25(OH)D_3, 25(OH)D_2 and free vitamin D levels were compared between vitamin D deficient and non-deficient groups. It is generally observed that the deficient group exhibited significantly lower 25(OH)D_3 levels and lower free vitamin D levels, possibly in combination with 25(OH)D_2 (Table 2). Conversely, the non-deficient group presents higher levels of

these metabolites, particularly 25(OH)D_3, reflecting normal vitamin D status. This underscores the role of vitamin D in maintaining overall health and suggests that 25(OH)D_3 is the key form linked to sufficient vitamin D levels.

Our study has some limitations. First, the sample size was relatively small. Second, although 50 participants had total 25(OH)D_3 levels below 20 ng/mL, indicating a considerable proportion of subjects with lower total 25D levels, the sample may not fully represent broader trends. Third, this study did not include a follow-up after vitamin D supplementation, which could have provided additional insights into distinguishing vitamin D deficiency. Additionally, the healthy participants were not severely deficient in vitamin D.

CONCLUSION

Despite these limitations, the study provides valuable insights into comparing free vitamin D and 25(OH)D_2 levels with 25(OH) D_3 levels in healthy Turkish individuals. Our findings indicate that 25(OH)D_3 and free vitamin D play a more significant role in determining vitamin D metabolite levels than 25(OH)D_2. Further research is necessary to explore whether variations in vitamin D metabolite levels are linked to specific vitamin D dosages and supplements, and to better understand the influence of dietary, sex, ethnic, and endocrine factors—such as PTH—on these physiological interactions.

Statement of Ethics: The study was approved by Biruni University Non-Interventional Research Ethics Committee (Approval No: 2020/43-20). Written informed consent was obtained from all participants.

Conflict of Interest Statement: The authors declare that there was no conflict of interest.

Funding Sources: This research received no external funding.

Data Availability Statement: The data of serum 25(OH)D_3, albumin and VDBP concentrations of 51 individuals were used for a previously published study of the authors in which VDBP gene polymorphisms were investigated [19].

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