

Dynamic of the seasonal levels of 25(OH)D in Bulgaria according to sex, age and winter status of vitamin D

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Abstract. Bulgaria is situated in the temperate climatic zone between 41° and 44° north. Between November and March the sun is low at the horizon and the skin production of vitamin D is very low. *The aim* of the study was to investigate the seasonal changes in 25(OH)D levels and their relationship to gender, age and the severity of vitamin D deficiency.

MATERIALS AND METHODS: Of the 433 subjects (tested in the winter) invited to participate in the seasonal 25(OH)D study - 315 (72.7%) were tested in the spring, 285 (65.8%) in the summer and 264 (61.0%) in the autumn. In 230 subjects data were available from all four seasonal measurements. One hundred twenty-five (54.3%) were women and 105 (45.7%) were men. The subjects were divided into three age groups: 1) young 20–44 years, $n = 107$ (46.5%); 2) middle age 45–59 years, $n = 75$ (32.6%); 3) elderly ≥ 60 years, $n = 48$ (20.9%). Serum 25(OH)D and PTH were measured.

RESULTS: The mean 25(OH)D levels in the whole group were: winter - 27.14 nmol/l (95%CI: 25.70–28.58), spring - 43.56 nmol/l (95%CI: 41.96–46.17), summer - 61.74 nmol/l (95%CI: 58.95–64.55), autumn - 52.75 nmol/l (95%CI: 50.63–54.88), $p < 0.001$. The absolute increase of vitamin D was higher in the males than in the females - $\Delta 30.38$ (95% CI: 28.11–32.39) vs. $\Delta 25.78$ (95% CI: 23.67–27.57), $p = 0.002$. The increase was higher in the younger individuals than in the elderly - $\Delta 30.23$ (95%CI: 28.19–32.26) vs. $\Delta 26.40$ (95%CI: 23.41–29.39), $p = 0.038$. The seasonal variations were higher in the subjects with vitamin D deficiency (< 25 nmol/l) in winter than in those with vitamin D insufficiency (25–50 nmol/l) - $\Delta 29.83$ (95%CI: 27.58–31.88) vs. $\Delta 26.20$ (95%CI: 24.19–28.07), $p = 0.015$.

CONCLUSION: There are significant seasonal variations in 25(OH)D levels in Bulgaria. The men, the younger individuals and those with deficiency have higher potential for an increase in 25(OH)D levels than the women, the elderly and those with insufficiency.

Keywords: Seasonal changes in 25(OH)D levels, sex, age, severity of vitamin D deficiency

The vitamin D is a term encompassing an array of metabolites of cholesterol, the principle being cholecalciferol, ergocalciferol, 25-hydroxy cholecalciferol, 1,25-dihydroxy cholecalciferol. The latter is

a steroid hormone that plays a key role in calcium and mineral homeostasis. Vitamin D receptors have been found in a vast number of cell types recently it has been proposed that the system of vitamin D as a wider health implication, for instance in the immune response and the cardiovascular health [1–3]. Therefore vitamin D deficiency might contribute to various morbidity and mortality.

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Vitamin D synthesis in humans depends on the skin insolation. The UV exposure for a given geographic latitude depends on the distance that the solar light travels through the atmosphere. An additional factor that changes the UV exposure especially in urban areas is air pollution. The annual UV exposure in the countries close to the equator is significantly higher than in the polar regions, where there are additionally seasonal variations. Therefore the insolation in a given population is determined by two major geographic factors – latitude and seasons. In most parts of the world, including Europe, vitamin D insufficiency is most prevalent in the winter months [4]. The hypovitaminosis D correlated inversely with UV exposure and therefore is dependent on latitude, air temperature and the hours of sunlight [5].

Bulgaria is situated in the temperate climatic zone between 41° and 44° north. The sun is low at the horizon from November through March and a large proportion of the UV light is absorbed by the atmosphere, leading to negligible skin vitamin D synthesis [6]. Other factors that may contribute to vitamin deficiency are female gender, advanced age, darker complexion, obesity and low dietary vitamin D [7–11]. The major source of vitamin D for the human organism is the skin synthesis from 7-Dehydrocholesterol with UVB (290–315 nm) exposure, while the alimentary vitamin D intake provides for only 10–20% of the daily requirements. Vitamin D₃ is further hydroxylated in the liver to 25-hydroxyvitamin D [25(OH)D], which is the primary form of vitamin D in the circulation [6].

According to the Committee of the Institute of Medicine (IOM, USA) the serum 25(OH)D level is the best indicator, reflecting both skin synthesis and alimentary intake of vitamin D [12]. Most experts define vitamin D deficiency as plasma or serum 25(OH)D levels lower than 20 ng/ml (50 nmol/l) [6, 13]. A similar definition was adopted at a consensus expert conference in 2012 as well though various factors might affect the 25(OH)D levels [14]. Further the low 25(OH)D levels are subdivided into deficiency or severe vitamin D deficit [25(OH)D < 25 nmol/l] and insufficiency or mild deficit [25(OH)D 25–50 nmol/l].

The aim of the study was to determine the seasonal variations in the 25(OH)D levels in a Bulgarian population with winter-time vitamin D insufficiency and deficiency, and to examine the role of the age, the gender and the severity of the vitamin D deficit.

1. Material and methods

The study was designed as a longitudinal observational, non-interventional study of the 25(OH)D levels in winter (initial data from the screening study), spring, summer and autumn. The study subjects were derived from a cohort of a national multicenter screening study of the prevalence of vitamin D deficiency in the Bulgarian population, carried out in January 2012 in 12 Bulgarian towns and the adjacent rural areas. Details on the initial study are published elsewhere [15].

A sample of 433 subjects with vitamin D deficiency ($n=247$) and insufficiency ($n=186$) from six geographic areas was randomly appointed and the subjects were invited to participate in the further study. All participants signed an informed consent, approved by the local Ethics Committee at the University Hospital of Endocrinology in Sofia. The participants were instructed not to take any vitamin D-containing preparation, calcium supplements or other PTH-influencing medication in order to explore the natural dynamics of 25(OH)D levels. Of the 433 invited subjects, 315 (72.7% turned up for the testing in spring, 285 (65.8%) – in summer and 264 (61.0%) in autumn. All four measurements were done in 230 subjects, who were divided into three age groups and further analyzed (Table 1).

All subjects were Caucasian, with light complexion and no history of hepatic or renal disease, or data of malabsorption, hypothyroidism, hyperparathyroidism. No questionnaire for daily sun exposure was applied in order to avoid induction of deviation from usual lifestyle in the studied subjects. None of the studied subjects travelled abroad during the period between the first and last study procedure. All subjects lived in 6 geographic areas of the country, between 41° and 44° North.

1.1. Cut off of hypovitaminosis D

To determine the cut-off point to define hypovitaminosis D based on PTH levels, we performed an adaptive regression of PTH on 25(OH)D level. Based on scatter plot, the relationship between PTH and 25(OH)D was not linear; however, it appears that the decrease in PTH levels ceases when vitamin D levels are beyond a turning point (inflection point). Similar to the reports of others, we have defined vitamin D deficiency as concentrations less than 20 ng/ml (50 nmol/liter), because PTH began to rise below this level [15–18].

Table 1
Distribution of the 230 participants according to gender and age

Age group (Years)	Female N=125 (54.3%)	Male N=105 (45.7%)	Total N (%)
20–44	56 (44.8%)	51 (48.6%)	107 (46.5%)
45–59	43 (34.4%)	32 (30.5%)	75 (32.6%)
≥60	26 (20.8%)	22 (20.9%)	48 (20.9%)

1.2. Laboratory method

A fasting morning venous blood sample was drawn in K2 EDTA blood tubes at the baseline and the three consecutive timepoints (January, April, July and October). The samples were centrifuged for 15 minutes at 2500 g and 4 deg C. The plasma was separated into 1 ml tubes and was frozen immediately at -20°C and transported to the central lab.

Determination of 25(OH)D was performed by a validated triple quadrupole tandem mass spectrometric (LC-MS/MS) method on TSQ Quantum Discovery Max LC-MS/MS system (Thermo Scientific). A 100 μL of human plasma were precipitated with 200 μL acetonitrile containing the internal standard, d3-25(OH)D3 and then 25(OH)D3, 25-(OH)D2 and d3-25(OH)D3 were extracted with 1.0 mL of hexane. After evaporation, the dry residue was reconstituted with 100 μL of mobile phase and 10 μL were injected for analysis. Chromatographic separation was performed on a 50×2.1 mm, 3.5 μm particles C18 analytical column with isocratic elution at a flow rate of 0.1 mL/min, utilizing a mobile phase consisting of 85% aqueous methanol, 0.005 mM ammonium acetate and 0.1% formic acid. Positive-ion electrospray ionization at 2.5 KV was performed with gases in arbitrary units as follows: sheath gas at 35, ion sweep gas at 0, auxillary gas at 2 units. Ion transfer capillary was set at 250°C , collision gas at 1.5 mTorr and selected reaction monitoring with collision energy at 10 was used to follow the predominant transitions at m/z 401.5 \rightarrow 383.5 for 25(OH)D3, at m/z 413.5 \rightarrow 395 for 25(OH)D2, and at m/z 404.5 \rightarrow 387 for d3-25(OH)D3. Scan width was 0.5 m/z , scan time was 0.5 s, and peak width was set at 0.7 for both quadrupole 1 and quadrupole 3. Method was validated according to industrial guidance requirements. Calibration curves were constructed using 6 point industrially certified human plasma calibration standards. Quality assurance utilized in-house prepared and commercial internal quality control samples, and participation in external proficiency testing program (DEQAS, UK)

with uninterrupted certification. Method selectivity was assessed with 6 different individual sources of human plasma, and confirmed with matrix effect (ME) averaging 91–93% for 25(OH)D3, 82–92% for 25(OH)D2, 84–89% for d3-25(OH)D3, and relative ME of 105–108% for 25(OH)D3, and of 97–104% for 25(OH)D2. Accuracy and precision (within-run and between-runs) were all within 7.5%. Extraction recoveries averaged 57–64% for 25(OH)D3, 69–73% for 25(OH)D2, 55–56% for d3-25(OH)D3; linearity range for both 25(OH)D3 and 25(OH)D2 was 3.0–300.0 nmol/L, $R^2 > 0.99$. Freeze-thaw stability was determined for three cycles each lasting 24 h, post-preparative stability was documented for 96 h at 10°C , short-term stability at ambient temperature was proven for 24 h in the dark and for 2 h at daylight; stock solution stability and long term stability in plasma - for 5 days at $4-8^{\circ}\text{C}$, and for 99 days at -20°C . With run time of less than 10 min, a throughput of over 100 samples per 24 h was achieved with instrument running unattended overnight.

PTH was determined by chemiluminescent immunoradiometric assay (IRMA) on DxI, (Beckmann Coulter Instruments) strictly following manufacturers' instructions, reference range 1.3–9.3 pmol/L. The analytical sensitivity of the method was 0.1 pmol/l and the functional sensitivity – 0.4 pmol/l. Quality assurance encompassed commercial internal quality control samples, and certified participation in the Bulgarian External Quality Assessment System (BEQAS).

1.3. Statistical analysis

The statistical analyses are done with SPSS 13.0. A descriptive analysis was done using grouping according to one or several variables. Suitable tests were performed to assess the level of significance of certain empirical characteristics and to validate the assumptions about their distribution. Empirical p -values were calculated based on these. The 95% was

assumed as a level of significance (unless stated otherwise), which is corresponding to 0.05 alpha risk of error. A GLM Repeated Measures ANOVA was performed to assess the seasonal dynamic of 25(OH)D and also the effect of age, gender and winter 25(OH)D levels to the observed patterns. The statistical significance of the studied within-subject effects was judged using Multivariate tests (Pillai's Trace, Wilks' Lambda, Hotelling's Trace and Roy's Largest Root). Pairwise Comparisons (LSD) were also applied to better understand the significance of the difference between available factor levels.

2. Results

The dynamic of the tested within subject effect was proven significant (all 4 used Multivariate tests indicated $p < 0.001$). At the same time the within-subject interactions with age groups, gender and winter 25(OH)D levels (second and higher order) were proven not statistically significant (all 4 used Multivariate tests indicated $p > 0.05$). Between subject effects of age, gender and winter 25(OH)D levels indicated significant results ($p < 0.05$), while their interactions (second and higher order) were estimated as non-significant ($p > 0.05$).

The differences between all four mean 25(OH)D were significant (winter 27.14 nmol/l (95%CI: 25.70–28.58); spring 43.56 nmol/l (95%CI: 41.96–46.17); summer 61.74 nmol/l (95%CI: 58.95–64.55); autumn 52.75 nmol/l (95%CI: 50.63–54.88~), $p < 0.001$. The mean weighted differences [$\Delta 25(\text{OH})\text{D}$] between the winter measurement and the three remaining seasonal measurements are presented in Table 2. The pairwise comparisons (using LSD test) indicated statistically significant difference between all [$\Delta 25(\text{OH})\text{D}$] (all $p < 0.001$). Thus the increase in the Summer was significantly higher than those in the Spring and the Autumn ($p < 0.001$) and this is visible for both genders.

Table 2

Mean weighed difference $\Delta 25(\text{OH})\text{D}$ between the winter measurement and the three other ones in the whole group and in the females and males separately. All differences are significant (all $p < 0.001$)

Group	$\Delta 25(\text{OH})\text{D}$ (nmol/l; 95%CI)		
	Spring/Winter	Summer/Winter	Autumn/Winter
All	17.55; 16.74–19.20	39.11; 36.50–40.86	26.96; 25.36–28.93
Female	15.5; 13.98–17.28	36.85; 33.06–38.93	24.97; 22.82–27.61
Male	20.0; 18.49–22.12	41.81; 38.14–44.58	29.33; 26.44–31.71

Table 3

Mean weighed averaged value of difference $\Delta 25(\text{OH})\text{D}$ for the three seasons vs. winter in the three age groups. $\Delta 25(\text{OH})\text{D}$ was higher in the young than in the and elderly ($p = 0.038$)

Age group	$\Delta 25(\text{OH})\text{D}$ (nmol/l)	95% Confidence Interval
20–44 ♂	$\Delta 30.23^*$	28.19–32.26
45–59 ♂	$\Delta 27.17$	24.76–29.57
≥ 60 ♂	$\Delta 26.40^*$	23.41–29.39

2.1. The gender as a factor

The increase in 25(OH)D was higher in the males than in the females for all seasons compared to the winter, $p < 0.05$ for all seasons, Table 2.

The calculated mean increase for the three seasons aggregated (Spring, Summer and Autumn) as compared to the winter was also higher in the males [All season $\Delta 25(\text{OH})\text{D}$ vs. Winter (nmol/l) - $\Delta 30.38$ (95% CI: 28.11–32.39) in the males and $\Delta 25.78$ (95% CI: 23.67–27.57) in the females - $p = 0.002$, LSD Pairwise Comparison].

2.2. The age as a factor

The increase in 25(OH)D levels in the three seasons vs. Winter was significantly higher in the young group as compared to the elderly ($p = 0.038$, LSD Pairwise comparisons), Table 3. The levels in the middle age group did not differ significantly from the other two ($p > 0.05$, LSD Pairwise comparisons).

2.3. The winter 25(OH)D levels (January) as a factor

The mean integrated increase in the 25(OH)D levels for the three seasons compared to those in winter was higher in the subjects with deficiency than in those with insufficiency [$\Delta 29.83$ (95%CI: 27.58–31.88) versus $\Delta 26.20$ (95%CI: 24.19–28.07), $p = 0.015$, LSD Pairwise Comparisons].

3. Discussion

Vitamin D is synthesized in the skin from 7-dehydrocholesterol by a photochemical reaction [10]. Due to its long plasma half-life and less restrictive regulation, the circulating 25(OH)D is the best indicator for the vitamin D stores of the body and its measurement serves to assess them [19].

A seasonal dynamic in 25(OH)D levels has been described with a peak in the summer months and nadir in the winter and early spring ones [20]. The seasonal vitamin D level changes may be explained by two major factors. The sunlight falls at a steeper angle during the winter and usually the clothing further restricts the sun-exposed skin area. Therefore the season, when measurement is taken should be kept in mind when interpreting the 25(OH)D levels.

In the current study we found a significant difference in the mean 25(OH)D levels between all the seasons. We observed a summer peak and significantly higher levels in the autumn than in the spring. A possible explanation of this curve is the lower vitamin D synthesis in spring and the measurement in autumn of the already synthesized during the summer but starting to decline 25(OH)D. Authors from Japan reported similar significant seasonal 25(OH)D variations - 27.4 ng/ml in July and 21.4 ng/ml in November [21]. Karohl C et al. also found a difference of 15 nmol/l between the 25(OH)D levels in summer and winter [22].

Vitamin D is lipid-soluble and is stored in fat tissue, the latter being more as a percentage of body weight in the women [23, 24]. That fact could explain the lower serum levels in women. The data on the dependence of the seasonal variations of 25(OH)D on gender are not unequivocal. Woitge HW et al. compared the 25(OH)D levels in winter and summer and found a significant difference in the women, but not in the men [25]. On the contrary, we observed a greater seasonal increment in 25(OH)D in the men than in the women. Similar results have been reported by Nanri A. et al. who demonstrated a higher summer 25(OH)D and autumn levels in the men, than in the women [20]. They reported also vitamin D insufficiency (<50 nmol/l) in 9% of the men in the summer and in 35.4% in the winter, and 9.8% in the summer and 62.2% in the winter in the women with a significant winter inter-gender difference. Bolland M. et al. [26] studied the serum 25(OH)D in winter and summer in a smaller group and reported a comparable increase in 25(OH)D levels in both genders (by 14.8% in the men and by 13% in the women). We observed a

similar decrease in the prevalence of vitamin D deficiency with a significant between-gender difference in winter - 37.8% vs. 48.6%, $p < 0.001$. Interestingly, the winter vitamin D deficiency prevalence in the men was similar in the two studies, while in women it was much higher in Japan, than in Bulgaria.

Romero-Ortuno R, et al. in Ireland (latitude 53°N) studied elderly subjects at mean age 73 years, two thirds of whom were women and demonstrated a mean 25(OH)D level of 40.3 nmol/l and only 6 nmol/l seasonal variation amplitude [27]. Lardner E. et al. measured 25(OH)D levels in postmenopausal women not receiving vitamin D supplementation or antioestrogenic therapy around the year. Only 4% of the studied women had sufficient vitamin D levels year-round. The authors found neither seasonal variations, nor correlation with the subjects' age [28]. Opposite to their findings, there was a clear correlation between the seasonal variations of vitamin D and the age in our study (41–44°N). Furthermore, the young subjects had a significantly higher increase in the 25(OH)D levels than the elderly. It might be assumed, that the young subjects synthesize more cholecalciferol than the elderly. A lifestyle difference with longer sunlight exposure in summer however might better explain the observed difference since the data is controversial as to whether there the young and elderly skin differ in the capacity for 25(OH)D synthesis.

The results of the National Health and Nutrition Examination Survey (NHANES 2001–2004) showed 25(OH)D levels below 75 nmol/l in 77% of the studied population [29], while that figure was only 55% for the period 1988–1994 [30]. We reported earlier that 25(OH)D levels were below 50 nmol/l in 75.8% of the Bulgarian population (20–80 years) [15]. In 21.3% there was a deficiency (<25 nmol/l) and in 54.5% - insufficiency (25–50 nmol/l). An interesting question was whether the initial 25(OH)D levels (deficiency or insufficiency) were related to its seasonal variations. A factor, that cannot be accounted for, but affects the vitamin D synthesis and metabolism, is the inter-individual variation in the vitamin D synthesis and metabolism [31]. Our results however showed greater seasonal variations in the subjects with more profound deficit (D deficiency) than in those with insufficiency. That observation cannot be explained by the individual differences in 25(OH)D synthesis, because otherwise parallel seasonal curves should be expected, rather than the observed pattern. A different clearance rate is a more probable candidate for an explanation or a difference in the storage/release of cholecalciferol and 25(OH)D

from the fat tissue. Further research might help get a better insight into this phenomenon.

4. Conclusion

There are significant seasonal variations in 25(OH)D levels in the Bulgarian population. The levels increase throughout spring and summer and decline in autumn. The capacity for vitamin D synthesis is higher in the men and in the younger subjects and those with lower winter levels.

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