Endocrine CONNECTIONS

The variation in free 25-hydroxy vitamin D and vitamin D-binding protein with season and vitamin D status

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Abstract

Purpose: Serum 25-hydroxy vitamin D [25(OH)D] varies greatly with season at northern latitudes. The purpose of this study was to determine if the seasonal variations in serum total 25(OH)D are followed by a concomitant variation in free 25(OH)D or if the variation is damped by alterations in the binding capacity of DBP.
Methods: Serum was collected from 540 healthy blood donors (60% men; mean age 41 ± 13 years) during 12 months and analyzed for total 25(OH)D, directly measured free 25(OH)D, vitamin D-binding protein (DBP) and albumin. Calculated free 25(OH)D was estimated.
Results: The UV-B radiation during the sampling month was positively correlated with the serum levels of total 25(OH)D ($r = 0.355$, $P < 0.001$), directly measured free ($r = 0.336$, $P < 0.001$) and calculated free 25(OH)D ($r = 0.275$, $P < 0.001$), but not with DBP and albumin. The percentage of free 25(OH)D was higher during the winter months than that during the summer months (0.020 ± 0.005% vs 0.019 ± 0.004%; $P = 0.007$) and higher in participants with a serum 25(OH)D below 25 nmol/L than that in participants with a serum 25(OH)D above 75 nmol/L (0.031 ± 0.007% vs 0.017 ± 0.003%; $P < 0.001$). iPTH was correlated with directly measured free 25(OH)D ($r = -0.226$; $P < 0.001$), but only weakly with calculated free 25(OH)D ($r = -0.095$; $P = 0.027$).
Conclusions: Directly measured free serum 25(OH)D was highly correlated with total serum 25(OH)D and followed the same seasonal variation, whereas the serum concentrations of DBP and albumin were stable. The fluctuation in free 25(OH)D was only marginally damped with an increase in the percentage of free 25(OH)D during the winter months and in participants with vitamin D deficiency.

Key Words
- free 25-hydroxy vitamin D
- vitamin D-binding protein
- 25-hydroxy vitamin D
- vitamin D deficiency
- parathyroid hormone

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Introduction

Vitamin D is essential for the calcium homeostasis and bone health and also of importance for the immune function, muscles and the cardiovascular system. Vitamin D3 is mainly produced in the skin after ultraviolet radiation, but smaller amounts of vitamin D2 and D3 are also obtained via food. To be activated, the prohormones undergo hydroxylation in two steps; firstly in the liver to become the pro-hormone 25-hydroxy vitamin D [25(OH)D] and secondly in the kidneys to become the active hormone 1,25-dihydroxy vitamin D, [1,25$(OH)_2$D].

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To assess a persons' vitamin D status, 25(OH)D is the metabolite that is measured in serum (1). Being a highly hydrophobic molecule, most circulating 25(OH)D is bound to either vitamin D-binding protein (DBP) (88%) or albumin (12%) and only a small fraction, less than 1%, circulates in a free unbound form (2). 25(OH)D binds with high affinity to DBP, but with lower affinity to albumin. The albumin-bound fraction of 25(OH)D plus free fraction has therefore been referred to as the bioavailable fraction of 25(OH)D (3, 4).

DBP is a 52–58 kDa glucoprotein produced in the liver, belonging to the albuminoid superfamily (5). First of all DBP is a carrier protein for the circulating vitamin D metabolites, but other roles for the protein have been described, such as the binding of fatty acids and endotoxins, chemotactic effects on neutrophil granulocytes, activating of macrophages and the sequestration of actin upon tissue damage (6). As the serum concentration of DBP is 20-fold higher than that of the vitamin D metabolites, only 2–5% of the circulating DBP is occupied by the vitamin D metabolites [25(OH)D and $1,25(OH)_2D$]. DBP prolongs the half-life of 25(OH)D, can act as a reservoir for 25(OH)D in situations of deficiency and also protect against vitamin D intoxication (7).

The concentration of free 25(OH)D can either be calculated using a formula, which includes the concentrations of DBP and albumin as described herein, or it can be directly measured (8). A problem with the calculation of the free concentration is that the DBP levels are not constant, but can vary in conditions such as pregnancy, liver cirrhosis, kidney disease and malnutrition (9). The concentrations of DBP and its' binding affinity for the vitamin D metabolites also vary among different populations depending on the polymorphism in the expression of the three different DBP isotypes (6, 10). Lower DBP concentrations have been observed in African-Americans compared with European-Americans when DBP was measured with an immunoassay using monoclonal DBP antibodies, but not when measured with a mass spectrometry or immunoassays using polyclonal DBP antibodies (11, 12, 13, 14, 15).

In a previous study, we analyzed serum 25(OH)D in a cohort of blood donors during 12 months and found that 50% of the participants had a 25(OH)D below 50 nmol/L during 50% of the year and that the levels varied greatly with season (16).

The primary aim of the present study was to determine if the seasonal variations observed in total 25(OH)D in serum is followed by a concomitant variation in free 25(OH)D or if the variation is damped by alterations in the binding capacity of DBP. We also wanted to compare the concentrations of directly measured with calculated free 25(OH)D and explore the associations between serum DBP and free 25(OH)D with demographic and life style-associated parameters.

Methods

Healthy blood donors

Healthy blood donors resident in Gothenburg, Sweden (57°41′N, 11°59′E) were invited to participate in the study when giving blood. Totally 540 blood donors were included, 40–60 in the middle of each month during a whole year. All blood samples were collected in the morning between 08:00 and 11:00 h.

Informed consent was obtained from all individual participants included in the study. The study was approved by the Regional Ethics Committee in Gothenburg and carried out in accordance with the Helsinki declaration.

All blood donors stated that they were in full physical health and answered questionnaires regarding medication, smoking habits, physical activity, sunbed use and sun holidays during the previous month. A sun holiday was defined as a journey to a location south of the latitude 43°N. Body weight and height were measured, and body mass index (BMI) was calculated.

Ethical approval

All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Laboratory analyses

The serum samples were frozen at –80°C immediately after collection.

Serum 25(OH)D (both D2 and D3) was analyzed with chemiluminescence immunoassay (CLIA) on a LIAISON instrument (DiaSorin Inc, Stillwater, MN, USA). The total coefficient of variance (CV) for serum 25(OH)D was 5–6%, with the highest variance in the lowest test range, and the limit of quantification (LoQ) was 12.5 nmol/L at a CV of 8%.

The free 25(OH)D concentration in serum was measured with enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Future Diagnostics Solutions, Wijchen, The Netherlands), hereafter, named

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directly measured free 25(OH)D. The analysis at the laboratory showed a total CV of 7.6% at a level of 4 pg/mL and a CV of 18% at 1.8 pg/mL, which was used as LoQ. Only two patients had a serum-free 25(OH)D below LoQ. The concentration of free 25(OH)D in serum was measured in pg/mL, but converted to pmol/L by using the formula 1 pg/mL = 2.5 pmol/L.

Serum DBP was measured with a monoclonal ELISA (R&D Systems). Our analysis showed a total CV of 7.4% at the level of 268 mg/L and a CV of 17.6% at 1.3 mg/L, which was to be used as LoQ.

Serum intact parathyroid hormone (iPTH) was analyzed with CLIA on an Abbott ARCHITECT instrument (Abbott Diagnostics Division) and serum $1,25(OH)_2D$ was analyzed on an IDS-iSYS instrument (Immunodiagnostic Systems Holdings, Boldon, UK). The serum levels of albumin, highly sensitive C-reactive protein (hsCRP), calcium, phosphate, creatinine, alanine aminotransferase (ALT), sexual hormone-binding globulin (SHBG), estradiol and testosterone were analyzed with standard laboratory techniques on a Cobas instrument (Roche Molecular Diagnostics).

The calculation of free 25(OH)D and bioavailable 25(OH)D in serum

Free 25(OH)D was calculated using the formula described by Bikle *et al.* (8):

$$\text{Calculated free } 25(\text{OH})\text{D} = \frac{\text{Total } 25(\text{OH})\text{D}}{1+\left(6\times10^{5}\times[\text{Albumin}]\right)+\left(7\times10^{8}\times[\text{DBP}]\right)}$$

$$\text{Bioavailable } 25(\text{OH})\text{D} = \left(6\times10^{5}\times[\text{Albumin}]+1\right) \times \text{calculated free } 25(\text{OH})\text{D}$$

$$[\text{Albumin}] = \text{serum albumin in g/L} \div 66{,}430\ \text{g/mol}$$

$$[\text{DBP}] = \text{serum DBP in g/L} \div 58{,}000\ \text{g/mol}$$

$$\text{The percentage of free } 25(\text{OH})\text{D} = \frac{\text{Free } 25(\text{OH})\text{D}}{\text{Total } 25(\text{OH})\text{D}}$$

Estimated UVB irradiation

Monthly sums of Commission Internationale de l'E' clairage (CIE)-weighted UV radiation (Wh/m^2), which mimics the erythemal effect of UV radiation, were calculated for Gothenburg (57°41′N, 11°59′E) for each month during a year using the Swedish Meteorological and Hydrological Institute's (SMHI) solar radiation model STRÅNG (http://strang.smhi.se/).

Statistical analyses

Statistical analyses were made using SPSS Statistics 23.0 (SPSS). Descriptive statistics are presented as mean ± S.D. In comparisons between groups, the independent samples *t*-test was used for continuous variables and the chi-square test for categorical variables. Correlations were calculated using Pearson correlation (r) for all parameters, except for hsCRP where Spearman correlation (r_S) was used due the skewed distribution of the parameter. All tests were two-tailed, and $P<0.05$ was considered statistically significant. Linear regression with a stepwise method was run with serum DBP as outcome. Covariates were the parameters significantly associated with serum DBP in the first analyses. The results from the linear regression are presented as unstandardized coefficients (B) and standard error (S.E.).

Results

Healthy blood doors

The characteristics of the 540 healthy blood donors are presented in Table 1.

The seasonal variation in vitamin D metabolites

The serum levels of free 25(OH)D, both directly measured and calculated and bioavailable 25(OH)D all followed the seasonal variation of total serum 25(OH)D, with a peak during the summer months and a low point during the winter months. There was no seasonal variation in serum DBP, serum albumin, serum calcium or serum $1,25(OH)_2D$. Serum phosphate also followed the seasonal variation of total 25(OH)D, whereas serum iPTH demonstrated an inverse pattern with a low point during the summer and a peak during the winter (Fig. 1).

The CIE-weighted UV radiation during the sampling month was positively correlated with the serum levels of total 25(OH)D ($r=0.355$, $P<0.001$), directly measured free 25(OH)D ($r=0.336$, $P<0.001$), calculated free 25(OH)D ($r=0.275$, $P<0.001$) and bioavailable 25(OH)D ($r=0.275$, $P<0.001$), but not with the serum levels of DBP ($r=-0.011$,

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Table 1 The characteristics of the 540 blood donors included in the study.

	All	Women	Men
	N (%)	*N* (%)	*N* (%)
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
Sex	540	215 (39.8)	325 (60.2)
Age (years)	40.9 ± 13.0	38.9 ± 12.8	42.3 ± 13.1
Body mass index (kg/m^2)	24.8 ± 4.0	24.2 ± 3.4	25.2 ± 4.3
Current smokers	24 (4.4)	9 (4.2)	15 (4.6)
Subjects who had used a sun-bed during the previous month	23 (4.3)	17 (7.9)	6 (1.8)
Subjects who had been on a sun-holiday during the previous month	36 (6.7)	15 (7.0)	21 (6.5)
Subjects taking vitamin D supplements	50 (9.3)	27 (12.6)	23 (7.1)
Subjects taking estrogens (contraceptives or HRT)		24 (11.2)	
Total serum 25(OH)D (nmol/L)	62.8 ± 26.3	65.6 ± 25.3	61.0 ± 26.8
Recommended level >50 nmol/L			
Directly measured free 25(OH)D in serum (pmol/L)	11.5 ± 4.3	11.7 ± 4.2	11.4 ± 4.4
Calculated free 25(OH)D in serum (pmol/L)	19.7 ± 10.7	19.8 ± 9.7	19.6 ± 11.3
Bioavailable 25(OH)D (nmol/L)	7.4 ± 4.1	7.2 ± 3.6	7.5 ± 4.4
The percentage of free 25(OH)D (%) (using directly measured free 25(OH)D)	0.019 ± 0.004	0.018 ± 0.004	0.020 ± 0.044
The percentage of free 25(OH)D (%) (using calculated free 25(OH)D)	0.032 ± 0.013	0.031 ± 0.010	0.033 ± 0.014
Serum DBP range 55.9–473 mg/L	253.1 ± 78.9	265.4 ± 87.8	244.9 ± 71.5
Serum albumin	41.5 ± 4.4	40.3 ± 3.8	42.2 ± 4.7
Ref. interval 36–48 g/L			
Serum 1,25(OH)$_2$D	116.6 ± 42.5	121.3 ± 46.1	113.5 ± 39.6
Ref. interval 36–216 pmol/L			
Serum iPTH	44.0 ± 18.6	45.0 ± 19.4	43.4 ± 18.1
Ref. interval 15–68 ng/L			
Serum calcium	2.4 ± 0.13	2.4 ± 0.12	2.4 ± 0.13
Ref. interval 2.15–2.5 mmol/L			
Serum phosphate (mmol/L)	1.3 ± 0.4	1.4 ± 0.4	1.3 ± 0.4
Ref. interval women 0.8–1.5; men 0.7–1.4 mmol/L			
Serum creatinine	81.2 ± 14.6	71.8 ± 10.8	87.5 ± 13.3
Ref. interval women 45–90; men 60–105 µmol/L			
Serum ALT	0.5 ± 0.4	0.4 ± 0.4	0.5 ± 0.3
Ref. interval women 0.15–0.75; men 0.15–1.1 µkat/L			
Serum hsCRP in median (IQR)	0.64 (0.32–1.2)	0.63 (0.33–1.28)	0.65 (0.31–1.15)
Ref. interval <5 mg/L			
Serum SHBG	60.6 ± 40.9	85.7 ± 51.8	44.0 ± 17.7
Ref. interval women 27–128; men 18–77 nmol/L			
Serum estradiol (women only) in median (IQR)		102.5 (18.3–277.8)	
Ref. interval dependent on menstrual cycle or menopausal status (pmol/L)			
Serum testosterone (men only) (nmol/L)			15.9 ± 5.5
Ref. interval 20–50 years 0.29–1.67, ≥50 years 0.1–1.42			

1,25(OH)$_2$D, 1,25-dihydroxy vitamin D; 25(OH)D, 25-hydroxy vitamin D; ALT, alanine aminotransferase; BMI, body mass index; DBP, vitamin D binding protein; HRT, hormone replacement therapy; hsCRP, highly sensitive c-reactive protein; iPTH, intact parathyroid hormone; IQR, interquartile range; SHBG, sexual hormone binding globulin.

P=0.840), albumin (r=0.045, P=0.292) or 1,25(OH)$_2$D (r=0.071, P=0.104).

The monthly serum concentrations of total 25(OH)D, measured free 25(OH)D, calculated free 25(OH)D, bioavailable 25(OH)D, 1,25(OH)$_2$D and DBP are shown in Fig. 1 and in Table 2.

Directly measured free 25(OH)D compared with calculated free 25(OH)D

The serum levels of directly measured free 25(OH)D (mean ± S.D. 11.5 ± 4.3 pmol/L corresponding to 4.6 ± 1.8 pg/mL) was significantly lower than the serum levels of calculated free 25(OH)D (mean ± S.D.

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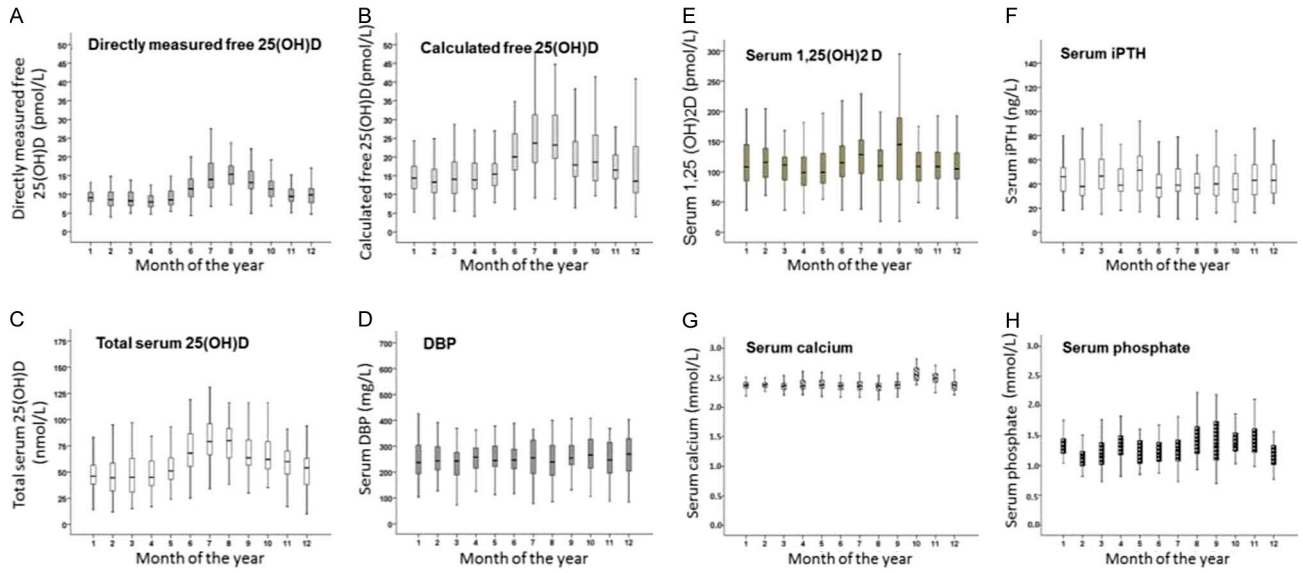

Figure 1
Boxplot diagram showing the variation in the vitamin D metabolites over the months of the year. (A) Directly measured free 25(OH)D. (B) Calculated free 25(OH)D. (C) Total serum 25(OH)D. (D) Vitamin D-binding protein. (E) Serum 1,25(OH)$_2$D. Regarding September the result is uncertain due to missing data from several of the controls. (F) Serum iPTH. (G) Serum calcium. (H) Serum phosphate.

19.7±10.7 pmol/L corresponding to 7.9±4.3 pg/mL, $P<0.001$). The correlation between directly measured and calculated free 25(OH)D was $r=0.695$, $P<0.001$.

The directly measured 25(OH)D showed a closer relationship with total serum 25(OH)D ($r=0.880$, $P<0.001$) than did the calculated free 25(OH)D ($r=0.670$, $P<0.001$) (Fig. 2). In addition, directly measured free 25(OH)D showed a stronger correlation with serum iPTH ($r=-0.226$; $P<0.001$) than calculated free 25(OH)D ($r=-0.095$; $P=0.027$), comparable with the association between total 25(OH)D and iPTH ($r=-0.257$; $P<0.001$).

The percentage of free 25(OH)D in serum

The percentage of free 25(OH)D, representing the fraction of total 25(OH)D, which circulates in a free

Table 2 The monthly serum variation in total 25(OH)D, directly measured free 25(OH)D, calculated free 25(OH)D, bioavailable 25(OH)D, 1,25(OH)$_2$D and DBP.

Month of the year	**Total 25(OH)D** (nmol/L)	**Directly measured free 25(OH)D** (pmol/L)	**Calculated free 25(OH)D** (pmol/L)	**Bio-available 25(OH)D** (nmol/L)	**1,25(OH)$_2$D** (pmol/L)	**DBP** (mg/L)
January	48.4±17.4	9.4±2.4	15.4±7.6	5.8±3.0	115.4±42.1	248.7±78.9
February	47.4±20.7	9.1±2.7	14.0±5.2	5.2±1.9	123.2±43.5	249.1±62.2
March	48.0±21.0	8.8±2.7	15.2±6.3	5.1±2.7	107.0±31.2	243.0±91.4
April	50.3±20.0	8.5±2.4	15.7±5.6	5.8±2.1	104.0±35.5	252.0±67.0
May	53.8±17.1	9.2±2.4	15.5±4.9	6.0±2.0	111.2±38.6	263.4±76.8
June	72.6±26.7	11.8±3.6	22.7±11.1	8.5±4.1	119.4±39.3	251.0±72.0
July	81.9±26.2	15.1±4.8	26.4±12.6	10.0±5.2	129.0±46.3	253.3±87.3
August	80.4±29.8	15.7±4.4	25.7±10.3	9.5±3.6	113.7±39.2	243.1±75.5
September	67.4±22.7	14.2±4.1	20.3±9.1	7.4±3.2	149.4±73.4	260.5±64.6
October	69.9±24.1	12.3±4.0	20.6±8.4	8.0±3.3	111.8±35.3	264.9±69.8
November	56.8±18.3	10.0±2.7	17.7±8.6	6.7±3.2	110.0±40.8	254.2±83.4
December	54.2±22.2	10.3±3.3	19.5±18.1	7.4±7.0	113.5±40.0	260.7±112.4
All months[#]						
Mean±S.D.	60.5±25.4	11.1±4.2	18.7±10.0	7.0±3.8	115.9±42.4	253.9±78.1
95% CI	18.0–116.0	5.3–22.2	6.3–42.5	2.0–16.8	37.5–218.7	104.0–400.0

[#]Values are adjusted for the fact that more subjects were included during the summer months.
1,25(OH)$_2$D, 1,25-dihydroxy vitamin D; 25(OH)D, 25-hydroxy vitamin D; DBP, vitamin D-binding protein.

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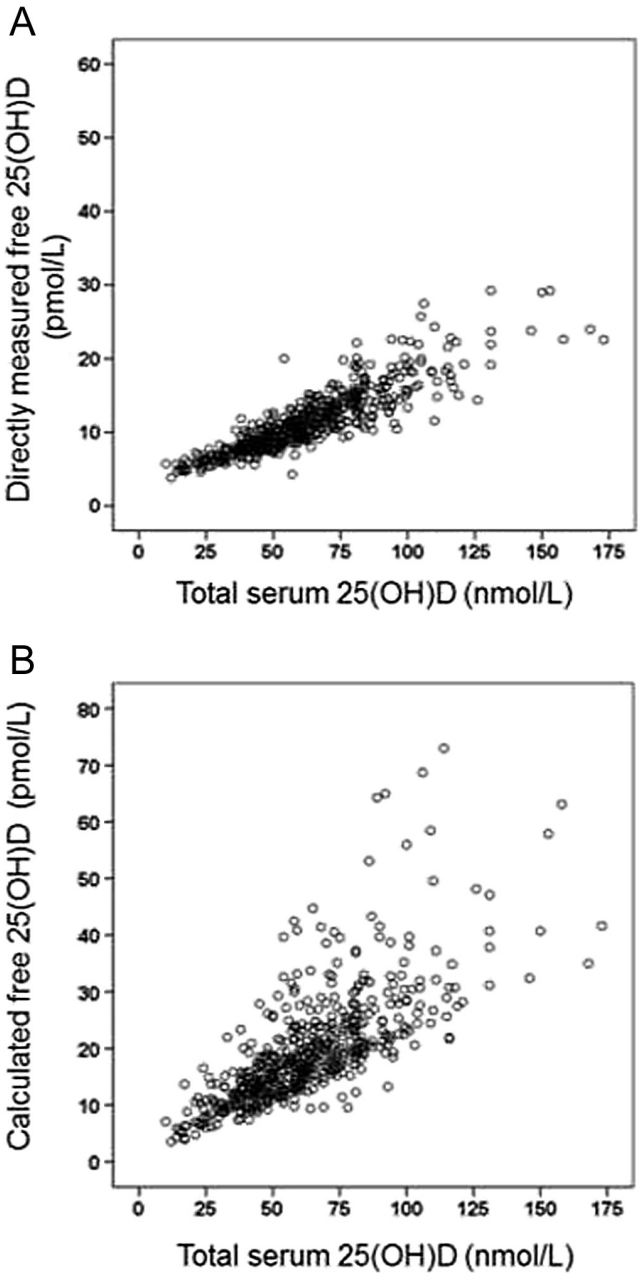

Figure 2
Scatter diagram showing the relation between total 25(OH)D and (A) directly measured free 25(OH)D and (B) calculated free 25(OH)D. (In Fig. 2A, two study subjects had a directly measured free 25(OH)D below the limit of quantification 1.8 pg/mL = 4.5 pmol/L.)

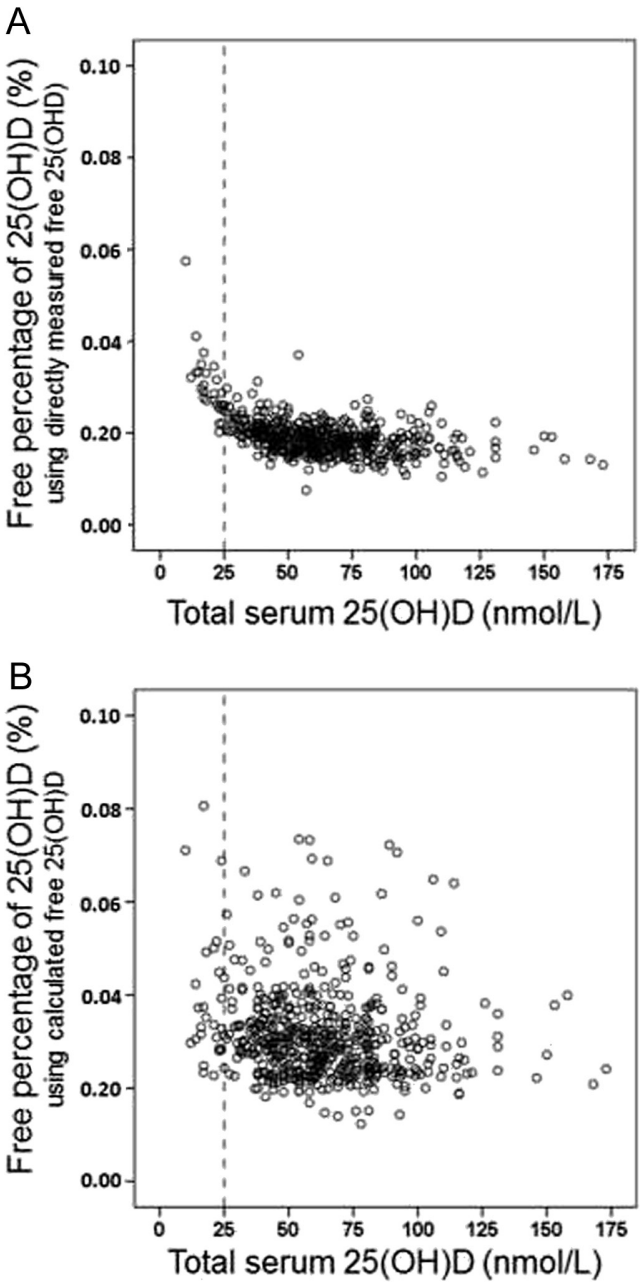

Figure 3
Scatter diagram showing the relation between total serum 25(OH)D and (A) directly measured free fraction of 25(OH)D in serum, with an increase in the free fraction in participants with a total serum 25(OH)D below 25 nmol/L and (B) calculated free fraction of 25(OH)D in serum.

unbound form, was calculated using the formula: (directly measured serum free 25(OH)D/serum total 25(OH)D). The percentage of free 25(OH)D was 0.019 ± 0.004% in this cohort.

The percentage of free 25(OH)D was negatively correlated with total serum 25(OH)D; $r = -0.474$, $P < 0.001$ (Fig. 3A). Thus, the percentage of free 25(OH)D was higher during the winter months than that during the summer months (0.020 ± 0.005% vs 0.019 ± 0.004%; $P = 0.007$) and higher in participants with a serum 25(OH)D below 25 nmol/L than that in participants with a serum 25(OH)D above 75 nmol/L (0.031 ± 0.007% vs 0.017 ± 0.003%; $P < 0.001$) (Table 3).

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Table 3 The difference in the serum concentration of free 25(OH)D and the free fractions between participants with vitamin D deficiency vs vitamin D sufficiency.

	Vitamin D deficiency (total serum 25(OH)D <25 nmol/L)	**Vitamin D Sufficiency** (total serum 25(OH)D ≥75 nmol/L)	**Significance**
	N=25	*N*=152	
	Mean ± S.D.	Mean ± S.D.	***P*-value**
Directly measured free 25(OH)D in serum (pmol/L)	5.6 ± 0.82	16.4 ± 4.1	<0.001
Calculated free 25(OH)D in serum (pmol/L)	7.4 ± 3.1	28.3 ± 11.4	<0.001
The percentage of free 25(OH)D (%) (using directly measured free 25OHD)	0.031 ± 0.007	0.017 ± 0.003	<0.001
The percentage of free 25(OH)D (%) (using calculated free 25(OH)D)	0.039 ± 0.015	0.030 ± 0.010	0.005
Serum DBP (mg/L)	203.4 ± 70.6	272.0 ± 86.0	<0.001
Serum albumin (g/L)	41.9 ± 3.2	41.5 ± 4.3	0.646
Serum 1,25 $(OH)_2$D (pmol/L)	80.5 ± 25.5	129.2 ± 47.4	<0.001
Serum iPTH (ng/L)	62.0 ± 32.4	38.7 ± 17.0	0.002

1,25$(OH)_2$D, 1,25-dihydroxy vitamin D; 25(OH)D, 25-hydroxy vitamin D; DBP, vitamin D binding protein; iPTH, intact parathyroid hormone.

The percentage of free 25(OH)D was also positively associated with BMI (r=0.206, P<0.001).

When using calculated free 25(OH)D in the formula for the percentage of free 25(OH)D (calculated serum free 25(OH)D/serum total 25(OH)D) the free percentage was significantly larger (0.032 ± 0.013%; P≤0.001), as expected. The correlation with total serum 25(OH)D was also weaker (r=–0.134, P=0.002) and the value of the percentage of free 25(OH)D of calculated free 25(OH)D increased less in vitamin D deficiency (Fig. 3B and Table 3).

Directly measured free 25(OH)D in serum in relation to demographics and lifestyle-related parameters

Directly measured free 25(OH)D was equally distributed over sex, age and smoking status, but negatively correlated with BMI (r=–0.175, P<0.001). The higher levels of total 25(OH)D in serum among the women could be due to their higher use of sun beds, as explained previously (16).

In addition, directly measured free 25(OH)D was higher in sun bed users compared with that in non-users (13.7 ± 4.8 vs 11.4 ± 4.3 pmol/L; P=0.014) and in participants who had been on a sun holiday during the last month (15.9 ± 5.9 vs 11.2 ± 4.0; P<0.001).

Serum DBP in relation to demographics, vitamin D metabolites and other laboratory parameters

The associations between DBP and demographic variables, vitamin D metabolites and other laboratory parameters are shown in Table 4.

Serum DBP was higher in women than that in men. The 95% confidence interval (CI) was 102–517 mg/L for the women and 92–382 mg/L for the men. Premenopausal women had a tendency to have higher serum DBP than postmenopausal women, but the difference did not reach the level of significance. Among women under age 50 years, the users of oral contraceptives had significantly higher serum DBP than non-users. The users of oral contraceptives also had significantly higher serum total 25(OH)D (87.5 ± 39.3 vs 64.9 ± 22.7; P=0.017), but lower free percentage of 25(OH)D (0.017 ± 0.003% vs 0.019 ± 0.004%; P=0.015) compared with non-users, whereas the difference in directly measured free 25(OH)D in serum did not reach level of significance (14.3 ± 6.1 vs 11.8 ± 4.1; P=0.076), maybe due to the relatively small number of cases.

Further serum DBP was positively correlated with SHBG and hsCRP and weakly negatively correlated with BMI.

Serum DBP was also positively correlated with serum levels of total 25(OH)D and negatively correlated with calculated free 25(OH)D) and iPTH, whereas no correlation with directly measured free 25(OH)D was found.

Linear regression was run with serum DBP as outcome and the following parameters as covariates: sex, age, BMI, use of contraceptives and the serum levels of 25(OH)D, 1,25$(OH)_2$D, iPTH, SHBG, testosterone and estradiol. In this analysis, serum DBP remained independently and significantly associated with serum SHBG (B=0.472, S.E.=13.792, P<0.001), total serum 25(OH)D (B=0.395, S.E.=0.129, P=0.002) and iPTH (B=–0.370, S.E.=0.183, P=0.043).

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Table 4 The association between DBP and demographics, vitamin D metabolites and other laboratory parameters.

	Difference in serum DBP mean (S.D.) (mg/L)	**Correlation with serum DBP**	***P*-value**
Demographics			
Age		$r=-0.068$	0.115
Women vs men	265.4±87.8 vs 245.0±71.5		0.005
Women <age of 50 years vs women ≥age 50 years	269.3±93.0 vs 250.5±62.6		0.116
Users of contraceptives vs non-users among women ≤50 years	328.5±122.9 vs 261.0±85.3		0.023
BMI		$r=-0.107$	0.013
Smokers vs non-smokers	261.1±59.4 vs 252.7±79.7		0.613
Vitamin D metabolites			
Total serum 25(OH)D		$r=0.192$	<0.001
Directly measured free 25(OH)D		$r=0.049$	0.225
Calculated free 25(OH)D		$r=-0.452$	<0.001
$1,25(OH)_2D$		$r=0.144$	0.001
iPTH		$r=-0.149$	<0.001
Other laboratory parameters			
Testosterone (men only)		$r=-0.062$	0.265
Estradiol (women only)		$r=-0.046$	0.532
SHBG		$r=0.268$	<0.001
hsCRP		$r_S=0.102$	0.017
ALT		$r=-0.035$	0.421
Creatinine		$r=-0.043$	0.314
Albumin		$r=-0.013$	0.762

$1,25(OH)_2D$, 1,25-dihydroxy vitamin D; 25(OH)D, 25-hydroxy vitamin D; ALT, alanine aminotransferase; BMI, body mass index; DBP, vitamin D binding protein; HRT, hormone replacement therapy; hsCRP, highly sensitive c-reactive protein; iPTH, intact parathyroid hormone; *r*, Pearson correlation coefficient; r_S, Spearman correlation coefficient; SHBG, sexual hormone binding globulin.

Discussion

We measured free 25(OH)D and DBP in serum in 540 healthy blood donors during 12 months to determine if the variation in total serum 25(OH)D with season is followed by a concomitant variation in free 25(OH)D. We found that directly measured free serum 25(OH)D was highly correlated with total serum 25(OH)D and followed the same seasonal variation, whereas the serum concentrations of DBP and albumin were stable. The fluctuation in free 25(OH)D was only marginally damped with an increase in the percentage of free 25(OH)D in participants with vitamin D deficiency and during the winter months, reflecting DBP ability to act as a reservoir for 25(OH)D. Our findings of stable concentrations of serum DBP during the year are supported by one earlier study from 1981 (17).

The free hormone hypothesis states that protein-bound hormones are inactive and that only the free unbound fraction of a hormone can enter the cells and exert its biological effects (18, 19). In the case of vitamin D, the pro-hormone 25(OH)D must enter the cells to be α-hydroxylated to $1,25(OH)_2D$. In the kidney, the intracellular uptake of 25(OH)D mainly occurs by megalin-mediated endocytosis of 25(OH)D bound to DBP and the uptake of 25(OH)D is thus facilitated by DBP (20). Megalin expression has also been found in other tissues such as the mammary glands, parathyroid gland, epididymis, lung and the placenta (21). Most other tissues are however dependent on the direct diffusion of the free unbound vitamin D forms through the cell wall and data suggest that DBP may actually have an inhibitory effect on 25(OH)D uptake here (7, 22). The seasonal variation in the concentration of free 25(OH)D shown in this study may thus have importance especially for extra-renal tissues, such as the immune cells.

When comparing directly measured free 25(OH)D with calculated free 25(OH)D, we found that the directly measured concentration was significantly lower than the calculated, a finding supported by earlier studies (15). Directly measured free 25(OH)D also showed a stronger correlation with serum iPTH, indicating that directly measured free 25(OH)D more adequately reflects the biologic activity of vitamin D compared with the calculated free 25(OH)D. The same findings and conclusion were reported by Schwartz and coworkers in an earlier study (10). In addition, we found that directly measured 25(OH)D better reflected DBP's ability to moderate the variation in free 25(OH)D by increasing the percentage of free 25(OH)D in situations of vitamin D deficiency

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and decreasing it in times of abundance. Based on these results, we consider the use of directly measured free 25(OH)D to be of greater value than the calculated free 25(OH)D. The discrepancies found between calculated and directly measured free 25(OH)D may be explained by that the affinity of 25(OH)D for DBP and albumin is not constant but varies depending on access to 25(OH)D and between individuals.

In the present study, the participants were Caucasians, ethnically homogenous and without any conditions known to affect the levels of DBP. Thus, the variation in the serum concentration of DBP was small.

Directly measured free 25(OH)D was strongly correlated with total 25(OH)D, and the negative association with serum iPTH was comparable between the directly measured and total 25(OH)D. Similar to our previous report on total 25(OH)D, directly measured 25(OH)D was positively associated with parameters reflecting the UV-B exposure, such as the UV-B irradiance during the sampling month, sun holidays and sun bed use (16). Based on this, we found no strong advantages in measuring free 25(OH)D instead of total 25(OH)D to evaluate the vitamin D status clinically.

The current study confirms the results of earlier studies with higher serum concentrations of DBP in women and in users of oral contraceptives and hormone replacement therapy, but we found no association between DBP and the levels of sex hormones in serum (23). Instead, we found that serum DBP was independently and positively associated with serum SHBG after adjusting for sex, age, BMI, estrogen use and levels of sex hormones. Both DBP and SHBG are proteins known to be induced by estrogens (24). Our findings, however, suggest a common regulatory pathway for DBP and SHBG, beyond the sex hormones.

Several earlier studies have reported a negative association between total serum 25(OH)D and BMI (25). We found that increasing BMI was associated with lower levels of total 25(OH)D, free 25(OH)D and DBP, but on the other hand, with an increased percentage of free 25(OH)D. The majority of the individuals in this cohort were however of normal weight (60.7%) and only a minority were obese (6.3%).

The present study has some limitations. Blood donors are a selected population of healthy individuals, and the results of the study may therefore not be representative of the general Swedish population. Gothenburg is situated at northern latitude, where the UV-B irradiance is too low during the winter months to allow the photolysis of vitamin D. As a result, the study may not be representative for populations living at southern latitudes. The measuring of free hormone concentrations has difficulties. The percentage of total 25(OH)D, which exists in free form is very low, and the assay needs to be highly sensitive and specific to avoid cross-reactivity. Furthermore, the relationship between the free fraction and the protein-bound fraction may not be the same in *in vitro* as *in vivo*. The method used to measure free 25(OH)D in the current study needs further evaluation. Total 25(OH)D was measured with CLIA and not with the gold standard method liquid chromatography–mass spectrometry (LC–MS/MS). Serum DBP was assessed with ELISA using a monoclonal antibody specific for DBP. Recent studies have found that this method has problems detecting the DBP variant GC1f, which is common in populations of African descent. In the present study, no assessment of DBP genotype was done. The study population was however of European ancestry, where the GC1f genotype is rare, so the use of a monoclonal ELISA should not have had any great impact on our results. Another study limitation was that serum estrogen in the premenopausal women was not measured on a standardized day of the menstrual cycle.

Conclusions

Directly measured free 25(OH)D in serum was strongly correlated with total 25(OH)D and followed the same seasonal variation, whereas the serum concentrations of DBP and albumin were stable during the year. The variation in directly measured free 25(OH)D was however damped with an increase in the percentage of free 25(OH)D in situations of vitamin D deficiency and a decrease in vitamin D abundance. Both directly measured and total 25(OH)D were negatively correlated with serum iPTH, but calculated free 25(OH)D was only weakly associated with iPTH. Serum DBP was higher in women and estrogen users and independently associated with SHBG.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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