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Original Article

Vitamin D deficiency and seasonal and inter-day variation in circulating 25-hydroxyvitamin D and parathyroid hormone levels in indoor daytime workers: a longitudinal study

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Running title: Fluctuation of vitamin D deficiency in indoor daytime workers

Abstract

Seasonal variation in circulating 25-hydroxyvitamin D (25OHD) levels related to seasonal and inter-day fluctuation in sunlight ultraviolet irradiation, may lead to misjudgments concerning 25OHD status in individual workers around threshold levels. Here, to examine seasonal and inter-day variations in plasma 25OHD, we conducted a longitudinal study involving indoor daytime workers. Subjects were four male indoor daytime workers aged 32-57 years working in Kawasaki City, Japan. Blood samples were obtained on six days within two two-week periods in February and October, 2008. Plasma 25OHD, serum intact parathyroid hormone (PTH) and 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D] were measured. Individual monthly mean 25OHD levels were 16%-56% higher in October than in February (p=0.03), while individual monthly mean intact PTH levels were 15%-41% lower in October (p=0.09). No seasonal change was observed in 1α,25$(OH)_2$D (p=0.62). Notably, nearly all measured 25OHD levels in February were lower than the reference value of 20 ng/mL. Our study identified the occurrence of seasonal variation in circulating 25OHD and intact PTH levels, even after accounting for inter-day variability, and hypovitaminosis D in wintertime in indoor daytime male workers in Japan. Due to this variability, single spot measurements of 25OHD may lead to misjudgment of workers' vitamin D status.

Keywords: vitamin D; measurement error; misclassification; intra-individual variability; working schedule; night work.

Introduction

Vitamin D deficiency resulting from insufficient exposure to sunlight, which causes rickets, osteomalacia, and other hypovitaminosis D-related disorders, is a common problem worldwide [1, 2]. Individuals who work indoors or at night may be insufficiently exposed to sunlight irradiation, thereby developing vitamin D deficiency [3, 4]. This problem may have been exacerbated by the development of a 24-hour society, in which work hours are spread across both day and night. Scientific and public concerns have recently focused on the higher risk of various health disorders related to nightshift work, including breast cancer, cardiovascular disease, and osteoporotic fractures [5-9]. For example, in a prospective study of nurses conducted in the U.S., nightshift work for 20 years or more was associated with an increased risk of hip and wrist fractures [8]. Although hypovitaminosis D resulting from night work is a suspected cause of these disorders, vitamin D status of night workers based on the measurement of blood metabolite levels has not been investigated, with the exception of one report concerning a single patient [4].

25-Hydroxyvitamin D (25OHD), a major component of circulating vitamin D metabolites whose biological half-life in blood (2-5 wk in normal subjects) [10-14] is substantially longer than that of vitamin D itself (24 h) [10], is commonly used as an indicator of individuals' vitamin D status [15]. Exposure to solar ultraviolet (UV) B irradiation changes 7-dehydrocholesterol to previtamin D by photosynthesis in the skin. Previtamin D is completely converted to vitamin D through thermal isomerization at body temperature within a 2-3 day period [16]. Once formed, vitamin D is transported in the blood bound to vitamin D-binding protein, converted into 25OHD

in the liver, and is then finally activated to 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D] in the kidney. Circulating 25OHD levels are regarded as the best indicator of vitamin D stores on account of its longer half-life and less restrictive regulation than 1α,25$(OH)_2$D, whose concentration is strictly regulated by the stimulatory effects of intact parathyroid hormone (PTH), calcium, and phosphorous, as well as by its relatively short biological half-life in blood (4-6 h) [10]. Vitamin D deficiency is defined by most experts as a plasma or serum 25OHD level of less than 20 ng/mL (50 nmol/L) [1, 17, 18]. Nakamura et al.[18] also concluded that a circulating 25OHD concentration of at least 20 ng/mL is required to achieve normal PTH levels and prevent low bone-mineral density, even in Japanese.

Despite the advantages of monitoring 25OHD over other vitamin D metabolites, circulating 25OHD levels vary seasonally due to seasonal fluctuations in sunlight UVB irradiation [3, 19]. Annual peak and nadir 25OHD levels occur in autumn and late-winter or spring, respectively, consistent with clinical observations indicating that rickets is common in spring, but rare in autumn [19]. In addition to seasonal variation of 25OHD levels, the magnitude of its intra-individual, inter-day variation might also be substantive. Such time-dependent variation in 25OHD may confound the diagnosis of workers vitamin D status; moreover, subsequent misclassification might attenuate or bias associations between circulating 25OHD levels and the risk of hypovitaminosis D-related diseases observed in epidemiologic studies. All previous studies assessing seasonal 25OHD variation or disease risk, however, have only documented seasonal differences in 25OHD by single-spot measurements in each examined month, and no data are available concerning the magnitude of inter-day variability in 25OHD or the

significance of seasonal differences vis-à-vis inter-day variation. Thus, it is unclear whether single-spot measurement of 25OHD is an appropriate means of accurately evaluating worker vitamin D status.

Here, to examine seasonal variation in plasma 25OHD levels considering within-subject inter-day variability, we conducted a longitudinal follow-up study with repeated measurements of plasma 25OHD concentrations in indoor daytime workers in Japan.

Methods

Subjects

Subjects were apparently healthy indoor daytime workers who commuted to Kawasaki City (35.6^{o}N, 139.6^{o}E), which is located adjacent to Tokyo, Japan, and were without exposure to specific hazardous factors such as chemicals, radiation, heat, noise, vibration and night work. A total of 12 non-fasting venous blood samples were obtained from each participant, typically between 10-11 a.m., on 6 different days within two 2-wk periods in February and October 2008. The rationale for this sampling timing was that the annual peak 25OHD level was reported to occurin October in indoor workers (and in November in outdoor workers), whereas the annual peak in solar ultraviolet irradiation was reported to occur in July, in at least one study [3]. This relatively long lag between peak exposure and 25OHD increase might be attributable to the properties of previtamin D or vitamin D, which are stored in skin and fat cells and are gradually released, in addition to the long half-life of circulating

25OHD in blood. Blood sampling was conducted such that it was balanced over a week, excluding weekends. Four male workers who completed all twelve visits for blood withdrawal were included in the analyses. According to an experimental field study in northern Norway, diet including fish liver did not increase plasma 25OHD level after meals[20]. Twelve spot urine specimens were also obtained from each study participant. Demographic and health information data were collected using self-administered questionnaires, including the Profile of Mood States (POMS, Japanese version) [21], a 65-item adjective scale, and hourly records of time spent outdoors. POMS presented participants with a series of mood adjectives; participants were asked to rate the degree of their mood state over the preceding seven days. All POMS tests were performed in the same quiet room. Each participant provided signed informed consent to participate, and the protocol was approved by the Institutional Review Board of the National Institute of Occupational Safety and Health, Japan.

Measurements

After blood withdrawal, plasma and serum were immediately separated by centrifugation and stored at -80 ^{o}C until analysis. Plasma total 25OHD and serum intact PTH were measured using a radioimmunoassay (25-hydroxyvitamin D ^{125}I RIA Kit, DiaSorin, Stillwater, MN, USA) [22] and an electrochemiluminescence immunoassay (ECLusys reagent PTH, Cobas system, Roche Diagnostics, Tokyo, Japan), respectively, at a commercial clinical laboratory (SRL, Tokyo, Japan). All samples were measured on the same day in one batch. Serum total 1α,25$(OH)_2$D was analyzed using a radioimmunoassay (1,25$(OH)_2$D RIA Kit, Immunodiagnostic Systems Ltd., Boldon, England) at another commercial laboratory (Mitsubishi Chemical Medience Corporation,

Tokyo, Japan), which also measured serum and urinary phosphorous and calcium content. Local laboratory reference ranges for 25OHD and 1α,25$(OH)_2$D by the respective assay manufacturers were 7-41 ng/mL and 20-60 pg/mL, respectively. For quality assurance, pooled plasma and serum samples from four volunteers were divided into aliquots, and were simultaneously analyzed in parallel for each analysis (n=5, for each batch) by laboratory analysts who were blinded to the control sample status. According to the control sample analysis, analytic CVs were 21.9% for 25OHD and 2.4% for intact PTH. Although serum 1α,25$(OH)_2$D was measured in two different batches (CV = 18.9% and 20.8%), no significant differences between the two mean levels were detected (p=0.54). The coefficients of intra- and inter-day variation in routine quality control data for 25OHD reported from the local laboratory ranged between 4.3-7.0% (at mean levels of 8.50, 14.10, and 50.92 ng/mL).

Statistical analyses

Subject characteristics were compared by season using the paired Welch's t test. Seasonal differences in 25OHD, intact PTH, and 1α,25$(OH)_2$D levels were analyzed in consideration of each inter-day variation as follows: the levels in each month were compared using the exact Wilcoxon rank sum test on an intra-individual basis, and subject averages (arithmetic mean) of 25OHD, intact PTH and 1α,25$(OH)_2$D in each season were then compared using the paired Welch's t test. All statistical analyses and plots of data were performed using the statistical analysis software R, version 2.9.0 [23)] and the spreadsheet software Excel, version 2003 (Microsoft Corporation, Redmond, WA, USA). All p-values were two-sided, and differences were considered significant at values of $p<0.05$. Statistical tests were partly cross-checked by the statistical analysis system SAS, version 9.1.3 (SAS

Institute Inc., Cary, NC, USA). We selected 20 ng/mL to serve as the reference value of circulating 25OHD, since the normal range of circulating 25OHD has been reported by most laboratories to be 20 to 100 ng/mL[1)]. In addition, a circulating 25OHD concentration of 20 ng/mL will not only prevent rickets and osteomalacia, but also dramatically suppresses PTH levels[18, 24)].

Results

Study participants were males, aged 32-57 years in February, who were not current smokers. Subject characteristics in February and October 2008 are summarized in Table 1. Anthropometric and psychological data showed no significant seasonal changes, except in Anger-Hostility. In addition, no differences in serum and urinary levels of calcium and phosphorous between February and October were detected.

Plasma 25OHD and serum intact PTH and $1\alpha,25(OH)_2D$ were detected in all 12 samples from each of the 4 subjects. Figure 1 shows data for 25OHD in each subject (A-D) by month. Nearly all 25OHD levels in February were below the reference value (20 ng/mL). Surprisingly, even in October, a few of the measured 25OHD values were below 20 ng/mL. We consistently found a 16%-56% relative increase in individual monthly mean 25OHD levels in October compared to February ($p=0.03$). Significant seasonal differences in 25OHD were found in Subjects A, C, and D ($p<0.02$) in addition to a marginal difference in Subject B ($p=0.052$). For intact PTH, the opposite trend was observed, as we consistently detected a 15%-41% relative decrease in individual monthly mean intact PTH levels in October compared to February ($p=0.09$) (Fig. 2). Significant or marginal seasonal

differences in intact PTH were found individually in Subjects B, C, and D ($p=0.006$, 0.02, and 0.054, respectively), but not in Subject A ($p=0.40$). In contrast, no consistent seasonal or individual changes were observed in $1\alpha,25(OH)_2D$ levels ($p=0.62$, paired Welch's t test; and $p>0.10$, exact Wilcoxon rank sum test, respectively), as shown in Fig. 3. Notably, within-subject inter-day CVs for 25OHD in each season were relatively small (4-10%) compared to those for intact PTH (10-26%) and $1\alpha,25(OH)_2D$ (12-32%).

Discussion

To our knowledge, this is the first study to report both inter-day variation in plasma 25OHD levels and seasonal variation in 25OHD considering inter-day variation on an intra-individual basis. Our analysis revealed a large amplitude seasonal difference in 25OHD between the months of February and October 2008 in indoor daytime workers, even after accounting for inter-day variation. In contrast to 25OHD, intact PTH circulating levels were high in February and decreased in October, while no consistent pattern of seasonal variation in $1\alpha,25(OH)_2D$ was observed. Insufficient levels of 25OHD were found mainly in wintertime in indoor daytime male workers.

The opposite patterns of seasonal variation in 25OHD and intact PTH observed in the present study, are consistent with a number of previous studies [3, 19, 25, 26] even though the size of our sample was small. Another longitudinal study also showed that 25OHD was higher in fall than in winter[27]. The relatively small inter-day variation in 25OHD may be predominantly attributable to its longer biological half-life in blood, in addition to the gradual release of vitamin D from skin and fat cells; whereas variation in intact PTH was larger due to its

shorter half-life. In contrast, the lack of a clear seasonal variation in 1α,25$(OH)_2$D is consistent with several previous studies[28-31], but contradicts a few studies which did report seasonal variation[19, 32]. In our subjects, seasonal variation may have been attenuated by inter-day variation in 1α,25$(OH)_2$D, as it is well known that 1α,25$(OH)_2$D synthesis is strictly regulated by PTH, calcium, and phosphorous. Whether variation in 1α,25$(OH)_2$D exists might also depend on the sufficiency of circulating 25OHD. Further, the observed 1α,25$(OH)_2$D variations may have been partly attributable to assay variation. Among the POMS scores, only Anger-Hostility was higher in February than in October; associations between vitamin D level and some mental health states have been reported[33, 34].

Several factors related to the seasonal and inter-day variations in 25OHD, intact PTH, and 1α,25$(OH)_2$D levels identified here can confound research results in a range of fields and settings. First, judgment of vitamin D deficiency on the basis of circulating 25OHD levels and a reference value (e.g., 20 ng/mL) will depend on the day and season of blood withdrawal. Even in the same month, individual 25OHD levels fluctuated above and below the reference value, even though the inter-day variations were relatively small. Accordingly, since 25OHD values were close to the reference value throughout the year, single-spot measurements of 25OHD may lead to misjudgment of workers' vitamin D status. The season of blood withdrawal and inter-day variability in 25OHD should be considered when determining whether a worker is at risk of vitamin D deficiency at any time during the year. Second, epidemiologic studies that measure circulating 25OHD should account for seasonal variation in their design. If one-sided measurement errors occur (e.g., blood samples from subjects were collected in autumn

and those from controls in late-winter, or vice versa), the resulting differential misclassification will likely fail to identify actual associations. Etiological evaluation would be more appropriate if blood is collected from all subjects in the same month (e.g., when testing differences in 25OHD levels between worker groups, such as daytime and nighttime workers). Alternatively, case subjects might be individually matched with controls by the month of blood withdrawal. At a minimum, the presence of seasonal fluctuation in the biomarker data should be monitored.

Individual daily UVB exposure records of our indoor daytime workers, which were calculated using self-reported times spent outdoors and hourly values of UVB index [W/m^2] (data not shown), show that individual exposure to sunlight UVB irradiation was scarce on weekdays and frequent on weekends. Going outside during the daytime on weekends and in the mornings and evenings on weekdays would therefore improve the vitamin D status of indoor workers. In this regard, a previous longitudinal study reported that annual 25OHD levels were lower in indoor than in outdoor workers [3].

Our study has several strengths. First, it was a longitudinal design with an adequate number of repeated measures per subject in each season (n=6), which enabled within-subject statistical testing between seasons and a reliable estimation of subject average in each examined month. To our knowledge, no previous observational study has repeatedly measured 25OHD in such a short time. Second, our longitudinal design with seasonal comparisons on an intra-individual basis obviated confounding by covariates such as anthropometric factors and unmeasured

confounders. Few studies have tested seasonal variation in 25OHD longitudinally on an intra-individual basis; most previous studies have only examined such variation cross-sectionally. Third, all analyses of 25OHD were conducted on the same day to exclude confounding caused by between-day assay variation[35, 36]. A few studies have demonstrated that the radioimmunoassay for 25OHD is more reliable than competitive protein-binding assays [26, 37], which is an important consideration as assay variation and standardization of 25OHD measurements are an important issue in this field of research[31, 35, 37, 38]. In addition to the possibility of clinical misinterpretation[39-41], non-differential measurement error and subsequent misclassification would lead to underestimation of relative risks. Quantitative measurement of such error and intra-individual inter-day variation would therefore be useful for estimating the true effects of risk factors such as 25OHD [42]. Although the measurement of 25OHD using high performance isotope-dilution liquid chromatography tandem mass spectrometry is considered to be the most reliable approach[38], the high cost of this equipment and the reagents limit their use.

Several limitations of this study also warrant mention. First, only two months were examined, October and February, and they may not have corresponded to the respective peaks and nadirs of the circannual fluctuation of each evaluated substance. If so, the resulting small differences in each substance may have caused the results of some statistical tests to be null. Second, the study consisted of a relatively small number of subjects, and the resulting lack of statistical power raises the possibility that the results were due to chance. Finally, although our results might not be generalizable to other worker groups, such as outdoor, nighttime, and female workers, the

peak 25OHD season in the present study was similar to that reported in the only previous study which followed both outdoor and indoor workers [3]. Third, analytical imprecision of 25OHD and subsequent non-differential measurement error might have attenuated seasonal variation to some extent.

In conclusion, our longitudinal study quantitatively identified the presence of seasonal variation in circulating 25OHD and intact PTH levels, even vis-à-vis their inter-day variability, and confirmed hypovitaminosis D in wintertime in indoor daytime male workers in Japan. The results presented here suggest that the season of blood withdrawal and inter-day variability in 25OHD should be considered when determining whether a worker is at risk of hypovitaminosis D at any time during the year. This proposition needs to be tested in studies with larger sample sizes. Further expansion and replication in other studies with sufficient statistical power are needed to confirm our results.

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Figure legends

Fig. 1 Seasonal and within-subject inter-day variation in serum 25-hydroxyvitamin D (25OHD) concentrations of four male indoor daytime workers (A-D) in Japan.

Fig. 2 Seasonal and within-subject inter-day variation in serum intact parathyroid hormone (PTH) concentrations of four male indoor daytime workers (A-D) in Japan.

Fig. 3 Seasonal and within-subject inter-day variation in serum 1α,25-dihydroxyvitamin D [$1\alpha,25(OH)_2D$] concentrations of four male indoor daytime workers (A-D) in Japan.

Tables

Table 1 Basic characteristics of subjects in February and October, 2008.

Variable	February mean (SD)	October mean (SD)	p-value*
Age [years]	43.0 (11.6)	43.5 (11.1)	0.18
Body mass index [kg/m^2]	22.8 (4.5)	22.6 (4.6)	0.29
Average sleeping time [hours]†,‡	5.55 (0.70)	5.83 (0.93)	0.35
Plasma 25OHD [ng/mL]†,§	17.7 (2.75)	22.1 (1.72)	0.03
Serum intact PTH [pg/mL]†,§	45.1 (14.0)	33.0 (4.38)	0.09
Serum 1α,25$(OH)_2$D [pg/mL]†,§	57.0 (6.09)	54.8 (4.66)	0.62
Serum calcium [mg/dL]†,§	9.80 (0.20)	9.76 (0.19)	0.12
Serum phosphorous [mg/dL]†,§	3.38 (0.45)	3.28 (0.26)	0.63
Urinary calcium [g/g creatinine]†,§	0.099 (0.025)	0.102 (0.012)	0.90
Urinary phosphorous [g/g creatinine]†,§	0.41 (0.16)	0.38 (0.073)	0.64
POMS standardized score			
Tension-Anxiety	48.4 (2.4)	55.5 (9.9)	0.45
Depression-Dejection	50.1 (4.5)	47.7 (3.3)	0.21
Anger-Hostility	47.1 (3.4)	44.1 (5.2)	0.04

Vigor	41.8 (9.1)	49.1 (5.7)	0.14
Fatigue	55.0 (3.1)	60.4 (6.7)	0.16
Confusion	50.5 (4.8)	57.8 (7.2)	0.76
Daily outdoor hours [minutes][§]	87.6 (51.4)	122.5 (67.1)	0.29
Mean daily outdoor temperature [^{o}C][¶]	5.4	13.0	-

*Paired Welch test (two-sided).

†n=6 for each subject in each month. Statistical tests were performed using subject averages.

‡Individual sleeping times were calculated from self-reported bedtime and hour of rising.

§Overall mean and SD of subject averages.

¶Observed in Yokohama City, Kanagawa Prefecture[43)].

Fig. 1

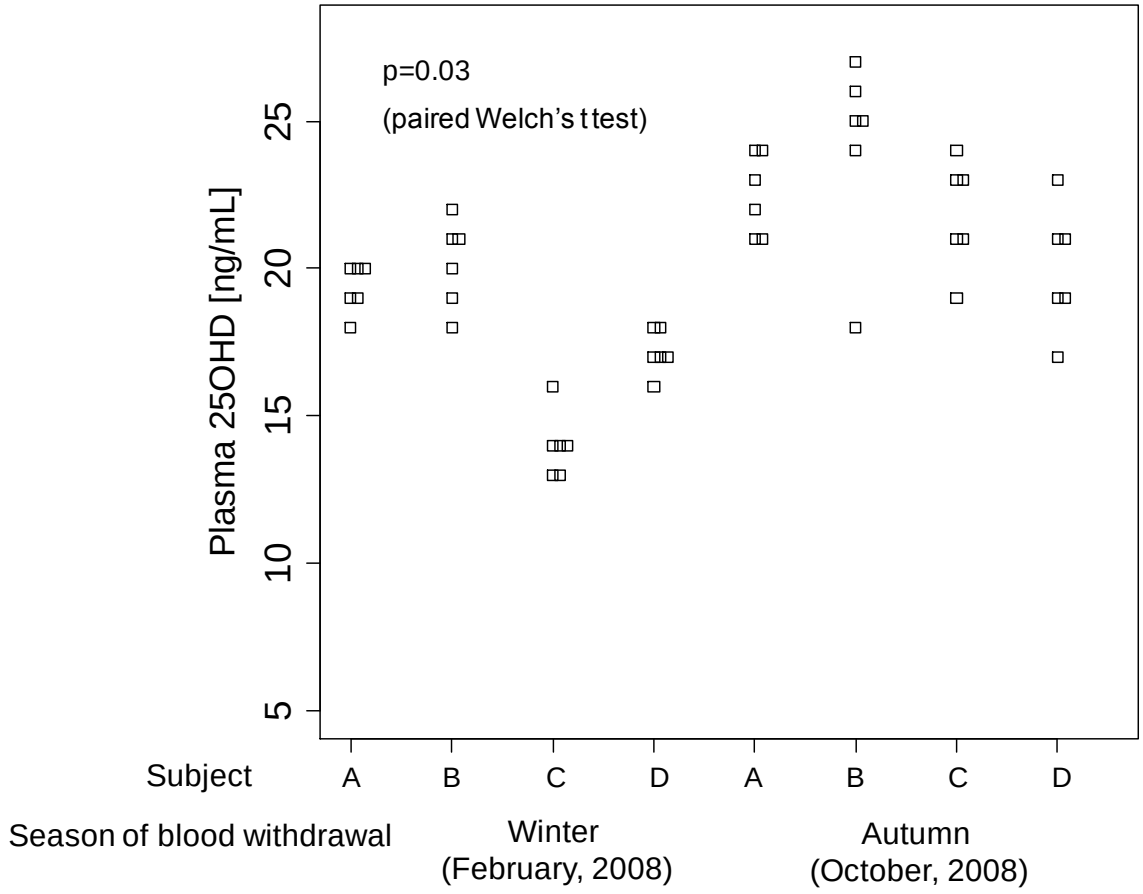

p=0.03
(paired Welch's t test)
Plasma 25OHD [ng/mL]
5
10
15
20
25
Subject
A
B
C
D
A
B
C
D
Season of blood withdrawal
Winter
(February, 2008)
Autumn
(October, 2008)

Fig. 2

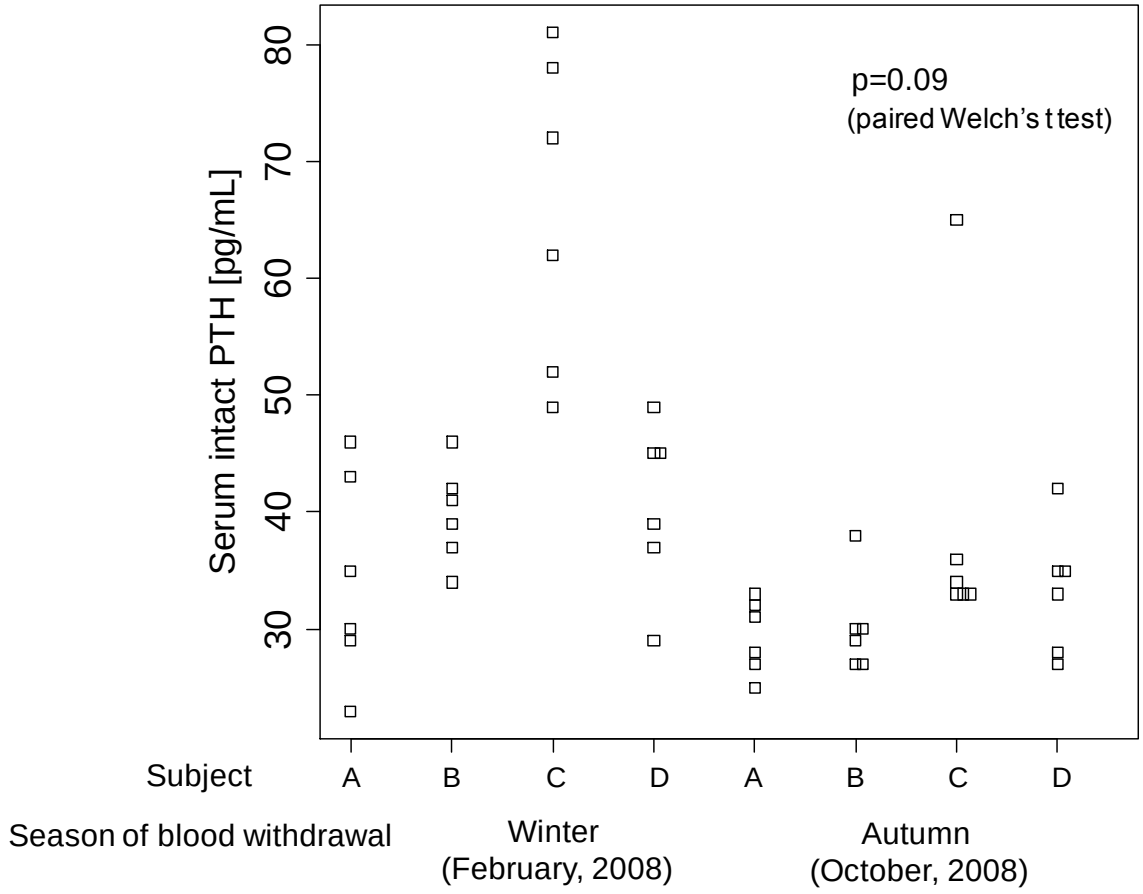
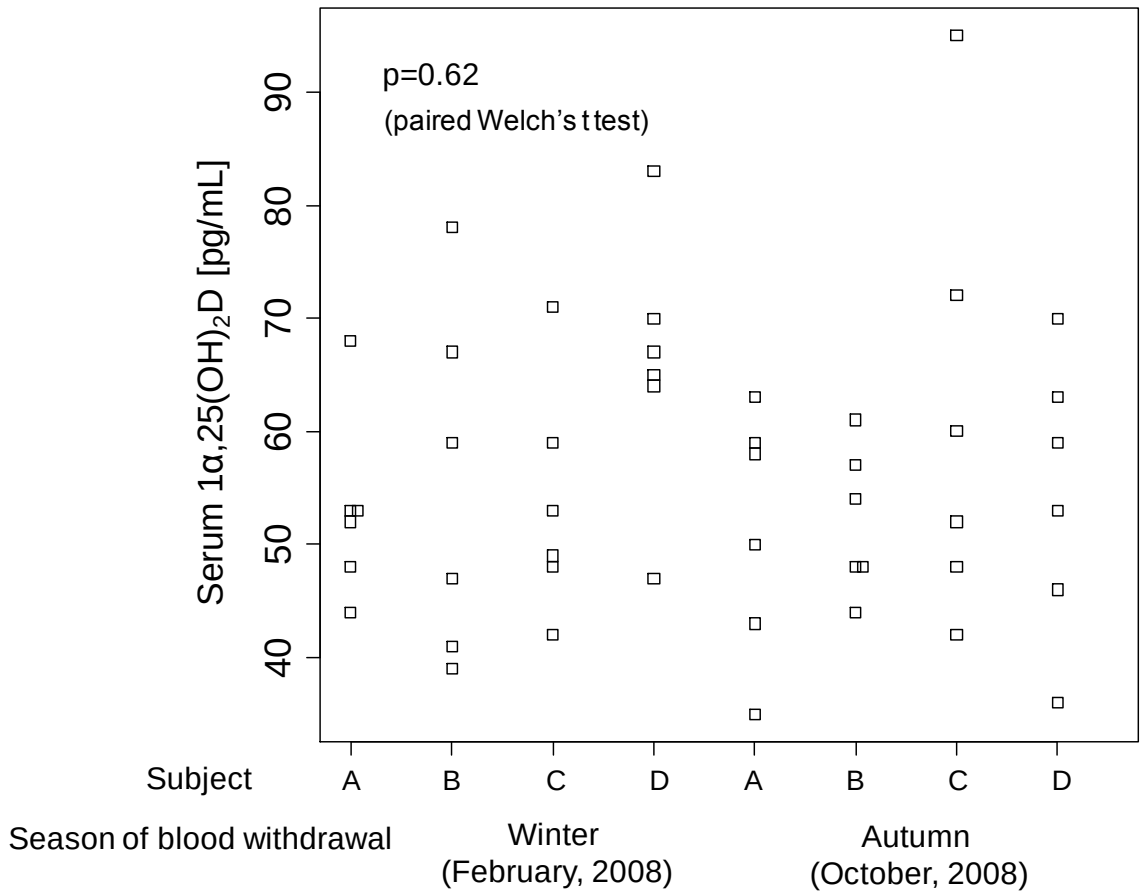

Serum intact PTH [pg/mL]
30
40
50
60
70
80
p=0.09
(paired Welch's t test)
Subject
A
B
C
D
A
B
C
D
Season of blood withdrawal
Winter
(February, 2008)
Autumn
(October, 2008)

Fig. 3


p=0.62
(paired Welch's t test)
Serum 1α,25(OH)2D [pg/mL]
40
50
60
70
80
90
Subject
A
B
C
D
A
B
C
D
Season of blood withdrawal
Winter
(February, 2008)
Autumn
(October, 2008)