Serum 25-hydroxyvitamin D is associated with fracture risk only during periods of seasonally high levels in women with a high body mass index

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ABSTRACT
Serum 25-hydroxyvitamin D (S-25OHD) is used to assess vitamin D status and is known to be affected by season and fat mass. Because these factors are often ignored when interpreting S-25OHD, assessment of vitamin D associations with disease outcomes may be distorted. We aimed to investigate the impact of season of blood draw and fat mass on the association of S-25OHD with fracture risk. We enrolled 5000 women, mean ± SD age 68 ± 7 years, with dual-energy x-ray absorptiometry (DXA) scans and blood collection in a population-based cohort. Proportional hazards regression, stratified by season and fat mass, was used to determine hazard ratios (HRs) of fracture according to categories of S-25OHD. Our secondary exposures were serum 1,25-dihydroxycholecalciferol (1,25-(OH)2 D3), the most active vitamin D metabolite and plasma parathyroid hormone (P-PTH). During an average of 9.2 years of follow-up, 1080 women had a fracture. Women with S-25OHD <30 nmol/L drawn during sunny months (May–October) had a multivariable-adjusted fracture HR of 2.06 (95% CI, 1.27–3.35) compared with those with S-25OHD >60 nmol/L; those with S-25OHD 30–40 nmol/L had an HR of 1.59 (95% CI, 1.12–2.26). In contrast, S-25OHD drawn during November through April was unrelated to fracture risk. The increased risk with low sunny season S-25OHD was seen only among women with body mass index (BMI) ≥ 25 kg/m² or fat mass index (FMI) ≥ 9.8 kg/m². High fat mass and low S-25OHD were independently related to lower S-1,-25-dihydroxycholecalciferol, which itself predicted fracture risk with samples collected during the sunny season. Irrespective of season, P-PTH was unrelated to fracture risk. We conclude that S-25OHD is associated with fracture risk only if drawn during periods of seasonally high levels in women with a high BMI. These results have implications for the evaluation of vitamin D status and can explain the lack of effect seen with vitamin D supplementation in many fracture trials. © 2021 The Authors. Journal of Bone and Mineral Research published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research (ASBMR).

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Introduction
Although vitamin D supplementation to treat rickets and osteomalacia is clearly effective,1 benefits among individuals with less apparent vitamin D deficiency, assessed by serum 25-hydroxyvitamin D (S-25OHD), are controversial. Trials for the prevention of osteoporosis and fractures have generally shown null effects2 and Mendelian randomization analyses have not found associations of S-25OHD with bone mineral density (BMD)3 or fracture risk.4 Vitamin D supplementation trials focusing on muscle function and risks of falls have been conflicting.5 In essence, the population that would truly benefit from vitamin D supplementation for fracture prevention is unknown.
A major source of vitamin D is synthesis in the skin from sunlight exposure, but at latitudes >40 degrees, UV-B radiation is insufficient during dark seasons. Mean decreases in S-25OHD concentrations from sunny season zenith to nadir in late winter are as high as 50%, a seasonality seen both at high latitudes and in temperate climates. There is evidence that interpretation of S-25OHD varies depending on season, because BMD is associated with levels drawn during sunny times of the year but not those drawn during dark periods. Adiposity may play an important role in this issue, because large amounts of vitamin D can be stored in fat for later release, and high fat mass leads to lower levels of both S-25OHD and serum calcitriol (serum 1,25-dihydroxyvitamin D [S-1,25-(OH)2D]), the most active vitamin D metabolite.

To clarify the implications of season of blood draw and adiposity on the interpretation of S-25OHD levels, we investigated the association of S-25OHD with fracture risk by season of blood draw and fat mass in a population-based cohort of women. In accordance with our cross-sectional BMD results, we hypothesized a stronger association between S-25OHD and fractures risk when the blood samples had been drawn during the sunny season. Because vitamin D deficiency not only leads to low S-1,25-(OH)2D but also increases circulating parathyroid hormone (PTH), regulating serum calcium through the release of bone calcium, we also examined the relationships of fracture risk with S-1,25-(OH)2D and PTH by season.

**Subjects and methods**

We used a previously described clinical subcohort of the population-based Swedish Mammography Cohort (SMC; https://www.simpler4health.se/?languageId=1), which included participants from central Sweden. Between November 2003 and October 2009, the 5022 women who were randomly chosen for the subcohort underwent a clinical examination that included dual-energy x-ray absorptiometry (DXA) measurements, blood and urine samples, fat biopsies, and anthropometric assessment. Previously, participants had responded to SMC lifestyle, food frequency and health questionnaires in 1987–1990, and 1 month before the clinical examination. The timeline for the assessments used in the analysis of the subcohort is depicted in Figure 1. The study has ethical approvals from Regional Ethical Review Boards; each participant provided written informed consent. When compared with national data, the full cohort well represents the Swedish population in terms of age distribution, educational level, prevalence of overweight and obesity, and smoking status and the subcohort has similar distributions of these characteristics with the exception of an on average higher educational level.

**Body composition**

BMD at the total hip, femoral neck, and lumbar spine (L1–L4), as well as total fat and lean body mass were measured using DXA (Lunar Prodigy; Lunar Corp., Madison, WI, USA; coefficients of variation (CVs) were 1%). Body mass index (BMI) was calculated as weight/height2 (kg/m2) and fat mass index (FMI) as (total DXA fat mass)/height2, with a high correlation between these indices (r = 0.95).

**Laboratory measurements**

Serum 25-hydroxyvitamin D3 (S-25(OH)D3) and serum 25-hydroxyvitamin D2 (S-25(OH)D2) (n = 5000) were assayed in a single batch by high-performance liquid chromatography–atmospheric-pressure chemical ionization–mass spectrometry (Vitas AS, Oslo, Norway; https://www.vitas.no/). Total S-25OHD was the sum of S-25OHD3 and S-25OHD2. We determined S-1,25-(OH)2D for 980 of the women using IDS-iSYS 1,25-VitD-Xp (Immunodiagnostic Systems, Gaithersburg, MD, USA). Women with S-25OHD <40 nmol/L were deliberately oversampled: we analyzed all with S-25OHD <30 nmol/L, a random half of those with S-25OHD 30–40 nmol/L, and a random sample of those with higher levels. To determine free S-25OHD3, we used the Free 25OH Vitamin D enzyme-linked immunosorbent assay (ELISA) (DIAsource, Ottignies-Louvain-la-Neuve, Belgium) as per manufacturer’s instructions. Plasma parathyroid hormone (P-PTH), fibroblast growth factor 23 (FGF23), calcium, creatinine, cystatin C, albumin, alanine aminotransferase (ALT), beta CrossLaps, and osteocalcin were analyzed using routine methods as described.

**Additional information**

We collected lifestyle information from the SMC questionnaires closest to the subcohort baseline, including smoking habits; leisure-time physical activity; education; marital status; postmenopausal estrogen therapy; bisphosphonate use, vitamin D and calcium supplement use; menopausal status; and parity. Nutrient intakes were estimated as cumulative averages from three food frequency questionnaires.
(FFQs). Diagnosis codes were collated from the National In-Patient Registry to calculate a weighted Charlson’s comorbidity index\(^9\) and identify those with previous fractures since 1964.

Fracture outcomes

Information on incident fracture events was obtained through linkage to the National In- and Out-Patient Registries using International Classification of Diseases and Related Health Problems, 10th Revision (ICD-10) codes starting with S12–S92 (hip fracture: S720–S722). Accordingly, we did not include fractures of the skull (ICD-10 code S02) because they often involve special injury mechanisms and (in contrast to other types of fractures) are unrelated to BMD.\(^16\) We retained all non-skull fractures in the analysis because comparable increases in the risks of low-impact and high-impact trauma fractures have been seen in association with decreasing BMD.\(^17\) We used a previously validated and accurate algorithm\(^18\) to distinguish incident fractures from readmissions. In the analysis of all non-skull fractures, only first fracture events after baseline were used in the analysis; for hip fracture, all first hip fracture events during follow-up were studied.

Statistical analysis

Based on the seasonal patterns of S-25OHD and UV radiation\(^19\) during November 2003 through September 2009 in Sweden at 60 degrees N (Figure 2), we defined season of blood draw as two 6-month periods: the “sunny” season with higher levels (May–October) and the “dark” season with lower levels (November–April). Average daily UV-radiation\(^19\) data were retrieved from the Swedish Meteorological and Hydrological Institute. We dichotomized BMI at 25 kg/m\(^2\) (i.e., normal weight vs. overweight/obese) and FMI (at the median 9.8 kg/ m\(^2\)). In descriptive analyses, we present baseline characteristics by S-25OHD, season, and BMI category.

Kaplan-Meier survival curves with log-rank tests were used to visualize times to first non-skull fracture. We used proportional hazards regression to compute hazard ratios (HRs) with 95% confidence intervals (CIs) to assess season-specific associations between categories of S-25OHD (<30 nmol/L, 40–50 nmol/L, 40–50 nmol/L, 50–60 nmol/L, >60 nmol/L) and fracture risk. Age- and month-adjusted models (using 2-month intervals to account for within-season variation in S-25OHD) as well as a multivariable model were used, with calendar date as the time scale and season of blood draw and body composition categories as stratifying variables. The proportional hazards assumption was assessed using Schoenfeld residuals plots. No deviation from proportionality was detected. Multiplicative interactions were considered using product terms and likelihood ratio tests.

Follow-up time for each participant accrued from baseline until fracture, death, or the end of the study period (December 31, 2017), whichever came first. To select covariates for a multivariable model we used current knowledge regarding fracture risk and directed acyclic graphs. The following baseline variables were selected: leisure physical exercise (<1, 1–2, 3, 4–5, 5+ h/week), education (<9, 9–11, 12+ years), marital status (living alone vs. married/cohabitee), total fat mass (continuous), total lean muscle mass (continuous), calcium intake (continuous), body height (continuous), current smoking, current vitamin D supplementation,\(^20\) ever bisphosphonate use, ever use of estrogen replacement therapy, weighted Charlson comorbidity index (continuous), previous hip fracture, previous fracture of any type, and estimated glomerular filtration rate (eGFR based on age, plasma creatinine, and cystatin C).

We also calculated HRs of fracture in four categories of S-1,25-(OH)\(_2\)D (percentiles <10, 10–20, 20–60, >60) and in four categories of P-PTH (<5.0, 5.0–10.0, 10.0–20.0, >20.0 pmol/L). Here, we used the age-season and multivariable models described above in the previous paragraph, with additional adjustment for S-25OHD, stratified by season. We also calculated multivariable-adjusted HRs of fracture within two categories of S-1,25-(OH)\(_2\)D (percentiles <10, 10–100) cross-classified by two categories of S-25OHD (<40, ≥40 nmol/L). Finally, to understand the associations of fat mass and S-25OHD with S-1,25-(OH)\(_2\)D, we conducted regression analyses of log-transformed variables with adjustment for age and month, stratified by season.

To assess the robustness of key findings, we calculated E-values,\(^21\) which assess how strong an unmeasured confounder would have to be to explain observed associations. Data analyses were conducted using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA) and R (R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org/).

Results

Baseline characteristics are displayed in Table 1. Women with low S-25OHD drawn during sunny and dark seasons had similar risk factor profiles, with higher BMI and fat mass, lower serum free S-25OHD, higher P-PTH levels, and less vitamin D and calcium supplement use than those with higher levels. S-25OHD was highly dependent on both season of blood draw and BMI (Figure 2). Levels were highest in samples taken in August, and lowest in February/March. S-25OHD was consistently lower in women with higher BMI. All women in the cohort had a White ethnicity.

Lifestyle factors were no different in women with low vitamin D status measured in the sunny versus the dark season, including leisure time physical activity, dietary calcium, vitamin D and

Figure 2. Seasonal variation in S-25OHD concentration among women above (red) and below (blue) body mass index of 25 kg/m\(^2\). Average UV-B radiation in the period 2003–2009 as measured by CIE-action spectrum per square meter;\(^19\) to mimic the erythemal effect of UV radiation are presented as a graph in yellow with values on the secondary vertical axis. Abbreviations: CIE, Commission Internationale de l’Eclairage; S-25OHD, serum 25-hydroxyvitamin D.
Table 1. Characteristics of the Swedish mammography cohort clinical by both sunny (May–October) and dark season (November–April) and by lower (<40 nmol/L) and higher (>40 nmol/L) S-25-OH-D concentrations

| Variable                          | Sunny season (May–October) (n = 186) | Dark season (November–April) (n = 577) | S-25-OH-D ≥40 nmol/L (n = 2066) | Dark season (November–April) (n = 2171) |
|----------------------------------|--------------------------------------|----------------------------------------|---------------------------------|----------------------------------------|
|                                  | (mean ± SD)                          | (mean ± SD)                            | (mean ± SD)                      | (mean ± SD)                            |
| Age (years)                      | 68.2 ± 7.6                          | 67.9 ± 6.7                             | 67.2 ± 6.8                      | 67.8 ± 6.6                             |
| Height (cm)                      | 163.0 ± 6.6                         | 163.3 ± 6.5                            | 163.8 ± 6.2                     | 163.5 ± 5.9                            |
| Body mass index (kg/m²)          | 26.6 ± 4.6                          | 27.2 ± 5.0                             | 25.5 ± 4.1                      | 26.0 ± 4.2                             |
| Total fat mass (kg)              | 28.5 ± 10.1                         | 29.6 ± 10.2                            | 26.0 ± 8.6                      | 27.2 ± 8.7                             |
| Fat mass index (kg/m²)           | 10.7 ± 3.7                          | 11.1 ± 3.9                             | 9.7 ± 3.2                       | 10.2 ± 3.3                             |
| S-25-OH-D total (nmol/L)         | 33.0 ± 6.2                          | 32.0 ± 6.5                             | 65.6 ± 16.2                     | 60.1 ± 14.2                            |
| S-25-OH-D$_3$ (nmol/L)           | 32.5 ± 6.3                          | 31.5 ± 6.6                             | 65.0 ± 16.3                     | 59.6 ± 14.3                            |
| S-Osteocalcin (μg/L)             | 0.5 ± 1.4                           | 0.5 ± 1.4                              | 0.5 ± 2.0                       | 0.6 ± 1.7                              |
| S-Epimer-D$_3$ (nmol/L)          | 0.7 ± 1.5                           | 0.4 ± 1.2                              | 1.5 ± 5.4                       | 1.0 ± 1.5                              |
| P-PTH (intact, pmol/L)           | 6.0 ± 2.2                           | 5.8 ± 2.2                              | 5.0 ± 1.8                       | 5.0 ± 1.9                               |
| P-Phosphate (mmol/L)             | 1.15 ± 0.14                         | 1.14 ± 0.15                            | 1.15 ± 0.13                     | 1.16 ± 0.22                             |
| e-GFR (ml/min)                   | 80.2 ± 16.3                         | 81.2 ± 15.2                            | 82.2 ± 14.7                     | 80.2 ± 15.4                            |
| ALP (μkat/L)                     | 0.24 ± 0.20                         | 0.24 ± 0.14                            | 0.23 ± 0.13                     | 0.24 ± 0.24                             |
| P-Calcium (mmol/L)               | 2.30 ± 0.11                         | 2.31 ± 0.11                            | 2.30 ± 0.11                     | 2.32 ± 0.11                             |
| S-CrossLaps (ng/L)               | 476 ± 191                           | 459 ± 197                              | 486 ± 186                       | 458 ± 195                              |
| S-Osteocalcin (μg/L)             | 26.2 ± 9.3                          | 25.5 ± 9.7                             | 24.6 ± 8.5                      | 25.2 ± 9.0                              |
| BMD total hip (g/cm$^2$)         | 0.89 ± 0.14                         | 0.92 ± 0.14                            | 0.92 ± 0.13                     | 0.92 ± 0.13                             |
| BMD femoral neck (g/cm$^2$)      | 0.84 ± 0.13                         | 0.87 ± 0.13                            | 0.88 ± 0.12                     | 0.87 ± 0.12                             |
| BMD lumbar spine L1–L4 (g/cm$^2$)| 1.11 ± 0.19                         | 1.13 ± 0.20                            | 1.12 ± 0.20                     | 1.12 ± 0.20                             |
| BMD total body (g/cm$^2$)        | 1.07 ± 0.10                         | 1.09 ± 0.10                            | 1.09 ± 0.10                     | 1.09 ± 0.10                             |
| Charlson’s comorbidity index (points) | 0.23 ± 0.56                   | 0.26 ± 0.66                             | 0.19 ± 0.58                     | 0.22 ± 0.61                             |
| Daily dietary intake             |                                     |                                        |                                |                                        |
| Energy (kcal)                    | 1780 ± 629                          | 1736 ± 544                             | 1815 ± 525                      | 1783 ± 539                              |
| Calcium (mg)                     | 1006 ± 319                          | 1000 ± 328                             | 1046 ± 323                      | 1032 ± 308                              |
| Vitamin D (μg)                   | 5.6 ± 4.1                           | 5.4 ± 2.9                              | 5.9 ± 2.4                       | 5.9 ± 2.7                               |
| Alcohol (ethanol, g)             | 6.1 ± 10.2                          | 5.4 ± 6.8                              | 6.6 ± 7.4                       | 6.0 ± 6.6                               |
| Variable                         | n (%)                                | n (%)                                  | n (%)                           | n (%)                                   |
| Previous fracture of any type    | 24 (13)                              | 100 (17)                               | 368 (18)                        | 397 (18)                                |
| Previous hip fracture            | 0 (0)                                | 6 (1)                                  | 32 (2)                          | 15 (1)                                  |
| Diabetes mellitus                | 11 (6)                               | 44 (8)                                 | 104 (5)                         | 89 (4)                                  |
| Leisure time physical activity   |                                     |                                        |                                |                                        |
| <1 h/week                        | 45 (24)                              | 111 (19)                               | 365 (18)                        | 303 (14)                                |
| 1 h/week                         | 34 (18)                              | 86 (15)                                | 386 (19)                        | 383 (18)                                |
| 2–3 h/week                       | 75 (40)                              | 256 (44)                               | 829 (40)                        | 967 (45)                                |
| 4–5 h/week                       | 22 (12)                              | 67 (12)                                | 263 (13)                        | 264 (12)                                |
| >5 h/week                        | 10 (5)                               | 57 (10)                                | 223 (11)                        | 254 (12)                                |
| Used postmenopausal estrogen      | 93 (50)                              | 333 (58)                               | 1295 (63)                       | 1355 (63)                               |
| Living alone                      | 66 (35)                              | 209 (36)                               | 659 (32)                        | 703 (32)                                |
| Nulliparous                      | 30 (16)                              | 75 (13)                                | 211 (10)                        | 230 (11)                                |
| Education                        |                                     |                                        |                                |                                        |
| <10 years                        | 82 (44)                              | 314 (54)                               | 1113 (54)                       | 1187 (55)                               |
| 10–12 years                      | 22 (12)                              | 53 (9)                                 | 175 (8)                         | 192 (9)                                 |
| >12 years                        | 82 (44)                              | 210 (36)                               | 778 (38)                        | 792 (36)                                |
| Current smoker                   | 33 (18)                              | 77 (13)                                | 171 (8)                         | 166 (8)                                 |
| Vitamin D supplement use          | 9 (5)                                | 21 (4)                                 | 255 (12)                        | 202 (9)                                 |
| Calcium supplement use            | 8 (4)                                | 20 (4)                                 | 232 (11)                        | 346 (16)                                |
| Bisphosphonate use               | 1 (0)                                | 1 (0)                                  | 40 (2)                          | 40 (2)                                  |

Abbreviations: μkat, SI unit of catalytic activity; BMD, bone mineral density; e-GFR, estimated glomerular filtration rate; P-ALT, plasma alanine aminotransferase; P-PTH, plasma parathyroid hormone; S-1,25-(OH)$_2$D$_3$, serum 1,25-dihydroxyvitamin D$_3$; S-25-OH-D$_3$, serum 25-hydroxyvitamin D$_3$; S-25-OH-D$_2$, serum 25-hydroxyvitamin D$_2$; S-25-OH-D$_3$, serum 25-hydroxyvitamin D$_3$; S-25-OH-D$_2$, serum 25-hydroxyvitamin D$_2$; SD, standard deviation; S-Epimer-D$_3$, serum 25-hydroxy-3-epi-vitamin D$_3$; S-FGF23, serum fibroblast growth factor.
energy intake, as well as alcohol use. We also found no important differences in the number of comorbidities at baseline, bone turnover markers, eGFRs, or P-ALT. However, mean BMD values were lower among women with 25OHD <40 nmol/L with blood collection during the sunny season, as described (Table S1).[^9]

During an average of 9.2 years of follow-up accruing 46,045 person-years, 1080 women had one or more non-skull fractures.

Figure 3. Kaplan-Meier curves of for any fracture by vitamin D status. Estimates for the sunny season (May–October) (A) and for the dark season (November–April) (B).

[^9]: Journal of Bone and Mineral Research
The distribution of fracture types at the first fracture event after baseline is displayed in Table S2. Kaplan-Meier curves for any fracture by vitamin D status and season (Figure 3, sunny season panel A and dark season panel B) illustrate the pattern. Fracture rates were higher only in women with S-25OHD <30 nmol/L and 30–40 nmol/L obtained during the sunny season (log-rank \( p = 0.0019 \)).

The rates and adjusted HRs for any fracture by S-25OHD and season of blood draw are shown in Table 2. Women with sunny season S-25OHD <30 nmol/L had a multivariable fracture HR of 2.06 (95% CI, 1.27–3.35) and those with levels 30–40 nmol/L an HR of 1.59 (95% CI, 1.12–2.26) compared with women who had S-25OHD >60 nmol/L. Findings were similar for hip fracture (n = 220), though with lower precision (Table 2). In contrast, S-25OHD drawn during the dark season was unrelated to fracture rates (\( p = 0.017 \) for seasonal interaction) and conventional analysis with seasonal adjustment (but no season stratification) showed no clear association (Table S3).

BMI strongly modified the association between S-25OHD and the fracture HR (Figure 4; \( p = 0.015 \) for interaction). A threefold to fourfold higher rate of fracture in those with sunny season S-25OHD <30 nmol/L and a twofold higher rate of fracture in those with 30–40 nmol/L was seen only in those with a BMI of 25 kg/m². Irrespective of body composition, dark season S-25OHD was unrelated to fracture risk. Findings for FMI were comparable (Figure S1).

These results were unchanged after exclusion of women with baseline eGFR <50 mL/min, women who used vitamin D supplements or bisphosphonate, those with serum calcium >2.5 mmol/L or P-PTH levels >6.9 pmol/L and after adjustment for baseline BMD (Table S4). Similar HR estimates for the lowest two categories of vitamin D status with samples collected in the sunny season were partially attenuated (by 25% to 30%) after adjustment for baseline BMD (Table S4). Similar HR estimates for the lowest two categories of S-25OHD were found in sensitivity analyses (Table S5) excluding fractures of the hand (n = 84), ankle (n = 83), foot (n = 41), and in multivariable models omitting adjustment for vitamin D supplement use or previous fractures (Table S5).

S-1,25-(OH)2-D3 was also associated with fracture risk, but there were indications that this was the case only if drawn during the sunny season (Table S6, \( p = 0.17 \) for interaction). Adjustment for S-25OHD only modestly attenuated these associations. Cross-classification of S-1,25-(OH)2-D3 and S-25OHD indicated doubled fracture rates with low concentrations of either metabolite drawn during the sunny (Figure S2A) but not the dark season (Figure S2B). P-PTH was not associated with fracture risk (Table S7), irrespective of season.

The impact of adiposity extended beyond the modification of fracture risks associated with low sunny season S-25OHD. BMI was negatively associated with S-25OHD (Table S8) more strongly for sunny season than for dark season measurements (\( p = 0.009 \) for interaction). BMI was also negatively associated with S-1,25-(OH)2-D3 even after adjustment for S-25OH.
In this large cohort study, we found that women with low S-25OHD during the sunny season had an increased fracture risk only if they had a higher fat mass. In contrast, women with low S-25OHD during the dark season were not at higher fracture risk, irrespective of fat mass. We also found that low S-1,25-(OH)2-D3 concentrations during the sunny season, but not the dark season, confer higher fracture risk. In this Swedish cohort, about 5% of all women were at increased fracture risk: 111 of 2252 women with S-25OHD <40 nmol/L during May through October and BMI ≥25 kg/m2.

Women with low vitamin D status during the summer will probably also have on average lower S-25OHD concentrations during the dark season, but the predictive ability of a low dark season value will be masked by the three to four times higher proportion of women whose levels are only temporarily low during winter. Accordingly, a dark season measurement will not be informative regarding vitamin D status over the whole year. The association of S-25OHD with bone health is also not adequately described by season-normalized levels at a particular time, because vitamin D status is a dynamic state that involves effects of both seasonal sunlight and fat mass. Those without a sufficient augmentation of 25OHD stores in the summer will be left with deficient concentrations throughout the year, an impact that is largely avoided in those with shorter periods of insufficiency only during late winter. Seasonal-adjustment of S-25OHD will not capture those at highest risk. Indeed, no clear associations with fractures were found with seasonal-adjustment (Table S2).

About 75% of total body vitamin D is stored in fat tissue in both lean and obese individuals. Due to a larger volume of distribution, higher fat mass negatively affects S-25OHD, as shown by studies of supplementation with vitamin D, UV B skin irradiation, and Mendelian randomization analysis. This implies that low accumulation of vitamin D stores during the sunny season will have a more negative effect on S-25OHD in overweight or obese subjects than in lean subjects. Indeed, we observed lower S-25OHD in overweight or obese women during the dark as well as the sunny season. We also found a similar association of BMI with S-1,25-(OH)2-D2, consistent with findings after supplementation with calcitriol. The implication is that volume of distribution is important for both S-25OHD and S-1,25-(OH)2-D2.

Vitamin D can potentially be released from fatty tissue into the circulation, dampening the reduction in levels that occur during prolonged periods without sun exposure. However, the resulting impact on circulating levels will be diminished by the high volume of distribution for vitamin D in the obese as well as by the impairment of adipocyte cellular vitamin D release. In addition, animal models show decreased 25-hydroxylase activity and renal 1-hydroxylase activity with obesity, whereas skin synthesis of cholecalciferol in obese humans seems normal.

Although with moderate precision, we found that the lowest decile of S-1,25-(OH)2-D3 in the sunny (but not dark) season conferred higher fracture rates, extending previous results by implying that serum levels need to be interpreted according to season. Our suggestive finding that both low S-1,25-(OH)2-D3 and low S-25OHD may independently predict future fracture risk is an indication that vitamin D status is not determined only by S-25OHD. Both metabolites have affinity for the vitamin D recept or, that for 1,25-(OH)2-D3 is about 100 times stronger, but this is countered by S-25OHD levels being about 300-fold to 500-fold higher than those for S-1,25-(OH)2-D3.

Our results may explain the overall lack of effect of vitamin D supplementation on BMD as demonstrated in a meta-analysis of randomized controlled trials (RCTs) because the appropriate target group for intervention has not been identified. Secondary analyses with individual data from two RCTs indicate a moderate 1% to 2% improvement in BMD over 1 to 2 years with vitamin D supplementation only in individuals with S-25OHD <30 nmol/L. Unfortunately, the participants in both trials were recruited during the dark season of the year. An interventional study of BMD among subjects with low S-25OHD during the sunny season would be of interest but a primarily designed randomized clinical trial comparing the placebo-controlled effect of vitamin D supplementation in those with low S-25OHD during both the sunny and the dark season with the effect in those with low S-25OHD during only the dark season will in practice be difficult to accomplish. Unlike cohort studies, biological

**Figure 4.** Multivariable hazard ratios for any type of fracture by S-25OHD, season, and BMI. The estimates were adjusted for age (continuous), month of blood draw (categories, two-month intervals), leisure time physical exercise (<1 h/week; 1 h/week; 2–3 h/week; 4–5 h/week; >5 h/week), education (≤9, 10–12, >12 years), marital status (living alone vs. married/cohabitee), total fat mass (continuous), total lean muscle mass (continuous), calcium intake (continuous), body height (continuous), current smoking, vitamin D supplementation, bisphosphonate use (yes/no), ever use of estrogen replacement therapy (yes/no), weighted Charlson comorbidity index (continuous), previous hip fracture (yes/no), previous fracture of any type (yes/no), and estimated glomerular filtration rate (continuous). Sunny season: May–October; dark season: November–April. The p value for interaction between season, BMI, and S-25OHD categories was 0.015. Total number of women and number of women with a fracture (within brackets) during follow-up in each category. Number of women in the higher risk categories are marked in bold. Abbreviations: BMI, body mass index; S-25OHD, serum 25-hydroxyvitamin D.

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**Discussion**

In this large cohort study, we found that women with low S-25OHD during the sunny season had an increased fracture risk only if they had a higher fat mass. In contrast, women with low S-25OHD during the dark season were not at higher fracture risk, irrespective of fat mass. We also found that low S-1,25-(OH)2-D3 concentrations during the sunny season, but not the dark season, confer high fracture risk. In this Swedish cohort, about 5% of all women were at increased fracture risk: 111 of 2252 women with S-25OHD <40 nmol/L during May through October and BMI >25 kg/m2.

Women with low vitamin D status during the summer will probably also have on average lower S-25OHD concentrations during the dark season, but the predictive ability of a low dark season value will be masked by the three to four times higher proportion of women whose levels are only temporarily low during winter. Consequently, a dark season measurement will not be informative regarding vitamin D status over the whole year. The association of S-25OHD with bone health is also not adequately described by season-normalized levels at a particular time, because vitamin D status is a dynamic state that involves effects of both seasonal sunlight and fat mass. Those without a sufficient augmentation of 25OHD stores in the summer will be left with deficient concentrations throughout the year, an impact that is largely avoided in those with shorter periods of insufficiency only during late winter. Seasonal-adjustment of S-25OHD will not capture those at highest risk. Indeed, no clear associations with fractures were found with seasonal-adjustment (Table S2).

About 75% of total body vitamin D is stored in fat tissue in both lean and obese individuals. Due to a larger volume of distribution, higher fat mass negatively affects S-25OHD, as shown by studies of supplementation with vitamin D, UV B skin irradiation, and Mendelian randomization analysis. This implies that low accumulation of vitamin D stores during the sunny season will have a more negative effect on S-25OHD in overweight or obese subjects than in lean subjects. Indeed, we observed lower S-25OHD in overweight or obese women during the dark as well as the sunny season. We also found a similar association of BMI with S-1,25-(OH)2-D2, consistent with findings after supplementation with calcitriol. The implication is that volume of distribution is important for both S-25OHD and S-1,25-(OH)2-D2.

Vitamin D can potentially be released from fatty tissue into the circulation, dampening the reduction in levels that occur during prolonged periods without sun exposure. However, the resulting impact on circulating levels will be diminished by the high volume of distribution for vitamin D in the obese as well as by the impairment of adipocyte cellular vitamin D release. In addition, animal models show decreased 25-hydroxylase activity and renal 1-hydroxylase activity with obesity, whereas skin synthesis of cholecalciferol in obese humans seems normal.

Although with moderate precision, we found that the lowest decile of S-1,25-(OH)2-D3 in the sunny (but not dark) season conferred higher fracture rates, extending previous results by implying that serum levels need to be interpreted according to season. Our suggestive finding that both low S-1,25-(OH)2-D3 and low S-25OHD may independently predict future fracture risk is an indication that vitamin D status is not determined only by S-25OHD. Both metabolites have affinity for the vitamin D receptor, that for 1,25-(OH)2-D3 is about 100 times stronger, but this is countered by S-25OHD levels being about 300-fold to 500-fold higher than those for S-1,25-(OH)2-D3.

Our results may explain the overall lack of effect of vitamin D supplementation on BMD as demonstrated in a meta-analysis of randomized controlled trials (RCTs) because the appropriate target group for intervention has not been identified. Secondary analyses with individual data from two RCTs indicate a moderate 1% to 2% improvement in BMD over 1 to 2 years with vitamin D supplementation only in individuals with S-25OHD <30 nmol/L. Unfortunately, the participants in both trials were recruited during the dark season of the year. An interventional study of BMD among subjects with low S-25OHD during the sunny season would be of interest but a primarily designed randomized clinical trial comparing the placebo-controlled effect of vitamin D supplementation in those with low S-25OHD during both the sunny and the dark season with the effect in those with low S-25OHD during only the dark season will in practice be difficult to accomplish. Unlike cohort studies, biological
mechanisms are not suitable to be examined in clinical trials and if only a low proportion in the population are exposed to low S-25OHD rendering higher risk of disease, Mendelian randomization studies are not suitable.

P-PTH in our study was unrelated to fracture risk and we have previously shown it to be unrelated to BMD.\(^9\) These findings strongly indicate that PTH in our context of calcium replete women is mainly a physiologic regulator of serum—but not bone—calcium homeostasis. Serum phosphate concentrations were not affected by vitamin D status, an indication that osteomalacia was not present.\(^1\)

Our novel observations are consistent with our previous cross-sectional findings that women with low summer but not winter S-25OHD are at an increased risk of low BMD and osteoporosis.\(^9\)

However, our new fracture findings were largely independent of differences in baseline BMD. Nonetheless, DXA measures only areal BMD and might miss other aspects of bone quality. In addition, we cannot rule out an impact of low S-25OHD on physical performance, especially in proximal muscle groups,\(^5\) leading to a higher risk of falls\(^5\) and as a consequence fractures.

Strengths of our study include the size and richness of the cohort, the population-based design, a time to event analysis with a clinically relevant and completely ascertained outcome, no loss to follow-up by use of the individual personal registration number to all Swedish citizens and complete registry data,\(^35,36\) and the use of S-25OHD measured with high accuracy.\(^39\) Settings with self-reported fractures will have underreporting of fracture cases, which can be selective based on fracture risk factors. One out of five women was identified by an incident fracture during follow-up, an expected proportion, because half of Swedish women will sustain a fracture after age 50 years. There was no indication that those who had low S-25OHD concentrations in the sunny season, compared to those with low concentrations in the dark season, were women with different lifestyle habits or especially frail individuals with derangements in biomarkers other than those belonging to the vitamin D axis. The proportion of women with low vitamin D status in our cohort during the sunny season is also similar to those in other Swedish population-based studies.\(^37,38\)

Although we studied only White women in Sweden, similar seasonal variations in S-25OHD have previously been reported in fair-skinned individuals of both sexes in other countries, and therefore our results are likely to have general implications. Dark-skinned individuals have on average throughout the year lower concentrations of total\(^36-38\) and free\(^39\) S-25OHD\(^39,41\) than fair-skinned persons. In addition, at high latitudes, they have lower S-1,25-(OH)\(_2\)-D and higher circulating PTH concentrations. Despite these differences they do not have lower serum concentrations of calcium or lower BMD and they have lower fracture rates.\(^42-44\) Our findings may therefore not apply to non-White groups with better conservation of calcium through mechanisms such as higher intestinal calcium absorption efficiency and lower urinary calcium excretion.\(^42-44\)

Selection mechanisms may have contributed to our observational findings; for example, women with low S-25OHD may have a different genetic constitution compared with those with low concentrations during winter. Our analyses were adjusted for important covariates without substantial impact on our estimates but residual confounding still remains a possible limitation. The observed multivariable HR of 2.06 for the association of S-25OHD <30 nmol/L with any fracture could be explained by an unmeasured confounder that was associated with both S-25OHD <30 nmol/L and any fracture by a risk ratio (E-value) of 2.7-fold each, but weaker confounding could not do so.\(^21\) The corresponding E-value is 4.3 for an unknown risk factor to “adjust away” our multivariable HR of 3.7 for fracture given the combination of S-25OHD <30 nmol/L and high fat mass. To widen the corresponding CIs for these two HRs to include 1.0, an unmeasured confounder would have to be associated with both the exposure and fracture by risk ratios ≥1.6-fold or ≥2.6-fold, respectively.\(^21\) No such risk factor for S-25OHD and fractures not already considered by us have been identified.\(^43,45,46\)

We used a single measurement of S-25OHD but we have previously found a high correlation \((r = 0.7)\) between summer and winter levels in our cohort, although absolute individual seasonal differences were substantial.\(^9\)

Our findings clearly have implications for the evaluation of vitamin D status in clinical practice and shed light on the lack of effect seen with vitamin D supplementation in many fracture trials.\(^42\) We conclude that sunny season concentrations of S-25OHD and fat mass are important when targeting interventional efforts with vitamin D supplementation for fracture prevention. This issue should also be considered in studies of other diseases possibly related to vitamin D status.

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Author contributions

Karl Michaëlsson: Conceptualization; data curation; formal analysis; investigation; methodology; resources; visualization; writing—original draft; writing—review & editing. Lisa Byberg: Data curation; investigation; methodology; visualization; resources; writing—review & editing. Bodil Svennblad: Data curation; formal analysis; investigation; methodology; writing—review & editing.

John Baron: Methodology; investigation; writing—review & editing.

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Disclosures

The authors declare no conflict of interest.
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