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Changes in plasma concentrations of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D during pregnancy: A Brazilian cohort

Vitamin D and Pregnancy

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Abstract

Purpose. To characterize the physiological changes in 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxivitamin D [1,25$(OH)_2$D] throughout pregnancy. *Methods.* Prospective cohort of 229 apparently healthy pregnant women followed at 5^{th}-13^{th}, 20^{th}-26^{th} and 30^{th}-36^{th} gestational weeks. 25(OH)D and 1,25$(OH)_2$D concentrations were measured by LC-MS/MS. Statistical analyses included longitudinal linear mixed-effects models adjusted for parity, season, education, self-reported skin color and pre-pregnancy BMI. Vitamin D status was defined based on 25(OH)D concentrations according to the Endocrine Society Practice Guideline and Institute of Medicine (IOM) for adults. *Results.* The prevalence of 25(OH)D <75 nmol/L was 70.4%, 41.0% and 33.9%; the prevalence of 25(OH)D <50 nmol/L was 16.1%, 11.2% and 10.2%; and the prevalence of 25(OH)D <30 nmol/L was 2%, 0% and 0.6%, at the first, second and third trimester, respectively. Unadjusted analysis showed an increase in 25(OH)D (β=0.869; 95%CI, 0.723-1.014; P<0.001) and 1,25$(OH)_2$D (β=3.878; 95%CI, 3.136-4.620; P<0.001) throughout pregnancy. Multiple adjusted analyses showed that women who started the study in winter (P<0.001), spring (P<0.001) or autumn (P=0.028) presented a longitudinal increase in 25(OH)D concentrations, while women that started during summer did not. Increase of 1,25$(OH)_2$D concentrations over time in women with insufficient vitamin D (50-75 nmol/L) at baseline was higher compared to women with sufficient vitamin D (≥75 nmol/L) (P=0.006). *Conclusions.* The prevalence of vitamin D inadequacy varied significantly according to the adopted criteria. There was a seasonal variation of 25(OH)D during pregnancy. The women with insufficient vitamin D status present greater longitudinal increases in the concentrations of 1,25$(OH)_2$D in comparison to women with sufficiency.

Keyword: Vitamin D; pregnancy; micronutrients; cohort; tropical country; seasons.

Introduction

Vitamin D deficiency or insufficiency is considered to be a global public health problem [1-2] with an estimated 1 billion people affected worldwide [3]. Pregnant women have been identified as a high-risk group for vitamin D deficiency [4], even in sunny regions [5-7]. Vitamin D is a pro-hormone with an important role in maintaining bone health [3]. Low vitamin D status during pregnancy has been associated with non-skeletal maternal and child outcomes such as preeclampsia, gestational diabetes mellitus, preterm birth, small for gestational age, and low birth weight [5,8]. These pregnancy complications continue to be important public health problems in Brazil [9-12].

Vitamin D can be obtained from foods and supplements, but the main source is sun exposure enabling synthesis in the skin induced by ultraviolet B radiation (wavelengths 290–315 nm) [3,13-14]. It is known that maternal vitamin D concentrations can be influenced by skin pigmentation, age, season, sunscreen use, latitude, physical activity, obesity status, atmospheric pollution, blood concentrations of parathyroid hormone, calcium, and phosphate [3,5,15-18].

Vitamin D from the skin and the diet is converted in the liver into 25-hydroxyvitamin D [25(OH)D] which is then metabolized in the kidneys to its active form, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] by the 1α-hydroxylase enzyme. Due to the short biological half-life of $1,25(OH)_2D$, vitamin D status is usually determined by measuring the 25(OH)D concentrations, which is the major circulating form of vitamin D in the blood [3,13-14].

During pregnancy there are physiological changes in $1,25(OH)_2D$ concentrations to ensure sufficient calcium required for fetal bone mineralization [19]. A gradual increase on plasma $1,25(OH)_2D$ concentrations during pregnancy and a modest contribution from synthesis in both the kidney and placenta have been reported [19-20]. However, the profile of the longitudinal changes in 25(OH)D concentrations as well the relationship between $1,25(OH)_2D$ and 25(OH)D throughout pregnancy remains unclear [20-22].

Despite these physiological changes in pregnant women the criteria for defining vitamin D deficiency and insufficiency are the same as for the general adult population and cutoff values remain controversial [4,14]. The Institute of Medicine (IOM) [14] recommends that at least 30 nmol/L of 25(OH)D is necessary to protect against rickets in children and osteoporosis in adults and that concentrations of $\geq$ 50 nmol/L corresponds to the level which meets the requirements of 97.5% of the population to obtain the recommended daily allowance of 600 IU/day [14]. The Endocrine Society Practice Guidelines [4] states that 50 nmol/L is required for optimal bone health but recommends concentrations $\geq$75 nmol/L for non-skeletal health benefits associated with vitamin D [4].

Few prospective studies have investigated changes in 25(OH)D and $1,25(OH)_2D$ concentrations over time during pregnancy [21-22]. Further, there are no studies in healthy pregnant Brazilian women. To the best of our knowledge, the association between 25(OH)D status in the first trimester of pregnancy and the change in $1,25(OH)_2D$ concentrations throughout gestation has not been assessed by previous studies. Therefore, considering the importance of adequate vitamin D status during pregnancy for positive gestational outcomes, the aim of this study was to estimate the prevalence of vitamin D inadequacy and to characterize the physiological changes in 25(OH)D and $1,25(OH)_2D$ concentrations among healthy pregnancies.

Methods

Design and study participants

This is a prospective cohort study conducted at a public health care center in Rio de Janeiro, Brazil. The recruitment was done between November 2009 and October 2011, and 299 women who met the following eligibility criteria agreed to participate in the study: being between 5th-13th weeks of gestation, free from chronic (except obesity) and infectious diseases, aged between 20 and 40 years, presenting a singleton pregnancy, residing in the study catchment area, and intending to continue prenatal care in the public health centre. The cohort comprised 229 apparently healthy pregnant women after exclusions, including: confirmed pre-gestational diagnosis of chronic non-communicable diseases (except obesity) (n=12), diagnosis of infectious or parasitic diseases (n=9), twin pregnancies (n=4), missed baseline evaluation data (n=20) or miscarriage (n=25). We further excluded women with insufficient volume to measure plasma 25(OH)D (n=30) or $1,25(OH)_2D$ (n=23) at the first trimester. Our sample comprised 199 and 178 women at the first trimester with available data on 25(OH)D and $1,25(OH)_2D$ concentrations, respectively. The women were followed at the first [(5-13 weeks (baseline)], second (20-26 weeks), and third trimesters (30-36 weeks) of pregnancy (**Online Resource Fig. 1**).

Biochemical analyses

A trained professional collected blood samples between 6:50 and 7:50 am into vacutainer tubes at each follow-up visit after a 12-hour fasting period. The samples were centrifuged (5,000 rpm for 5 minutes). The plasma was separated and prepared from blood collected into tubes containing EDTA, and stored at -80°C for subsequent analysis.

Plasma concentrations of 25(OH)D and 1,25$(OH)_2$D were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the LC Thermo Cohesive System coupled to Thermo Quantum Ultra Mass Spectrometer (Thermo Fisher; San Jose, CA, USA). The analyses were performed by Quest Diagnostics Nichols Institute (San Juan Capistrano, CA, USA), which is part of the Hormone Standardization Program conducted by the Centers for Disease Control and Prevention. The LC-MS/MS is considered to be the gold standard for measuring vitamin D status due to its high sensitivity and specificity [4,23]. The analytical measurement range was 19-960 pmol/L and 10-640 nmol/L for 1,25$(OH)_2$D and 25(OH)D, respectively. The coefficient of variation for all analyses was <10%. The 25(OH)D and 1,25(OH)2D are stable in plasma for more than 10 years when stored under appropriate conditions as in the present study [24-25].

Definition of vitamin D status

Vitamin D status was defined based on plasma 25(OH)D concentrations. Participants were categorized as vitamin D deficient (<50 nmol/L), insufficient (50-<75 nmol/L), and sufficient (≥75 nmol/L) according the Endocrine Society Practice Guidelines [4]. We also used the cutoffs of vitamin D status based on IOM: deficient (<30 nmol/L), insufficient (30-<50 nmol/L) and sufficient (≥50 nmol/L) [14]. Vitamin D status was also analyzed as a binary variable according to the Endocrine Society Practice Guidelines as inadequate/adequate (<75/≥75 nmol/L) [4] and IOM [14] as inadequate/adequate (<50/≥50 nmol/L).

Covariate assessment

Socioeconomic, demographic, reproductive and lifestyle variables were obtained through interviews with structured questionnaires administered at the baseline (5–13 weeks of gestation). The following variables were collected: age (years), self-reported skin color (white/mixed/black), education (years), monthly per capita family income (R$), parity (nulliparous/parous), first trimester smoking habit (yes/no), first trimester alcohol intake (yes/no) and leisure physical activity before pregnancy (yes/no). The season was classified according to the date of recruitment as follows: winter (June 21st to September 21st; spring (September 22nd to December 20th); summer (December 21st to March 19th); or autumn (March 20th to June 20th).

Gestational age was measured from the first ultrasonography (USG) (n=174) or using the reported date of the last menstrual period if the first USG was not performed prior to the 24th week of gestation (n=25). Height (cm) was measured at the beginning of pregnancy using a portable stadiometer (Seca Ltd., Hamburg, Germany). Pre-

gestational body mass index (BMI, weight [kg]/height [m²]) was calculated and categorized (<25/≥25 kg/m²) based on self-reported pre-gestational weight obtained at the first follow-up visit at the 1st trimester of gestation.

Total vitamin D (IU/day) and calcium (mg/day) intakes were estimated using a semi-quantitative food frequency questionnaire (FFQ) validated for the adult population of Rio de Janeiro [26]. The questionnaire was administered in the third trimester and referred to intakes during the prior 6 months. The FFQ included 82 food items and had eight frequency options: more than three times a day, two to three times a day, once a day, five to six times a week, two to four times a week, once a week, one to three times a month, and never or hardly ever. Tables of Nutritional Composition of Food Consumed of Brazilian Institute of Geography and Statistics were used for the analysis [27]. The FFQ did not take into consideration the use of supplements. Rather, information on vitamin D supplementation was self-reported in all trimesters.

Statistical analyses

The characteristics of the sample were described using mean and standard deviations (SD) for continuous variables and absolute (n) and relative frequencies (%) for categorical data.

Baseline characteristics of women with complete 25(OH)D and $1,25(OH)_2D$ information during pregnancy were compared to those women who presented losses to follow-up with missing information for at least one of these variables. In addition, baseline characteristics were presented for each variable and stratified according to 25(OH)D status for both the Endocrine Society Practice Guidelines and the IOM criteria. ANOVA was used to compare means according to 25(OH)D status and the Bonferroni test was employed as the post hoc test. The Chi-squared test was used to compare proportions.

Pearson coefficient correlations were calculated between 25(OH)D and $1,25(OH)_2D$ in the first, second and third trimesters. Linear mixed-effects (LME) regression models were performed to evaluate the longitudinal variation of 25(OH)D and $1,25(OH)_2D$ concentrations during pregnancy. The models included information from all individuals who had data from at least one time point on 25(OH)D and $1,25(OH)_2D$ concentrations. The LME models enable the inclusion of time-dependent and time-independent variables and unbalanced time intervals and the correlation between repeated measures was taken into account [28-29]. Gestational age (weeks) was included in the LME models as the time variable for both random and fixed effects.

Interactions between season at recruitment and gestational week were tested when assessing the longitudinal behavior of 25(OH)D concentrations. We also tested interactions between gestational age and first trimester vitamin D status to explore the effect on $1,25(OH)_2D$ longitudinal variation. The LME models were

adjusted for parity, season, education, self-reported skin color and pre-pregnancy BMI based on biological plausibility and statistical significance (P<0.2) of the associations on the bivariate analysis with the study outcome.

A P<0.05 was regarded as significant. All analyses were performed in STATA 12.0 (Stata Corporation, College Station, TX) [30].

Results

Losses to follow-up analysis comparing socio-demographic, lifestyle and reproductive characteristics between women with plasma 25(OH)D and $1,25(OH)_2D$ data in all trimesters and those who had plasma 25(OH)D data in at least one trimester showed no significant differences (P>0.05) between groups (**Data not shown**).

The overall mean 25(OH)D concentrations at the first trimester was 65.0 (17.7 SD) nmol/L which increased to 78.7 (22.0 SD) nmol/L and 84.1 (24.5 SD) nmol/L during the second and third trimesters, respectively. The mean of $1,25(OH)_2D$ pmol/L during the first, second and third trimesters were 173.4 (77.9 SD), 227.6 (91.9 SD) and 257.5 (92.3 SD), respectively. The $1,25(OH)_2D$ mean concentrations were significantly different only in the first-trimester among women within the sufficient category according to Endocrine Society Practice Guidelines. A higher prevalence of vitamin D deficiency was observed among pregnant women who started the study in winter and spring compared to those who started in summer and autumn according to both criteria (**Table 1**).

The prevalence of pregnant women with plasma 25(OH)D concentrations <75 nmol/L was 70.4%, 41.0% and 33.9%; the prevalence of women with 25(OH)D concentrations <50 nmol/L was 16.1%, 11.2% and 10.2%; while 2%, 0% and 0.6%, of women had 25(OH)D concentrations <30 nmol/L at the first, second and third trimester, respectively (**Figure 1**).

Plasma 25(OH)D and $1,25(OH)_2D$ concentrations were weakly but significantly correlated only in the first trimester of pregnancy (**Online Resource Fig. 2a, b, c)**. The concentrations of 25(OH)D and $1,25(OH)_2D$ significantly increased throughout gestation in the unadjusted model (β=0.869; 95% CI=0.723-1.014; P<0.001 and β=3.878; 95% CI=3.136-4.620; P<0.001, respectively) (**Fig. 2a, b**). Women who started the study during the summer or autumn seasons had higher mean 25(OH)D concentrations during the first trimester compared to women whose pregnancy began during winter or spring (**Fig. 3**). The longitudinal change in concentrations of 25(OH)D during pregnancy were modified by the season at recruitment in the adjusted model. Women that started the study in winter (P<0.001), spring (P<0.001) or autumn (P=0.028) presented a longitudinal increase in 25(OH)D concentrations, while women that started during summer did not (**Fig. 4 and Online Resource Table 1**).

Different patterns of $1,25(OH)_2D$ concentrations during the course of gestation were also observed according to vitamin D status at baseline considering the cutoffs proposed by the Endocrine Society Practice Guidelines. Women with insufficient concentrations of vitamin D at baseline had greater longitudinal increases in $1,25(OH)_2D$ in comparison to women with sufficiency in the adjusted model (P=0.006), but not among women with deficient 25(OH)D concentrations (P=0.364) (**Fig. 5 and Online Resource Table 1)**. We did not observe interactions between vitamin D status at baseline according to IOM and $1,25(OH)_2D$ concentrations during pregnancy (**data not shown**).

Discussion

The present study has three main findings. First, it was observed that the prevalence of vitamin D inadequacy varied significantly according to the adopted criteria. The prevalence of vitamin D inadequacy was high, especially in the first trimester using the Endocrine Society Practice Guidelines (<75 nmol/L). It was moderate when the inadequacy criterion from the IOM (<50 nmol/L) was considered, but virtually non-existent when the IOM deficiency criterion was employed (<30 nmol/L). Second, it was observed that the longitudinal patterns of 25(OH)D concentrations during pregnancy were modified by the season at recruitment. Finally, when we stratified the participants according to vitamin D status at baseline, pregnant women who had vitamin D insufficiency (50-<75 nmol/L) had a greater increase in $1,25(OH)_2D$ concentrations throughout pregnancy compared to women with 25(OH)D adequacy (≥75 nmol/L).

There is no consensus for the definition of vitamin D inadequacy for pregnant women and results should be interpreted cautiously. We used the cut-off values proposed by the Endocrine Society Practice Guidelines [4] and the IOM [14] to allow comparisons among studies. Our results revealed that the prevalence of 25(OH)D <75 nmol/L, <50 nmol/L and <30 nmol/L at the first trimester was 70.4%, 16.1% and 2.1%, respectively. Studies with pregnant women that have used the 25(OH)D cut points of <75 nmol/L also found a high prevalence of vitamin D inadequacy in countries such as Korea (91.4%) [31], Spain (64.1%) [32], the United States (54.4%) [33], and other sunny regions such as Thailand (75.5%) [7], and Australia (80.4%) [6]. Results from a systematic review and meta-analysis revealed that the prevalence of 25(OH)D <50 nmol/L during pregnancy was greater in regions such as the Americas (64%), Europe (57%), Eastern Mediterranean (46%), South-East Asian (87%) and Western Pacific countries (83%), in comparison to the present study [34]. In contrast, it is worth noting that only 2% of women in this cohort presented 25(OH)D concentrations <30 nmol/L, a more restrictive cut-off point that classifies vitamin D deficiency according to the IOM criteria. A higher prevalence of 25(OH)D <30 nmol/L have been reported in

205 countries such as Australia (15%), Belgium (12%), the Netherlands (23%) and India (60%) [2]. Kiely et al. (2016)
206 [35] observed in a large cohort from Ireland that 17% of the pregnant women had 25(OH)D <30 nmol/L, while
207 Haggarty et al. (2013) [36] observed in a cohort study from Scotland that 21.5% had 25(OH)D values <25 nmol/L.
208 The mean 25(OH)D concentration observed in our study (65.0 nmol/L) in early pregnancy is higher than other
209 larger cohorts with pregnant women from countries located at higher altitudes such as Ireland (56.7 nmol/L) and
210 Scotland (40.2 nmol/L) [35,36], but is comparable to other studies in sunny regions [2,6,7].

211 We found only one study comprising healthy pregnant women in Brazil that has addressed a vitamin D
212 research question. This study was a clinical trial with 26 pregnant adolescents in the placebo group and 30 in the
213 supplemented group (calcium and vitamin D) and found plasma 25(OH)D concentrations of 57.9 (20.7 SD) nmol/L
214 and 59.5 (20.6 SD) nmol/L, respectively at baseline (second trimester of gestation) [37]. For the same period of
215 gestation those concentrations were lower than the observed in our sample [78.7 (22.0 SD) nmol/L].

216 Some other factors could explain the higher prevalence of vitamin D inadequacy observed in this cohort,
217 Air pollution has been inversely associated with 25(OH)D concentration [5]. Air pollution is known to absorb
218 ultraviolet B (UVB) rays and thereby limit cutaneous synthesis of vitamin D [5,38). Rio de Janeiro is known to have
219 high pollution with particulate matter less than 10 μm in diameter (PM10) estimated to be 67 $\mu g/m^3$ in 2010 [39].
220 This value is above the World Health Organization recommendations (<20 $\mu g/m^3$ annual average concentrations)
221 [40]. We hypothesize that air pollution in Rio de Janeiro plays a role in the observed high prevalence of vitamin D
222 inadequacy in this cohort.

223 We found a mean vitamin D intake of only 186.8 IU/day, which is well below the recommendations set
224 forth for pregnant women by both the Endocrine Society Practice Guidelines (1500 - 2000 IU/day) [4] and the IOM
225 (600 IU/day)[14]. Inadequate dietary intake during pregnancy has been observed in other countries such as the
226 United States [41], Iran [5] and Thailand [42]. Few food items naturally contain vitamin D in the Brazilian diet.
227 Also, there is no national mandatory food fortification or supplementation program for vitamin D [23]. The mean
228 calcium intake (760.2 mg/day) was also below the IOM recommendation (1,000 mg/day) for pregnant women.
229 Similar results of low vitamin D and calcium intake were also observed in a systematic review and meta-analysis
230 during pregnancy in regions such as the United States, Europe and Australia [43]. Finally, none of the women from
231 our sample reported vitamin D and calcium supplementation during pregnancy. In Brazil, vitamin D and calcium
232 supplementation are not usual among women receiving pre-natal care in public service [44].

233 Brazil is a racially diverse country, resulting in a mixed skin pigmentation of the population, which can
234 affect endogenous production of vitamin D via sun exposure [18,45]. In the current study, 26.7% self-reported to be

blacks and 46.2% mixed. Low vitamin D status has been found among obese adults, because vitamin D can be partially sequestered by body fat [46]. However, we did not find associations between vitamin D status with vitamin D and calcium intake, skin pigmentation, or pre-pregnancy BMI.

Physiological adjustment of vitamin D metabolism occurs during pregnancy. The activity of the 1α-hydroxylase enzyme is increased in the kidney, placenta and decidua [47-48], and an elevation in plasma vitamin D-binding protein (DBP) is observed [19]. The kidney has megalin which internalizes 25(OH)D-DBP resulting in the release of 25(OH)D for its conversion to $1,25(OH)_2D$ [19,49]. Moreover, the expression of vitamin D receptor (VDR) may be increased in the placenta and decidua during pregnancy [50]. These changes are important for the maternal and fetal requirements of $1,25(OH)_2D$ during this period [4].

Few prospective studies have previously measured longitudinal changes in vitamin D concentrations in pregnant women. During this period an increase in $1,25(OH)_2D$ is well reported while changes in 25(OH)D concentrations remain controversial [20-22]. Lee et al. [22] evaluated a sample of 275 Korean pregnant women and found that the mean 25(OH)D concentration during the first trimester was significantly lower than in the second and third trimesters, even after adjusting for season. Lundqvist et al. [51] reported that 25(OH)D concentrations increased slightly over the duration of pregnancy in 184 Swedish women. However, when the authors considered the months of recruitment, a lower rise of 25(OH)D concentrations during pregnancy was observed between women that started the study in summer, and a peak-shaped pattern during winter. On the other hand, Zhang et al. [21] evaluated 30 Irish women at several pregnancy weeks (15, 20, 24, 28, 32, 36 and 40) and reported that 25(OH)D concentrations decreased during this period. However, these women were recruited only during summer. Fernandez-Alonso et al. [52] also observed a decrease in 25(OH)D concentrations from first to third trimester in 148 Spanish pregnant women considering the effect of season. We found that women who started the study during spring, winter and autumn increased 25(OH)D concentration over this period. The women that began the study during summer had high 25(OH)D concentrations, and thus presented no change during pregnancy.

The increase of $1,25\ (OH)_2D$ can be probable attributed to an increase on the metabolism of 25(OH)D and thus, resulting in the decreased in the 25(OH)D [19,49,53]. However, we suggest that the elevation of DBP [19] during pregnancy could prolong the 25(OH)D half-life during this period as a form of protection [53-54]. Thus, besides the influence of season women that begin the study with higher or lower concentrations of 25(OH)D can maintain or increase this metabolite during pregnancy, respectively [53-54].

Brazil is a tropical country with abundant sunlight and the State of Rio de Janeiro, situated at approximately 23ºS latitude, favors conditions for cutaneous vitamin D production. The ultraviolet B radiation is high throughout

the year and reaches maximum values in summer [23,55]. Therefore, high concentrations of 25(OH)D are expected between women who started the study during summer is plausible, even in a sunny region, as has been reported in other studies [15,42]. Furthermore, in the summer, people are more exposed to the sunlight, and do more outdoor leisure activities, such as going to the beach, which, besides being a cultural habit, is also accessible to the low-income population. The seasonal variation of vitamin D is even greater in regions with higher latitudes and well-defined seasons, as for example in pregnant woman resident in European countries as Ireland (52°N) or Spain (40°N), with lower concentrations of vitamin D in winter [21,52,56].

In a systematic review and meta-analysis involving twenty studies, the authors found that serum $1,25(OH)_2D$ was not related with 25(OH)D in pregnant women at term, and that the 25(OH)D concentrations were not different from the concentrations found in non-pregnant women, though the $1,25(OH)_2D$ concentrations were twice as high than the non-pregnant women [20]. Nevertheless, this study considered pregnant women at term and was comprised of cross-sectional data only. Hollis et al. (2011) [41] in a randomized controlled trial with vitamin D supplementation found an association between circulating $1,25(OH)_2D$ concentrations and circulating 25(OH)D in 148 pregnant women evaluated at 12, 16, 20, 24, 28, 32 and 36 weeks. However, concentrations of 25(OH)D of at least 100 nmol/L were needed to enable a maximum $1,25(OH)_2D$ increase during this period. Young et al. (2012) [57] observed that 25(OH)D measured at mid-gestation (~26 weeks) was inversely associated with $1,25(OH)_2D$ at delivery in a sample with 168 pregnant adolescents.

In the present study, we did not find a longitudinal association between 25(OH)D and $1,25(OH)_2D$.We observed that pregnant women with vitamin D insufficiency at baseline according to Endocrine Society Practice Guidelines had a greater increase in $1,25(OH)_2D$ concentrations throughout pregnancy when compared to women with sufficient 25(OH)D concentrations. These results indicate that there is a possible mechanism to meet the additional demands of vitamin D among pregnant women with low concentrations of 25(OH)D in early pregnancy. This may be due to secondary hyperparathyroidism associated with low plasma 25(OH)D, thereby increasing the renal production of $1,25(OH)_2D$ [3-4]. Another possible mechanism is that there are four different 1α-hydroxylase enzymes with distinct concentrations of regulation, one of which sufficiently converts even small amounts of vitamin D efficiently to 25(OH)D [58-59]. Moreover, considering that there is an increased activity of 1α-hydroxylase enzyme [47-48], DBP concentrations [19] and VDR expression during gestation [50], we suggest that these changes are more pronounced among pregnant women with 25(OH)D inadequacy. This may be a form of protection to prevent further reductions in the 25(OH)D concentration during the course of pregnancy. We believe

294 that with a larger sample size this pattern would have also occurred in women with vitamin D insufficiency at
295 baseline according to cut points by IOM.

296 The current study has some limitations. First, information on individual sun exposure was not collected.
297 Instead, we considered the season at recruitment. Second, the loss to follow up was 14.6% and 13.5% for 25(OH)D
298 and $1,25(OH)_2D$, respectively. Despite the losses to follow-up, we did not identify significant differences in socio-
299 demographic, lifestyle and reproductive characteristics between pregnant women with vitamin D data in all
300 trimesters or those with data in at least one trimester. Other limitation of this study is the lack of assessment of
301 serum PTH, calcium, phosphorus and DBP. On the other hand, this cohort study has important strengths. Plasma
302 $1,25(OH)_2D$ and 25(OH)D concentrations were evaluated in each of the three pregnancy trimesters, and when
303 performing the longitudinal models, our analyses were adjusted for several important confounders. 25(OH)D and
304 $1,25(OH)_2D$ were also analyzed by the gold standard method (LC-MS/MS).

305 Low concentrations of vitamin D during pregnancy may be associated with increased risk of adverse
306 maternal outcomes as gestational diabetes mellitus and low birth weight [5,8]. Further, there is a high correlation
307 between maternal and fetal cord blood 25(OH)D concentrations [34]. Studies have suggested that low vitamin D
308 status during pregnancy is associated with short and long-term newborn health consequences [5,8,60-61]. According
309 to the theory of developmental origins of diseases (fetal programming), nutrition allows early life adaptation when
310 there is an adverse environment. Thus, low concentrations of vitamin D during pregnancy can have effects
311 throughout life via fetal programming [62], as for example negative impact in brain health, inflammation and
312 respiratory disorders [61,63-64].

313 The prevalence of vitamin D inadequacy was high throughout pregnancy. Our results indicate that there is a
314 need to examine the vitamin D status of pregnant women residing in other areas of Brazil who may have less sun
315 exposure. There were different patterns of longitudinal 25(OH)D concentrations according to the season at
316 recruitment. Although an increase in $1,25(OH)_2D$ was observed, these changes were not sufficient to reach vitamin
317 D sufficiency in a high proportion of women at the level of the current recommendations for adults. Pregnant
318 women who had insufficient 25(OH)D at the beginning of pregnancy had a higher increase in $1,25(OH)_2D$ across
319 trimesters than those women with sufficient baseline 25(OH)D concentrations, after controlling for important
320 confounders. The findings from this prospective cohort conducted in apparently healthy women from a tropical and
321 sunny region contributes to the understanding of 25(OH)D and $1,25(OH)_2D$ changes during pregnancy and
322 highlights the importance of vitamin D sufficiency in early pregnancy. Our study has the potential for generating
323 new evidence since there are few studies that prospectively evaluated plasma $1,25(OH)_2D$ and 25(OH)D

concentrations in healthy pregnant women. Further studies are necessary to set the appropriate 25(OH)D cut points to compensate for the increased physiological requirements of vitamin D during pregnancy

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Ethical standards

The Research Ethics Committees of the Municipal Secretariat of Health and Civil Defense of the State of Rio de Janeiro (Protocol number: 0012.0.249.000-09) approved the present study. Written consent from all participants was obtained freely and spontaneously, after all necessary clarifications were provided in accordance with principles of the Declaration of Helsinki.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Baseline characteristics according to vitamin D status in women followed at a public health center in Rio de Janeiro, Brazil, 2009-2012.

**Notes: ***P-value refers to ANOVA test or to chi-squared test.

[a, b, c] Different letters at the same line indicate statistically significant difference (P<0.05) between categories of vitamin D using Bonferroni post hoc test. 25(OH)D=25-hydroxyvitamin D; $1,25(OH)_2D$=1,25-dihydroxyvitamin D; BMI=Body Mass Index.

Fig. 1 Frequency of vitamin D status according to trimester of pregnancy in women followed at a public health center in Rio de Janeiro, Brazil, 2009-2012.

Notes: [a] Vitamin D status according to the Endocrine Society Practice Guidelines, vitamin D inadequacy refers to the sum of vitamin D deficient and insufficient (25(OH)D <75 nmol/L). [b] Vitamin D status according the Institute of Medicine, vitamin D inadequacy refers to the sum of vitamin D deficient and insufficient (25(OH)D <50 nmol/L).

Fig. 2 Changes in plasma 25(OH)D and 1,25$(OH)_2$D concentrations during pregnancy in women followed at a public health center in Rio de Janeiro, Brazil, 2009-2012.

Notes: [a] Fitted values were predicted using an unadjusted longitudinal linear regression model between 25(OH)D concentration (nmol/L) and gestational age (weeks) (n=225 groups, n=565 observations). [b] Fitted values were predicted using an unadjusted longitudinal linear regression model between 1,25$(OH)_2$D concentration (pmol/L) and gestational age (weeks) (n=214 groups, n=522 observations). CI=confidence interval; 25(OH)D=25-hydroxyvitamin D; 1,25$(OH)_2$D=1,25-dihydroxyvitamin D. The group refers to the number of women with at least one data point in time and observation refers to the total number of data points in time for all women.

Fig. 3 Mean in plasma 25(OH)D concentration according to first, second and third trimester of pregnancy and season of recruitment into the study among women followed at a public health center in Rio de Janeiro, Brazil, 2009-2012.

Notes: Results are presented as mean and standard deviation (error bars); winter=June 21st to September 21st; spring=September 22nd to December 20th; summer=December 21st to March 19th; autumn=March 20th to June 20th; 25(OH)D=25-hydroxyvitamin D.

Fig. 4 Changes in plasma 25(OH)D concentration during pregnancy according to seasons at recruitment in women followed at a public health center in Rio de Janeiro, Brazil, 2009-2012.

Notes: Interaction between season at first trimester and gestational age: summer (n=42: reference); winter (n=51): β=1.441; 95% CI, 1.066 to 1.816, P<0.001; spring (n=54): β=1.126; 95% CI, 0.758 to 1.493, P<0.001; autumn (n=52)*:* β=0.398*;* 95% CI, 0.044 to 0.752, P=0.028. Longitudinal model adjusted for parity, education, self-reported skin color and pre-pregnancy Body Mass Index. The shaded grey area represents the 95% CI. The black lines at the bottom of the figure represent the scatter of the data. winter=June 21st to September 21st; spring=September 22nd to December 20th; summer=December 21st to March 19th; autumn=March 20th to June 20th. 25(OH)D=25-hydroxyvitamin D; 1,25$(OH)_2$D=1,25-dihydroxyvitamin D; β=Longitudinal Linear Regression Coefficient; CI=Confidence Interval.

Fig. 5 Changes in plasma 1,25$(OH)_2$D concentration during pregnancy according to vitamin D status at baseline in women followed at a public health center in Rio de Janeiro, Brazil, 2009-2012.

Notes: Interaction between vitamin D insufficiency (50-<75 nmol/L) (n= 97) and gestational age: β=2.365; 95% CI, 0.675 to 4.054; P=0.006. Longitudinal model adjusted for seasons at recruitment, parity, education, self-reported skin color and pre-pregnancy Body Mass Index. The shaded grey area represents the 95% confidence interval. The black lines at the bottom of the figure represent the scatter of the data. 1,25$(OH)_2$D=1,25-dihydroxyvitamin D; β=longitudinal linear regression coefficient, CI=Confidence Interval.

Online Supporting Material

Online Resource Fig. 1 Flowchart of the selection process of study final sample of pregnant woman followed at a public health center in Rio de Janeiro, Brazil, 2009-2012.

Notes: [a] 25(OH)D, total number of observations (data)=565 and total number of groups (women=225). [b] $1,25(OH)_2D$, total number of observations=522 and total number of groups=214. All women with information for $1,25(OH)_2D$ also present data from 25(OH)D concentrations. 25(OH)D=25-hydroxyvitamin D; $1,25(OH)_2D$=1,25-dihydroxyvitamin D. The group refers to the number of women with at least one data point in time and observations refers to the total number of data points in time for all women.

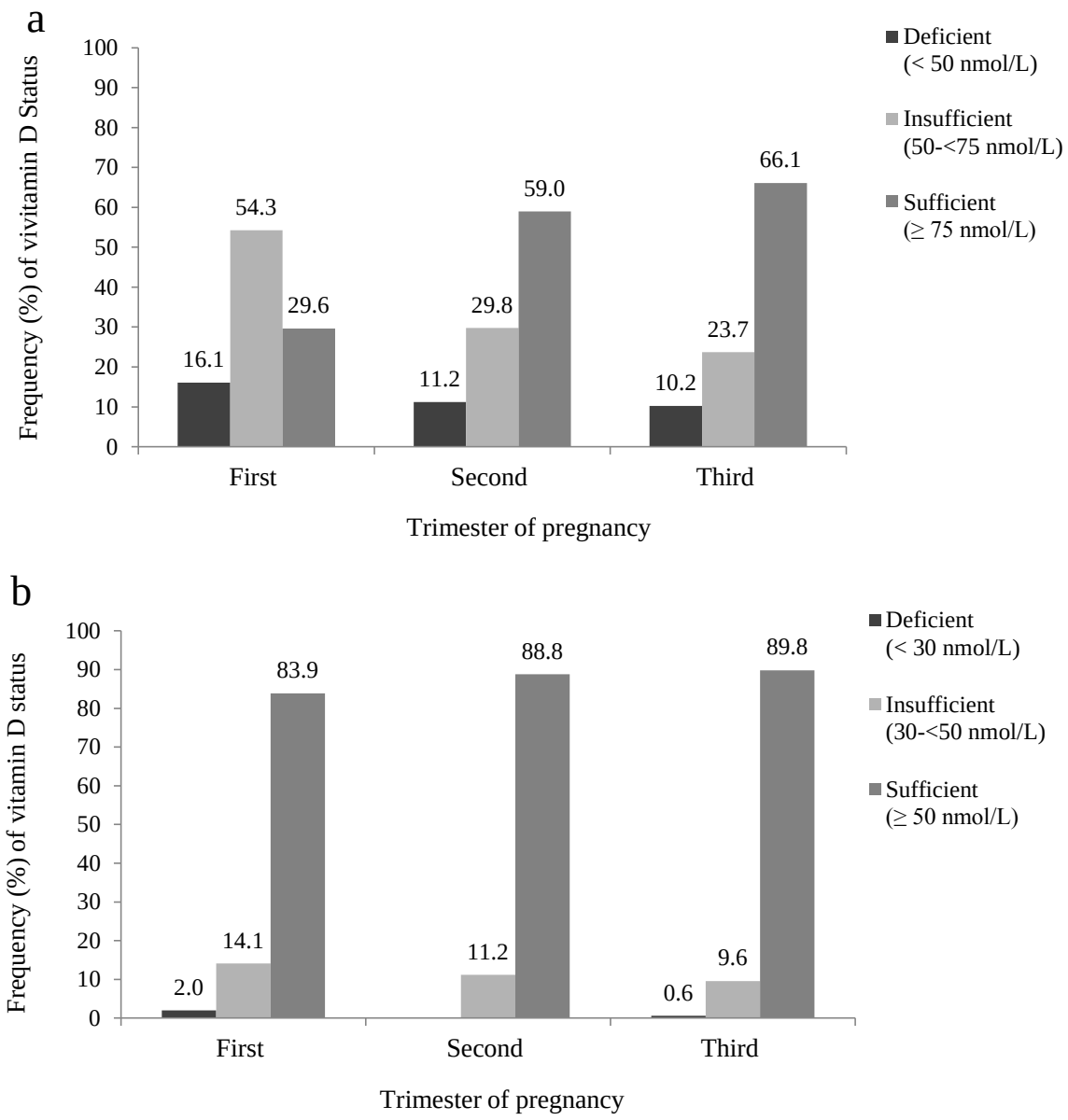
Online Resource Fig. 2 Correlation between 1,25$(OH)_2$D and 25(OH)D concentrations during first (a), second (b), and third (c) trimester in women followed at a public health center in Rio de Janeiro, Brazil, 2009-2012.

Notes: [a] first trimester (n=178), [b] second trimester (n=177), [c] third trimester (n=167). 25(OH)D=25-hydroxyvitamin D; 1,25$(OH)_2$D=1,25-dihydroxyvitamin D.

Online Resource Table 1. Confounders estimates in the longitudinal model of plasma 25(OH)D and 1,25$(OH)_2$D concentrations during pregnancy in women followed at a public health center in Rio de Janeiro, Brazil, 2009-2012.

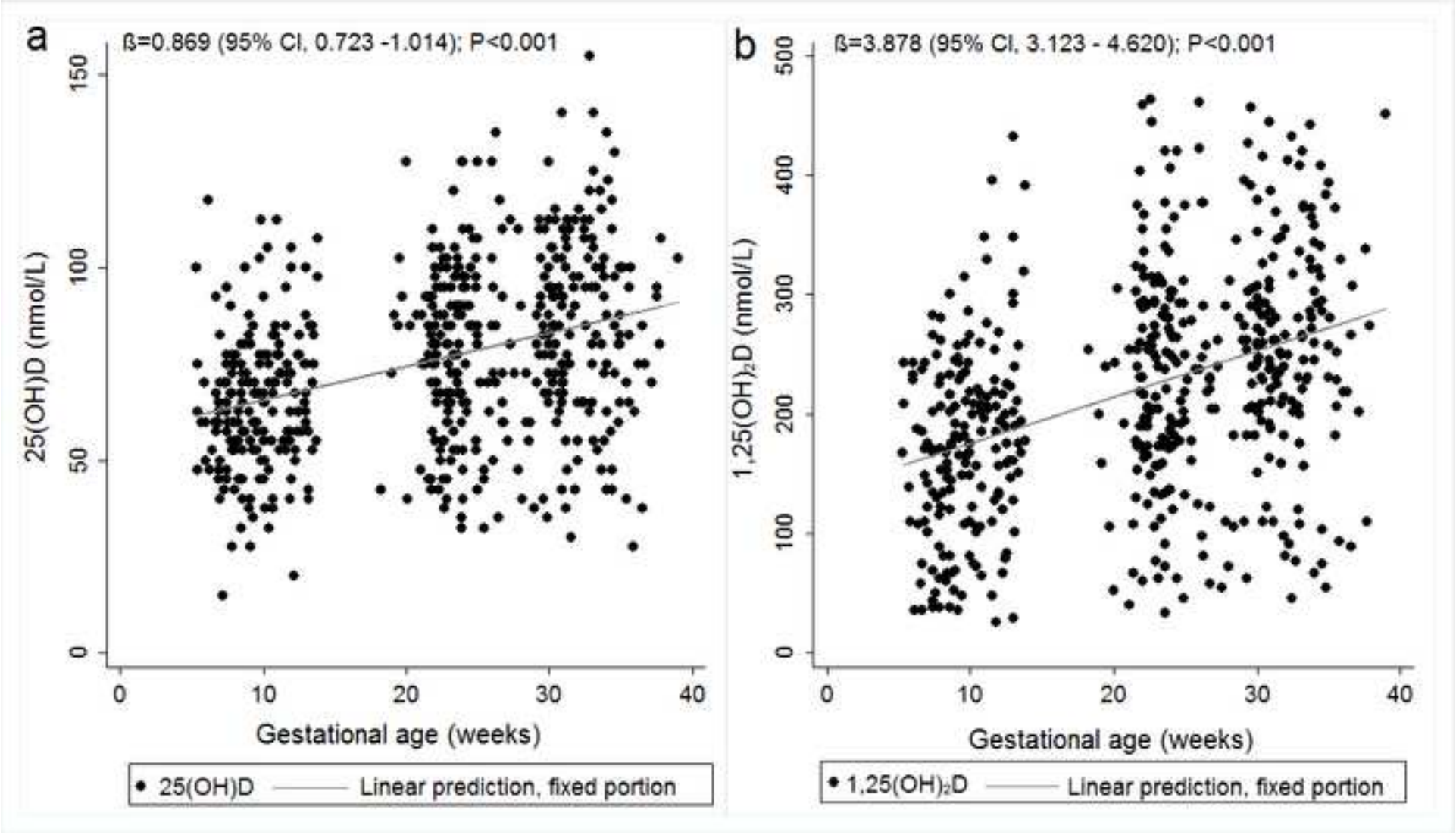
Notes: Longitudinal linear regression coefficient (β), 95% confidence interval (CI) and P were calculated using linear mixed effects; winter=June 21st to September 21st; spring=September 22nd to December 20th; summer=December 21st to March 19th; autumn=March 20th to June 20th.; 25(OH)D=25-hydroxyvitamin D;1,25$(OH)_2$D=1,25-dihydroxyvitamin D; BMI=Body Mass Index.

Figure

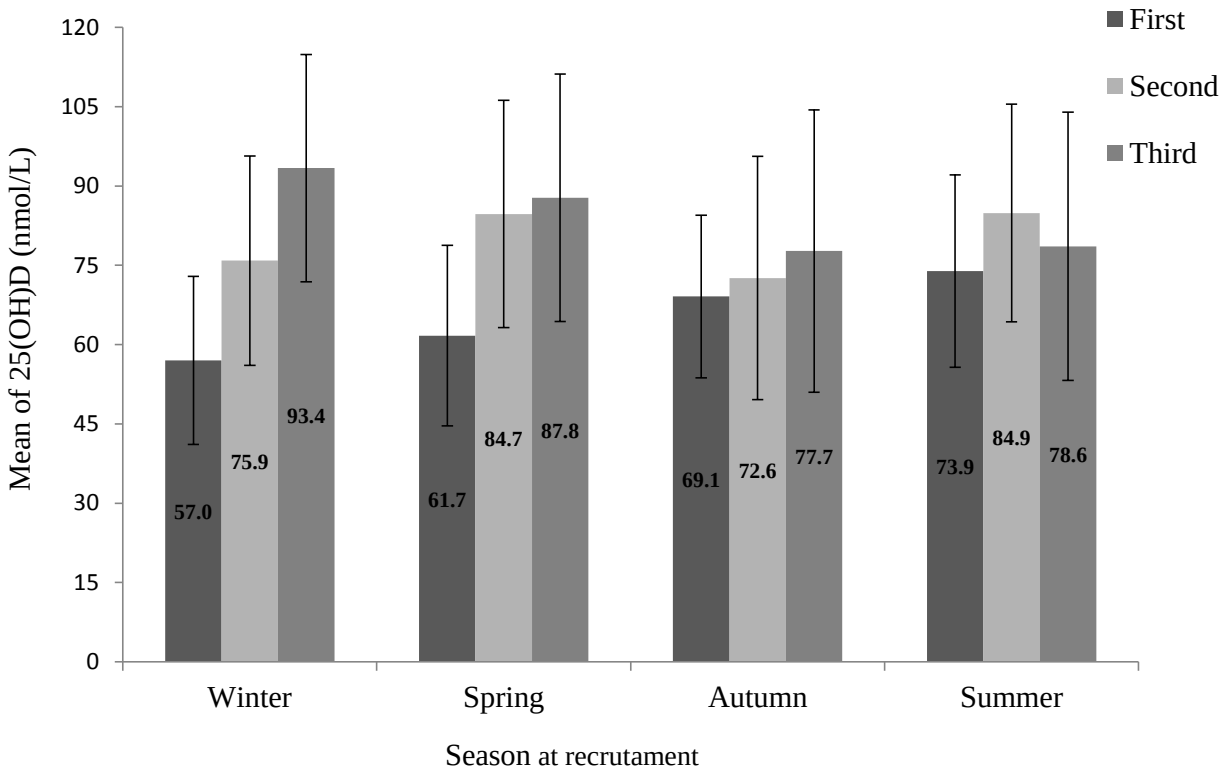

a
Frequency (%) of vivitamin D Status
100
90
80
70
60
50
40
30
20
10
0
16.1
54.3
29.6
11.2
29.8
59.0
10.2
23.7
66.1
First
Second
Third
Trimester of pregnancy
Deficient (< 50 nmol/L)
Insufficient (50-<75 nmol/L)
Sufficient (≥ 75 nmol/L)
b
Frequency (%) of vitamin D status
100
90
80
70
60
50
40
30
20
10
0
2.0
14.1
83.9
11.2
88.8
0.6
9.6
89.8
First
Second
Third
Trimester of pregnancy
Deficient (< 30 nmol/L)
Insufficient (30-<50 nmol/L)
Sufficient (≥ 50 nmol/L)

Figure

Click here to download Figure figure 2 ab.tif

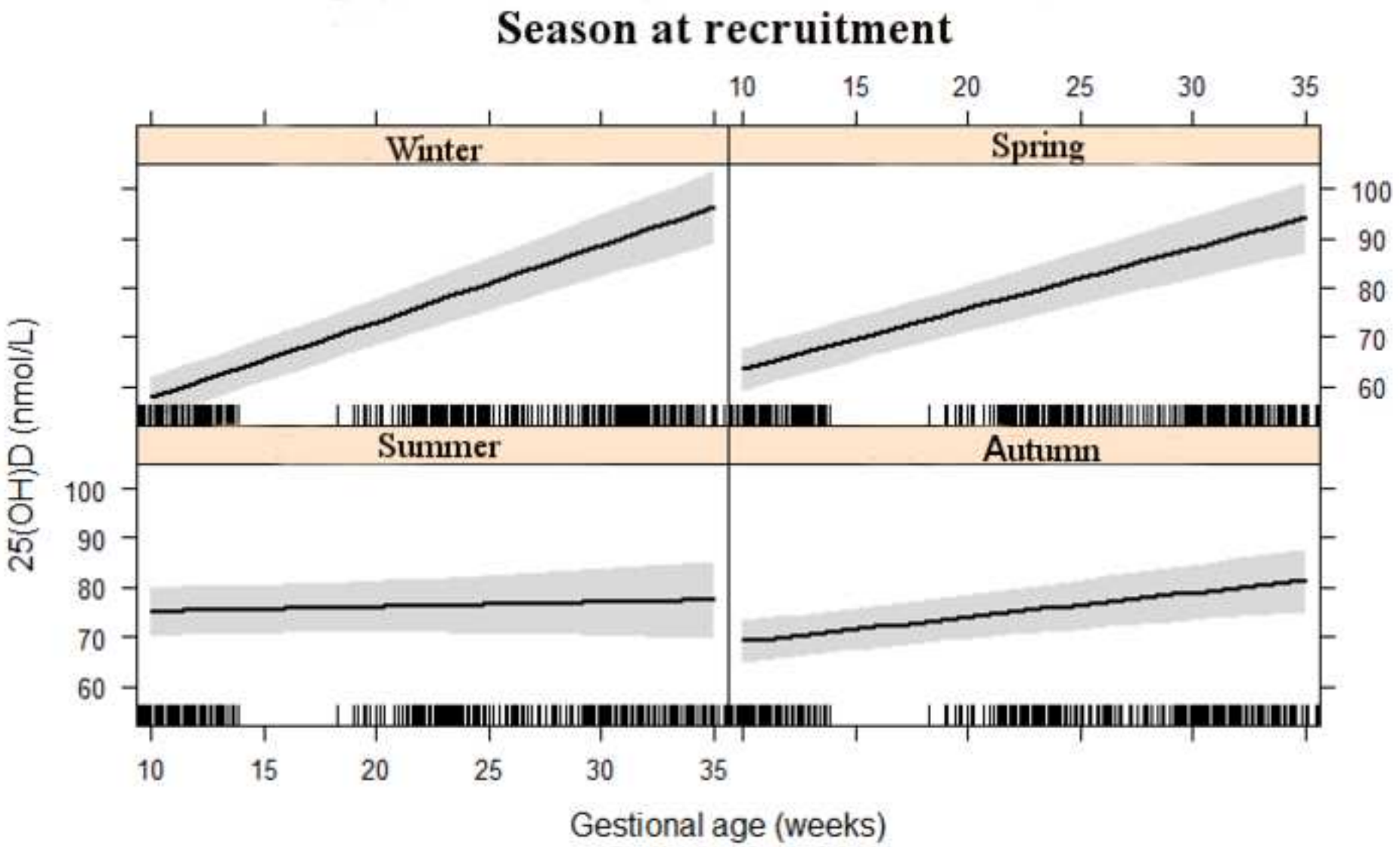


Figure


First
Second
Third
Mean of 25(OH)D (nmol/L)
120
105
90
75
60
45
30
15
0
57.0
75.9
93.4
61.7
84.7
87.8
69.1
72.6
77.7
73.9
84.9
78.6
Winter
Spring
Autumn
Summer
Season at recrutament

Figure

Click here to download Figure Fig4..png



Figure

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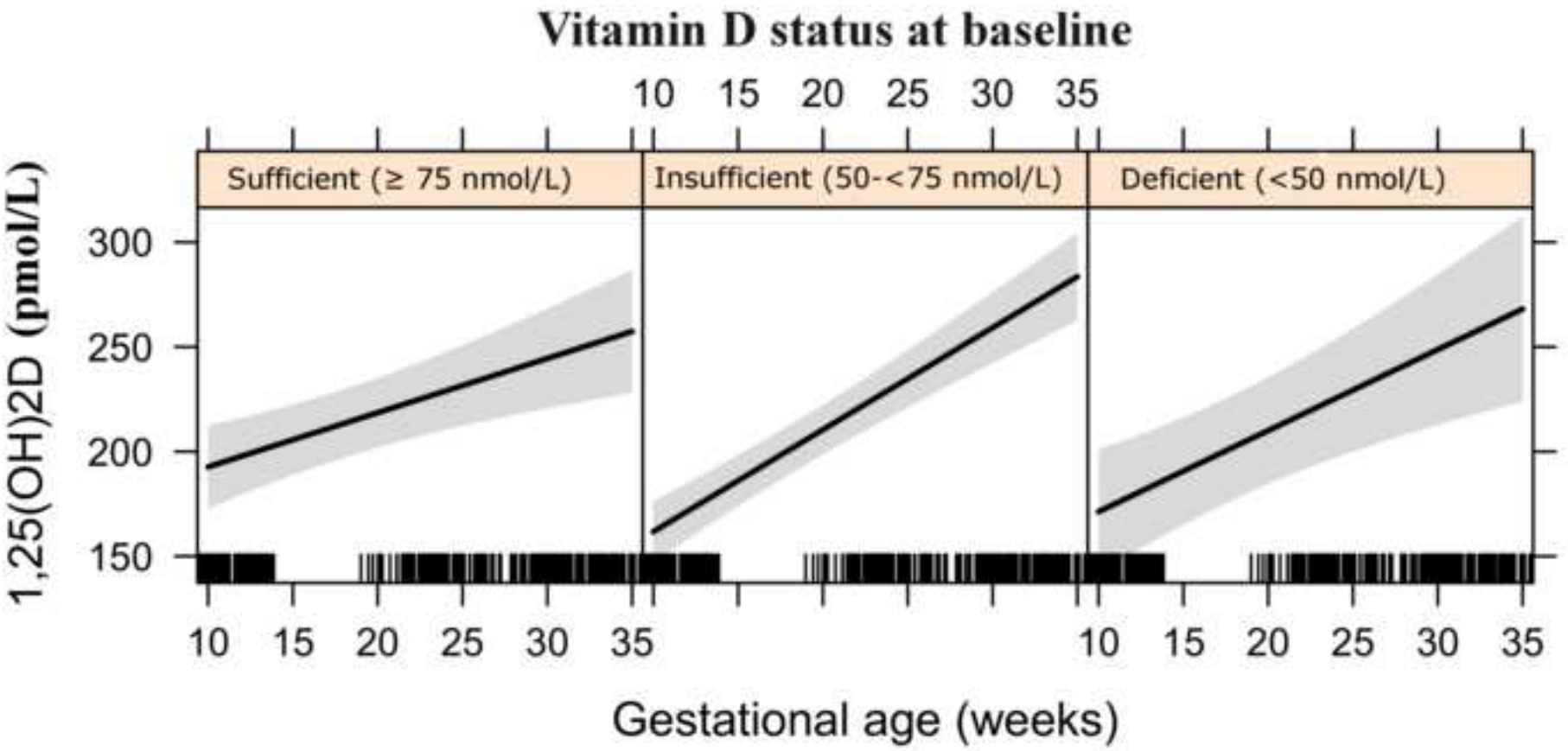

Table

Variables	Total n=199	Vitamin D status at baseline							
		Endocrine Society Practice Guidelines			P-value*	Institute of Medicine			P-value*
		Deficient < 50 nmol/L n=32	Insufficient 50-<75 nmol/L n=108	Sufficient ≥ 75 nmol/L n=59		Deficient < 30 nmol/L n=4	Insufficient 30-<50 nmol/L n=28	Sufficient ≥ 50 nmol/ n=167	
		Mean (SD)							
25(OH)D (nmol/L)									
First trimester	65.0 (17.7)	39.6 (8.0) [a]	61.2 (6.7) [b]	85.8 (11.3) [c]	<0.001	22.5 (6.1) [a]	42.1 (4.5) [b]	69.9 (14.6) [c]	<0.001
Second trimester	78.7 (22.0)	61.3 (17.6) [a]	76.2 (19.3) [b]	94.9 (18.7) [c]	<0.001	35.0 (2.5) [a]	64.8 (15.8) [b]	82.6 (21.0) [c]	<0.001
Third trimester	84.1 (24.5)	70.5 (20.9) [a]	81.1 (25.0) [a]	96.1 (22.3) [b]	<0.001	40.0 (3.5) [a]	73.5 (19.4) [a]	86.3 (25.1) [b]	0.004
$1,25(OH)_2D$ (pmol/L)									
First trimester	173.4 (77.9)	160.4 (65.1) [a]	158.9 (73.6) [a]	203.7 (82.5) [b]	0.001	115.2 (79.2)	166.8 (62.4)	175.4 (79.8)	0.384
Second trimester	227.6 (91.9)	229.1 (97.5)	224.7 (93.1)	221.3 (88.3)	0.948	231.2 (50.7)	228.8 (104.3)	223.5 (91.2)	0.965
Third trimester	257.5 (92.3)	241.2 (111.5)	267.7 (90.7)	260.4 (72.9)	0.512	180.0 (115.3)	265.2 (84.8)	226.9 (112.1)	0.328
Age (years)	26.6 (5.5)	26.8 (5.6)	26.7 (5.5)	26.3 (5.5)	0.876	28.5 (8.1)	26.6 (5.3)	26.6 (5.5)	0.795
Per-capita family income (R$)	538.7 (328.8)	599.5 (403.9)	506.3 (314.7)	495.8 (295.2)	0.452	741.5 (326.7)	578.5 (415.2)	527.0 (312.4)	0.348
Dietary vitamin D intake during pregnancy (IU/day)	186.8 (109.9)	169.6 (91.7)	187.4 (108.8)	196.4 (122.7)	0.607	241.3 (139.4)	161.3 (84.8)	190.4 (113.2)	0.320
Dietary calcium intake during pregnancy (mg/day)	774.5 (361.1)	748.5 (313.3)	760.3 (366.0)	819.5 (382.5)	0.589	752.4 (504.3)	748.0 (299.3)	780.2 (371.2)	0.912
		n (%)							

Vitamin D supplementation during gestation					1.000				1.000
No	199 **(**100)	32 (100)	108 (100)	75 (100)		4 (100)	28 (100)	167 (100)	
Yes	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)	
Calcium supplementation during gestation					1.000				1.000
No	199 (100.0)	32 (100.0)	108 (100.0)	59 (100.0)		4 (100.0)	28 (100.0)	167 (100.0)	
Yes	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)	
Season at recruitment					<0.001				0.010
Winter	51 (25.6)	15 (46.9)	30 (27.8)	6 (10.2)		2 (50.0)	13 (46.4)	36 (21.6)	
Spring	54 (27.2)	12 (37.5)	29 (26.9)	13 (22.0)		2 (50.0)	10 (35.7)	42 (25.1)	
Summer	42 (21.1)	2 (6.2)	21 (19.4)	19 (32.2)		0 (0.0)	2 (7.2)	40 (24.0)	
Autumn	52 (26.1)	3 (9.4)	28 (25.9)	21 (35.6)		0 (0.0)	3 (10.7)	49 (29.3)	
Pre-pregnancy BMI ($kg/m^{2)}$ [b]					0.900				0.872
<25	119 (59.80)	18 (56.2)	65 (60.2)	36 (61.0)		2 (50.0)	16 (57.1)	101 (60.5)	
≥25	80 (40.20)	14 (43.8)	43 (39.8)	23 (39.0)		2 (50.0)	12 (42.9)	66 (39.5)	
Alcohol consumption					0.224				0.203
No	158 (79.4)	29 (90.6)	84 (77.7)	45 (76.3)		0 (0.0)	3 (10.7)	38 (22.8)	
Yes	41 (20.6)	3 (9.4)	24 (22.3)	14 (23.7)		4 (100.0)	25 (89.3)	129 (77.2)	
Smoking habit					0.687				0.671
No	186 (93.5)	31 (96.9)	100 (92.6)	55 (93.2)		4 (100.0)	27 (96.4)	155 (92.8)	
Yes	13 (6.5)	1 (3.1)	8 (7.4)	4 (6.8)		0 (0.0)	1 (3.6)	12 (7.2)	
Education (years)					0.207				0.207
<8	60 (30.2)	8 (25.0)	29 (26.8)	23 (39.0)		9 (25.0)	29 (26.9)	33 (39.0)	
≥8	139 (69.8)	24 (75.0)	79 (73.2)	36 (61.0)		24 (75.0)	79 (73.1)	36 (61.0)	
Self-reported skin color					0.268				0.306

White	54 (27.1)	4 (12.5)	35 (32.4)	15 (25.4)		0 (0.0)	4 (14.3)	50 (29.9)	
Mixed	92 (46.2)	18 (56.2)	47 (43.5)	27 (45.8)		3 (75.0)	15 (53.6)	74 (44.3)	
Black	53 (26.7)	10 (31.3)	26 (24.1)	17 (28.8)		1 (25.0)	9 (32.1)	43 (25.8)	
Leisure physical activity before pregnancy					0.213				0.342
No	148 (75.1)	26 (83.9)	82 (76.6)	40 (67.8)		4 (100.0)	22 (81.5)	122 (73.5)	
Yes	49 (24.9)	5 (16.1)	25 (23.4)	19 (32.2)		0 (0.0)	5 (18.5)	44 (26.5)	
Parity					0.322				0.393
Nulliparous	78 (39.2)	16 (50.0)	42 (38.9)	20 (33.9)		2 (50.0)	14 (50.0)	62 (37.1)	
Parous	121 (60.8)	16 (50.0)	66 (61.1)	39 (66.1)		2 (50.0)	14 (50.0)	105 (62.9)	