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Calcium plus vitamin D supplementation during pregnancy interacts with polymorphisms in the promoter region of the *VDR* gene to affect postpartum bone mass of Brazilian adolescent mothers: A randomized controlled trial

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ABSTRACT

Objective: We investigated whether calcium plus vitamin D supplementation interacts with polymorphisms in the *VDR* gene promoter region to affect changes on maternal bone mass from 5 to 20 wk postpartum in Brazilian adolescent mothers.
Methods: Pregnant adolescents (14–19 y) randomly received calcium plus cholecalciferol (600 mg/d + 200 IU/d, n = 30) or placebo (n = 26) from 26 wk of pregnancy until parturition. Bone mineral content (BMC), bone area (BA), and bone mineral density (BMD) at total body, lumbar spine, total hip, and femoral neck were evaluated at 5 and 20 wk postpartum. Serum 25-hydroxyvitamin D (25[OH] D) and parathyroid hormone concentrations were measured. Real-time polymerase chain reaction was used for genotyping rs7139166 (1521 pb G > C) and rs4516035 (1012 pb A > G). Interactions between supplementation and polymorphisms were adjusted for significant covariates.
Results: Changes in serum 25(OH)D from pregnancy to postpartum differed between supplemented and placebo groups for mothers carrying 1521 GG/1012 AA genotypes ($P = 0.004$). Only in the placebo group, mothers carrying 1521 GG/1012 AA had greater reduction in total BMD *z* score, femoral neck BMC, and BMD from 5 to 20 wk postpartum compared with those with 1521 GC/1012 AG ($P < 0.05$). In the placebo group, total hip BA decreased from 5 to 20 wk postpartum in adolescents with 1521 GG/1012 AA, but increased in those with 1521 GC/1012 AG ($P < 0.05$), in contrast to the supplemented group.
Conclusion: Calcium plus vitamin D supplementation during pregnancy interacted with polymorphisms in the *VDR* gene promoter region affecting postpartum bone loss. The increased supply of calcium and vitamin D appeared to minimize postpartum bone loss particularly in adolescents with 1521 GG/1012 AA.

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Introduction

The recognized increased calcium (Ca) demand during pregnancy and lactation is primarily achieved by maternal physiological adaptations, including highly efficient intestinal absorption during pregnancy and lactation-associated renal Ca conservation and bone loss [1,2]. These mechanisms seem to operate in both adult and adolescent mothers. However, in adolescents, there is evidence that these physiological adaptations may not be sufficient to ensure adequate maternal bone accumulation, together with appropriate fetal growth and milk production [3,4], especially when habitual intake of calcium is low [4,5]. Furthermore, it is possible that, in adolescents, maternal bone loss during lactation is not completely recovered after weaning [4].

Calcium supplementation during pregnancy does not seem to prevent bone loss during lactation in adult women [6,7] and depending on the population, it may even result in higher bone loss [8]. In Gambian mothers with very low habitual calcium diets (~300 mg/d), supplementation with Ca during pregnancy (1500 mg/d, from 20 wk of pregnancy until parturition) resulted in lower bone mineral content (BMC), bone area (BA), and bone mineral density (BMD) at the hip throughout 12 mo of lactation, and also in higher bone mobilization of the lumbar spine and distal radius during lactation, compared with the placebo [8]. In contrast, in Brazilian adolescent mothers consuming low Ca diets (~600 mg/d) it was observed that supplementation with Ca and vitamin D (Ca/D) during pregnancy (600 mg/d Ca + 200 IU/d cholecalciferol, from 26 wk of pregnancy until parturition) resulted in higher lumbar spine BMC, BA, and BMD at 20 wk postpartum, and a reduced rate of bone mass loss at the femoral neck during the first 20 wk postpartum, compared with the placebo [9]. It is possible that these conflicting results may reflect differences in maternal biological maturity and habitual intake of Ca, and also differences in genetic background between populations.

It is estimated that the majority of the variability in peak bone mass (60%–80%) is determined by genetic factors [10]. Calcium homeostasis strongly depends on gene expression of the VDR, the nuclear receptor and the transcription factor known to regulate the expression of genes important for calcium and bone metabolism [11,12]. In adolescents, several studies have associated Ca and bone metabolism with genetic polymorphisms in the *VDR* gene [13–20], however, few studies focused on the promoter region of the *VDR* gene [17–19]. The polymorphisms 1012 pb (rs4516035, A > G) and 1521 pb (rs7139166, G > C) are located in the promoter region of the *VDR* gene and thus may affect its transcriptional activity. There is evidence from studies in adolescents that these polymorphisms influence serum 25-hydroxyvitamin D (25[OH]D) concentrations [17] and are associated with BMC, BMD, and BMD *z* score, depending on habitual calcium intake [18].

The present study reports the secondary outcome of a randomized controlled trial study (International Trial Registry: NCT01732328) testing the effect of supplementation with Ca (600 mg/d) plus cholecalciferol (200 IU/d) from 26 wk of gestation until parturition on bone mass postpartum of Brazilian adolescent mothers with low calcium intake [9]. We herein aimed to investigate whether Ca/D supplementation interacts with polymorphisms in the promoter region of the *VDR* gene to affect changes on maternal bone mass from 5 to 20 wk postpartum.

Materials and methods

Participants and sample collection

Detailed descriptions of the inclusion and exclusion criteria, supplementation protocol, and results of the trial on maternal bone outcomes at 5 and 20 wk postpartum were previously published [9]. Briefly, pregnant adolescents were recruited during prenatal care (between gestational weeks 26 and 29) at the Maternity School of the Federal University of Rio de Janeiro, Brazil, from September 2009 to June 2011. The participation of volunteers occurred after full explanation of all study procedures. Written informed consent was obtained from each adolescent and her parent or legal guardian. The trial was approved by the Ethical Committee of Maternity School of the Federal University of Rio de Janeiro.

Participants were included if they were between the ages of 13 and 19 y, primiparous, carrying a single fetus, and intended to breastfeed. Exclusion criteria were diagnosis of chronic diseases affecting bone health, complications during pregnancy, smoking, use of supplements other than iron and folate, and no intention to breastfeed. Details on screening, enrollment, dropouts, and final number of participants analyzed are shown in Figure 1. Sample size was calculated based on maternal bone response as the primary outcome of treatment, as previously described [9]. At entry into to the study, the pregnant adolescents were single-blinded and randomly assigned to receive a commercially available supplement (Rexall Sundown, Inc, Bohemia, NY, USA) that contained 600 mg Ca (as calcium carbonate) plus 200 IU vitamin D (as cholecalciferol, vitamin D_3) or placebo, for daily usage, from an average of 26 wk of pregnancy until parturition. Randomization was done by a member of the research team in a 1:1 ratio within permuted blocks of size 10. Adherence to the intervention (83% of Ca plus vitamin D capsules and 87% of placebo capsules) was considered satisfactory and was similar in supplemented and placebo groups ($P = 0.24$, independent-samples *t* test).

Morning fasting blood samples (10 mL) were collected at 26 wk of pregnancy and at 5 and 20 wk postpartum. Aliquots of whole blood and serum were stored at −80°C until analysis. Maternal bone measurements were assessed at 5 and 20 wk postpartum.

Dietary intake, hormones, and bone mass

Detailed descriptions of the methods for analysis of dietary intake of Ca and vitamin D, serum concentrations of 25(OH)D and parathyroid hormone (PTH), and dual-energy x-ray absorptiometry (DXA) measurements have been published previously [9].

Briefly, dietary Ca intake was analyzed based on a Brazilian food database [21] by using the Avanutri program (Version Revolution 4.0). Vitamin D intake was analyzed according to the US Department of Agriculture database [22] for nonprocessed foods, and according to the nutritional information on the labels for processed foods. Serum 25(OH)D and PTH concentrations were analyzed by using a chemiluminescent enzyme-labeled immunometric assay (Liaison, Diasorin). Serum calcium was measured by colorimetric assay (Bioclin Quibasa, Brazil). Changes in serum 25(OH)D, PTH, and Ca concentrations were calculated as differences between measurements obtained at postpartum period (both at 5 and 20 wk) and those at 26 wk of pregnancy. BMD of total body, lumbar spine (L1-L4, LS), and hip (total and femoral neck) were assessed using DXA with a Lunar iDXA densitometer and the enCore 2008 version 12.20 software (GE Healthcare, Madison, WI, USA). Maternal bone mass adequacy (BMD *z* score) for all bone sites evaluated was obtained by comparing with an age-, sex-, and race-matched reference, according to the manufacturer's database. Changes in bone measurements of total body, lumbar spine, total hip, and femoral neck were calculated as differences between measurements obtained at 20 and 5 wk postpartum.

VDR *genotyping*

DNA was extracted from whole blood using a commercial kit (PureLink Genomic DNA Mini Kit, Invitrogen, Carlsbad, CA, USA). The single nucleotide polymorphisms (SNPs) rs7139166 (1521 pbG > C) and rs4516035 (1012 pbA > G) at the promoter region of the *VDR* gene were studied. Real-time polymerase chain reaction (PCR) allelic discrimination TaqMan assay (Applied Biosystems, Foster City, CA, USA) was used for genotyping analysis. The total reaction volume was 10 μL distributed between the DNA, primers, and probes (Applied Biosystems), TaqMan Master Mix (Applied Biosystems), and Milli-Q water. Probes and primers were designed by Applied Biosystems: Assay ID C__28950953_10 for rs7139166 and assay ID C___2880805_10 for rs4516035. Real-time PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System. Assay conditions for both SNPs were 95°C for 10 min and 40 cycles of amplification (95°C for 15 sec and 60°C for 1 min). For each cycle, the software determined the fluorescent signal from the VIC- or FAM-labeled probe (Applied Biosystems). Results were analyzed using the SDS 2.3 software (Life Technologies, Carlsbad, CA, USA).

Statistical analysis

Comparison between groups (supplemented versus placebo) was performed by Student's *t* test for general characteristics at baseline (26 wk pregnancy) and by χ^2 test for genotype frequency distribution. Genotypic

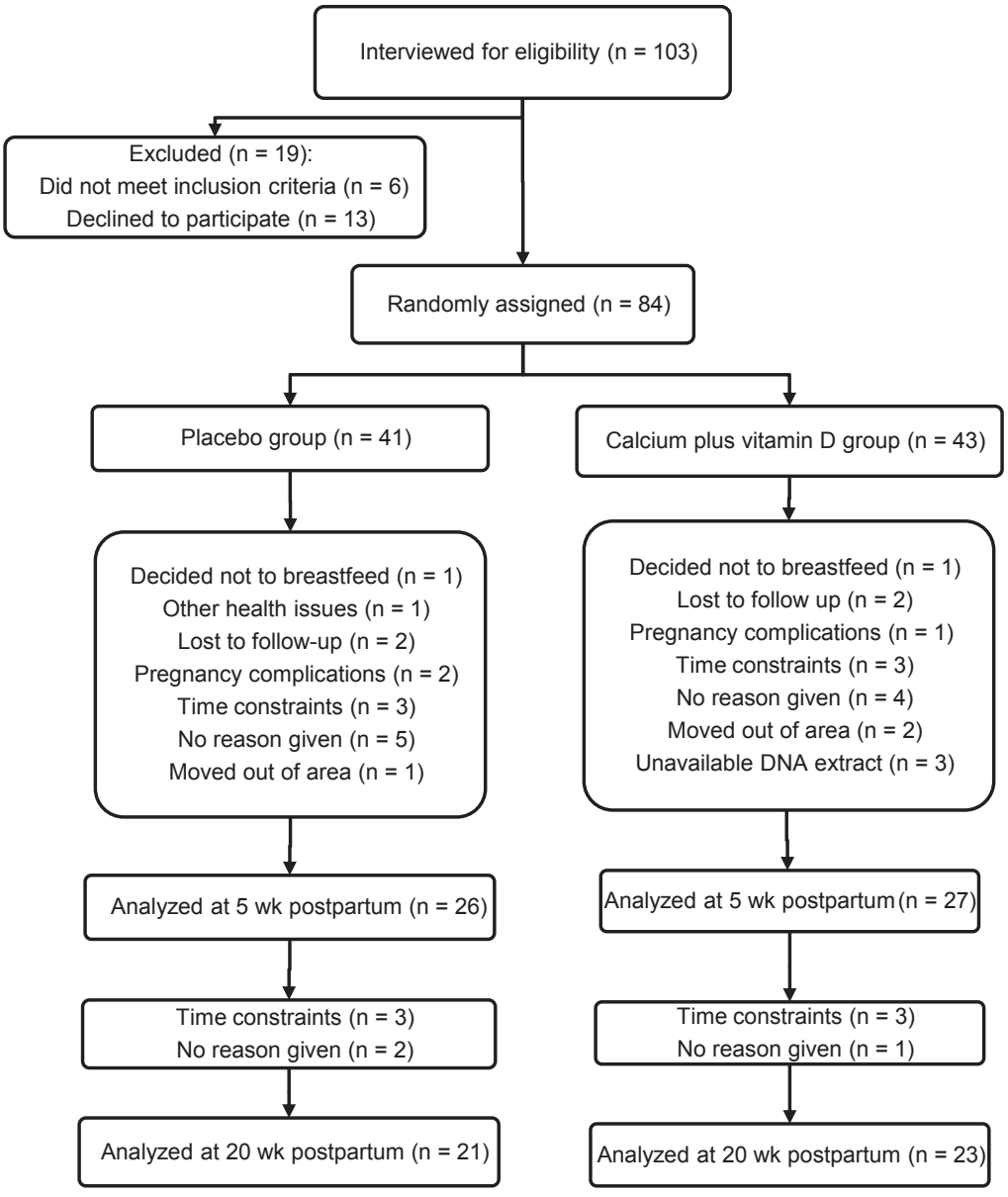

Fig. 1. Flow diagram of recruitment, random assignment, losses, and follow-up of study participants.

frequencies for both SNPs were assessed for Hardy-Weinberg equilibrium using the χ^2 test. Linkage disequilibrium between SNPs was also evaluated using the χ^2 test. The effect of supplementation, genotype, and their interaction on bone measurements at 5 and 20 wk postpartum and on bone changes from 5 to 20 wk postpartum were evaluated using a univariate general linear model, followed by a least significant difference (LSD) post hoc test, with each bone measurement as dependent variable, intervention and genotypes as fixed factors, and significant confounding variables as covariates. Significant covariates were identified as previously described [9]. Briefly, significant covariates were identified for each bone measurement (dependent variable) by using multiple linear regression models with backward elimination of those that were nonsignificant considering the following potential confounders (independent variables): chronologic age, body weight, body height, season (four categories), years since menarche, percentage of total capsules offered and consumed (percentage of compliance), days postpartum, dietary calcium intake during pregnancy, intervention group, breast-feeding practice (four categories), and return of menstruation (yes or no). The effect of supplementation, genotype, and their interaction on serum concentration changes of 25(OH)D (adjusted for season at 26 wk of pregnancy) and PTH were analyzed by two-way analysis of variance followed by LSD post hoc test. Statistical analyses were performed with SPSS 17.0 for Windows software (SPSS Inc., Chicago, IL, USA). Results are reported as mean ± SD or as mean ± SE. Values at $P \leq 0.05$ were considered significant. P values between 0.06 and 0.10 were considered trends.

Results

The general characteristics of pregnant adolescents in the groups receiving placebo or Ca/D supplement at baseline are presented in Table 1. At 26 wk of pregnancy, adolescents were on average 17 y old and 4.9 y past the onset of menarche. Dietary Ca intake represented, on average, 46% of that recommended for pregnant and lactating adolescents (1300 mg/d) [23] and was significantly higher in the placebo group than in the supplemented group ($P < 0.05$; Table 1). Thus, dietary Ca intake was systematically considered as a potential confounder in all the statistical comparisons between the groups. Dietary vitamin D intake was similar between the groups and accounted for only

Table 1
General characteristics of the adolescent mothers at the beginning of the study (26 wk of pregnancy)

Characteristics	Placebo (n = 26)	Calcium plus Vitamin D (n = 27)	*P* value
Chronologic age (y)	17.1 ± 0.9	16.6 ± 1.5	0.14
Time elapsed since menarche (y)	5.0 ± 1.7	4.9 ± 2.2	0.80
Dietary calcium intake (mg/d)	743 ± 457	450 ± 191	**0.003**
Dietary vitamin D intake (IU/d)	25 ± 37	23 ± 29	0.83
Genotypes rs7139166 (1521 pb)			0.71
GG	13 (50%)	13 (48%)	
GC	11 (42%)	10 (37%)	
CC	2 (8%)	4 (15%)	
Genotypes rs4516035 (1012 pb)			0.71
AA	13 (50%)	13 (48%)	
AG	11 (42%)	10 (37%)	
GG	2 (8%)	4 (15%)	

For continuous variables, values are presented as mean ± SD. For categorical variables, values are n values with percentages of the total in parentheses. Comparisons between groups were determined based on independent-samples *t* test (continuous variables) or χ^2 test (categorical variables).
Bold values indicate statistical significance ($P < 0.05$).

about 4% of the recommended intake for pregnant and lactating adolescents [23]. No differences in genotype or allele distributions were observed between placebo and supplemented groups (χ^2; $P > 0.05$; Table 1). The distribution of genotypes determined by polymorphisms rs7139166 (1521 pb) and rs4516035 (1012 pb) followed the Hardy-Weinberg equilibrium ($P > 0.05$) and were in complete linkage disequilibrium ($P < 0.0001$). Thus, our results were treated as the genotype combination 1521 GG/1012 AA, 1521 GC/1012 AG and 1521 CC/1012 GG. Due to the low frequency of 1521 CC/1012 GG, we focused on the comparisons between 1521 GG/1012 AA and 1521 GC/1012 AG genotypes.

The isolated effect of Ca/D supplementation on the bone measurements at 5 and 20 wk postpartum and on bone changes from 5 to 20 wk postpartum has been previously described [9]. No isolated effects of genotype were observed in the bone parameters at 5 (Table 2) and 20 wk (data not shown), except for femoral neck BMC at 20 wk postpartum that tended to be higher in mothers (from both groups) carrying the 1521 GG/1012 AA genotypes compared with those carrying 1521 GC/1012 AG genotypes (4.26 ± 0.10 and 4.01 ± 0.11 g, respectively; $P = 0.09$, post hoc test). Similarly, no isolated effects of genotype were observed for bone mass changes from 5 to 20 wk postpartum, except for the femoral neck BMC ($P = 0.05$; Table 3). Combining data from adolescent mothers receiving placebo and supplement, those carrying the 1521 GG/1012 AA genotypes had a higher decrease in femoral neck BMC from 5 to 20 wk postpartum than those carrying 1521 GC/1012 AG genotypes (−0.19 ± 0.02 and −0.11 ± 0.02 g, respectively; $P = 0.02$, post hoc test).

There was no interaction between genotypes and Ca/D supplementation on bone measurements at 5 (Table 2) and 20 wk postpartum (data not shown). However, significant interactions between genotype and supplementation were observed for bone changes from 5 to 20 wk postpartum ($P \leq 0.03$; Table 3). Among adolescent mothers who received the placebo, those with 1521 GG/1012 AA genotypes had a greater reduction from 5 to 20 wk postpartum in total BMD *z* score, and femoral neck BMC and BMD compared with those carrying 1521 GC/1012 AG genotypes ($P \leq 0.04$, post hoc test; Table 3), whereas this was not observed in the supplemented group. Additionally, in the placebo group, adolescents with 1521 GG/1012 AA genotypes had a decrease in total hip BA from 5 to 20 wk postpartum compared with those with 1521 GC/1012 AG genotypes ($P = 0.03$, post hoc test; Table 3), whereas the opposite was observed for adolescents in the supplemented group ($P = 0.05$, post hoc test; Table 3). No isolated effects of polymorphisms on serum concentrations of 25(OH)D were observed at 26 wk of pregnancy, or at 5 and 20 wk postpartum. However, significant interactions between supplementation and polymorphisms were observed on changes in 25(OH)D concentrations from 26 wk of pregnancy to 5 wk postpartum ($P = 0.01$; Table 4). In the placebo group, adolescent mothers with 1521 GG/1012 AA genotypes had a reduction in serum 25(OH)D from 26 wk of pregnancy to 5 wk postpartum that was significantly different from the increase in those with 1521 GC/1012 AG genotypes ($P = 0.01$, post hoc test; Table 4). In

Table 2
Interaction between calcium plus vitamin D supplementation and SNPs rs7139166/rs4516035 on changes in bone measurements of adolescent mothers at 5 wk postpartum

Bone parameters	Placebo			Calcium plus vitamin D			SNP	*P* value	SNP × Suppl interaction
	GG/AA	GC/AG	CC/GG	GG/AA	GC/AG	CC/GG		Suppl	
Total body									
BMC (g)	2065 ± 59	2080 ± 63	2220 ± 146	2172 ± 60	2052 ± 69	2154 ± 107	0.44	0.95	0.48
BA (cm^2)	1986 ± 18	1987 ± 20	1975 ± 46	1994 ± 19	1936 ± 21	2020 ± 34	0.28	0.97	0.21
BMD (g/cm^2)	1.041 ± 0.026	1.047 ± 0.029	1.118 ± 0.66	1.082 ± 0.025	1.059 ± 0.031	1.060 ± 0.048	0.74	0.96	0.51
BMD *z* score	−0.53 ± 0.28	−0.49 ± 0.30	0.52 ± 0.70	0.07 ± 0.29	−0.26 ± 0.32	0.04 ± 0.51	0.41	0.75	0.51
Lumbar spine									
BMC (g)	50.7 ± 2.4	47.1 ± 2.6	52.7 ± 6.0	55.7 ± 2.4	50.0 ± 2.7	52.5 ± 4.4	0.18	0.40	0.79
BA (cm^2)	46.7 ± 1.0	45.4 ± 1,1	46.2 ± 2.5	50.1 ± 1.0	47.2 ± 1.1	50.5 ± 1.8	0.12	0.01	0.66
BMD (g/cm^2)	1.075 ± 0.040	1.025 ± 0.043	1.142 ± 0.101	1.114 ± 0.040	1.044 ± 0.045	1.089 ± 0.74	0.28	0.97	0.79
BMD *z* score	−0.99 ± 0.30	−1.32 ± 0.32	−0.41 ± 0.76	−0.64 ± 0.30	−0.97 ± 0.34	−0.75 ± 0.55	0.43	0.74	0.78
Total hip									
BMC (g)	28.3 ± 1.1	27.6 ± 1.2	28.1 ± 2.7	28.7 ± 1.1	26.6 ± 1.2	30.2 ± 2.0	0.37	0.70	0.66
BA (cm^2)	27.8 ± 0.3	27.7 ± 0.4	27.0 ± 0.8	27.3 ± 0.3	26.7 ± 0.4	27.6 ± 0.6	0.68	0.50	0.37
BMD (g/cm^2)	1.013 ± 0.039	0.994 ± 0.042	1.062 ± 0.098	1.049 ± 0.038	0.998 ± 0.044	1.091 ± 0.072	0.45	0.64	0.93
BMD *z* score	−0.01 ± 0.30	−0.18 ± 0.33	0.43 ± 0.76	0.24 ± 0.30	−0.13 ± 0.34	0.61 ± 0.56	0.40	0.66	0.95
Femoral neck									
BMC (g)	4.5 ± 0.1	4.2 ± 0.1	4.6 ± 0.3	4.5 ± 0.1	4.2 ± 0.2	4.7 ± 0.2	0.12	0.87	0.97
BA (cm^2)	4.3 ± 0.1	4.2 ± 0.1	4.1 ± 0.2	4.1 ± 0.0	4.2 ± 0.1	4.2 ± 0.1	0.86	0.80	0.36
BMD (g/cm^2)	1.055 ± 0.035	1.004 ± 0.038	1.148 ± 0.88	1.072 ± 0.035	1.009 ± 0.040	1.113 ± 0.065	0.10	0.92	0.91
BMD *z* score	4.27 ± 0.06	4.22 ± 0.07	4.09 ± 0.16	4.15 ± 0.06	4.24 ± 0.08	4.25 ± 0.12	0.10	0.95	0.92

BA, bone area; BMD, bone mineral density; BMC, bone mineral content; SNP, single nucleotide polymorphism
All values are presented as mean ± SE. *P* values were obtained by univariate general linear model, followed by least significant difference post hoc test, using significant covariates as previously described [9]

Table 3
Interaction between calcium plus vitamin D supplementation and SNPs rs7139166/rs4516035 on changes (Δ) in bone measurements of adolescent mothers from 5 to 20 wk postpartum

Bone parameters	Placebo			Calcium plus vitamin D			SNP	*P* value	SNP × Suppl interaction
	GG/AA	GC/AG	CC/GG	GG/AA	GC/AG	CC/GG		Suppl	
Total Body									
ΔBMC (g)	−23.11 ± 6.74	−11.15 ± 7.28	−16.58 ± 15.97	−14.71 ± 6.73	−19.10 ± 7.46	−0.05 ± 12.70	0.62	0.52	0.43
ΔBA (cm^2)	3.51 ± 5.64[a]	−10.76 ± 6.16[a,b]	−11.82 ± 12.78[a,b]	−11.13 ± 5.47[b]	3.17 ± 6.05[a]	9.77 ± 10.11[a]	0.95	0.33	**0.03**
ΔBMD (g/cm^2)	−0.012 ± 0.004	0.001 ± 0.004	0.000 ± 0.009	−0.005 ± 0.004	−0.009 ± 0.004	−0.009 ± 0.007	0.53	0.37	0.10
ΔBMD *z* score	−0.15 ± 0.04[b]	−0.02 ± 0.05[a]	0.00 ± 0.10[a,b]	−0.06 ± 0.05[a,b]	−0.16 ± 0.05[b]	−0.15 ± 0.08[a,b]	0.89	0.23	**0.03**
Lumbar spine									
ΔBMC (g)	0.52 ± 0.53	0.11 ± 0.61	0.52 ± 1.24	−0.16 ± 0.55	−0.08 ± 0.57	1.84 ± 1.01	0.84	0.43	0.52
ΔBA (cm^2)	0.60 ± 0.40	−0.01 ± 0.42	0.70 ± 0.89	0.33 ± 0.38	0.40 ± 0.42	0.56 ± 0.73	0.72	0.99	0.70
ΔBMD (g/cm^2)	−0.004 ± 0.009	0.002 ± 0.010	−0.009 ± 0.020	−0.006 ± 0.009	−0.008 ± 0.009	0.026 ± 0.016	0.64	0.51	0.30
ΔBMD *z* score	−0.03 ± 0.07	−0.02 ± 0.08	−0.08 ± 0.16	−0.12 ± 0.07	−0.08 ± 0.07	0.21 ± 0.13	0.48	0.58	0.26
Total hip									
ΔBMC (g)	−0.99 ± 0.18	−0.28 ± 0.19	−1.13 ± 0.40	−0.66 ± 0.18	−0.65 ± 0.19	−0.70 ± 0.34	0.56	0.11	0.13
ΔBA (cm^2)	−0.05 ± 0.10[b]	0.26 ± 0.10[c]	−0.10 ± 0.21[a,b,c]	0.22 ± 0.09[a,c]	−0.04 ± 0.10[b]	−0.17 ± 0.18[a,b]	0.30	0.79	**0.02**
ΔBMD (g/cm^2)	−0.032 ± 0.006	−0.026 ± 0.007	−0.038 ± 0.013	−0.030 ± 0.006	−0.023 ± 0.006	−0.014 ± 0.011	0.61	0.19	0.52
ΔBMD *z* score	−0.25 ± 0.06	−0.20 ± 0.06	−0.27 ± 0.13	−0.33 ± 0.06	−0.18 ± 0.06	−0.13 ± 0.10	0.25	0.64	0.44
Femoral neck									
ΔBMC (g)	−0.23 ± 0.03[c]	−0.09 ± 0.03[a,b]	−0.28 ± 0.07[c,d]	−0.15 ± 0.03[a,c]	−0.13 ± 0.03[a,b,d]	−0.02 ± 0.06[b]	**0.05**	0.01	**0.008**
ΔBA (cm^2)	0.00 ± 0.02	0.05 ± 0.02	−0.04 ± 0.05	0.01 ± 0.02	−0.01 ± 0.02	0.00 ± 0.04	0.48	0.85	0.19
ΔBMD (g/cm^2)	−0.054 ± 0.006[b]	−0.032 ± 0.007[a,c]	−0.071 ± 0.013[b]	−0.037 ± 0.006[a]	−0.032 ± 0.006[a]	−0.007 ± 0.011[c]	0.14	**0.01**	**0.008**
ΔBMD *z* score	−0.38 ± 0.05	−0.26 ± 0.06	−0.45 ± 0.12	−0.34 ± 0.05	−0.22 ± 0.06	−0.07 ± 0.10	0.08	**0.02**	0.13

BA, bone area; BMD, bone mineral density; BMC, bone mineral content; LSD, least significant difference; SNP, single nucleotide polymorphism
All values are mean ± SE. *P* values were obtained by univariate general linear model, followed by least significant difference post hoc test, using significant covariates as described previously [9]. Different superscript letters (a, b, c, d) in the same row indicate significant differences between subgroups ($P \leq 0.05$; LSD post hoc test).
Bold values indicate statistical significance ($P \leq 0.05$).

the supplemented group, the increase in serum 25(OH)D from 26 wk of pregnancy to 5 wk postpartum was not significantly different between genotypes. Furthermore, in the same period, changes in serum 25(OH)D concentrations were significantly different between supplemented and placebo groups only for those mothers with 1521 GG/1012 AA genotypes ($P = 0.004$; post hoc test; Table 4), being higher in the supplemented group.

Similarly, no isolated effect of polymorphisms on serum concentrations of PTH was observed at 26 wk of pregnancy, and at 5 and 20 wk postpartum. However, interactions between supplementation and polymorphisms were observed on serum concentrations of PTH at 5 ($P = 0.07$; Table 4) and 20 wk postpartum ($P = 0.03$; Table 4). In the placebo group, serum PTH concentrations at 5 and at 20 wk postpartum tended to be lower ($P = 0.08$, post hoc test; Table 4) in adolescent mothers with 1521 GG/1012 AA genotypes compared with those carrying 1521 GC/1012 AG genotypes. In the supplemented group, no significant difference between genotypes was observed at 5 wk postpartum. However, at 20 wk postpartum, adolescent mothers with 1521 GG/1012 AA genotypes had higher serum PTH

Table 4
Interaction between calcium plus vitamin D supplementation and SNPs rs7139166/rs4516035 on serum concentrations of 25(OH)D, PTH, and calcium of the adolescent mothers from 26 wk of pregnancy to 20 wk postpartum

Biochemical indices	Placebo			Calcium plus vitamin D			SNP	*P* value	SNP × Suppl interaction
	GG/AA	GC/AG	CC/GG	GG/AA	GC/AG	CC/GG		Suppl	
Serum 25(OH)D (nmol/L)									
26 wk pregnancy	60.6 ± 5.7	52.1 ± 6.2	69.5 ± 14.7	55.7 ± 5.8	66.3 ± 6.5	56.3 ± 10.3	0.86	0.82	0.19
5 wk postpartum	50.8 ± 8.4	69.6 ± 9.2	60.4 ± 21.4	76.7 ± 8.4	71.1 ± 9.6	84.5 ± 15.1	0.70	0.11	0.39
20 wk postpartum	47.6 ± 7.1	59.8 ± 7.3	57.6 ± 15.8	70.5 ± 6.7	62.2 ± 7.4	82.9 ± 12.8	0.62	**0.05**	0.31
Change from 26 wk pregnancy to 5 wk postpartum	−10.4 ± 7.5[b]	18.7 ± 8.2[a]	−9.1 ± 19.3[a,b]	22.3 ± 7.6[a]	3.8 ± 8.6[a,b]	28.5 ± 13.5[a]	0.80	0.06	**0.01**
Change from 5–20 wk postpartum	2.3 ± 6.4	−21.2 ± 6.7	2.4 ± 14.3	−13.1 ± 6.1	−13.7 ± 6.8	−3.9 ± 11.7	0.12	0.53	0.22
Serum PTH (pg/dL)									
26 wk pregnancy	31.7 ± 4.7	45.9 ± 5.1	28.6 ± 12.1	44.3 ± 4.7	36.5 ± 5.4	37.0 ± 8.5	0.57	0.52	0.10
5 wk postpartum	37.7 ± 10.5	65.0 ± 11.4	64.9 ± 26.8	37.7 ± 10.5	41.6 ± 12.0	59.2 ± 19.0	0.86	0.99	**0.07**
20 wk postpartum	49.0 ± 8.9[a,b]	72.3 ± 9.4[a]	38.1 ± 20.0[a,b]	73.3 ± 8.5[a]	44.9 ± 9.4[b]	44.9 ± 16.3[a,b]	0.39	0.91	**0.03**
Change from 26 wk pregnancy to 5 wk postpartum	5.3 ± 8.1	20.2 ± 8.8	36.3 ± 20.7	22.1 ± 8.1	11.14 ± 9.3	22.2 ± 14.7	0.54	0.84	0.26
Change from 5–20 wk postpartum	15.2 ± 10.5	11.9 ± 11.0	−26.8 ± 23.4	8.8 ± 10.0	−1.7 ± 11.0	−10.2 ± 19.1	0.20	0.92	0.67
Serum calcium (mmol/L)									
26 wk pregnancy	2.04 ± 0.07	1.97 ± 0.08	2.05 ± 0.18	1.95 ± 0.07	2.04 ± 0.08	2.12 ± 0.13	0.85	0.74	0.54
5 wk postpartum	2.06 ± 0.06	2.18 ± 0.07	2.10 ± 0.15	2.08 ± 0.06	2.11 ± 0.07	2.10 ± 0.11	0.83	0.51	0.75
20 wk postpartum	2.13 ± 0.06	2.22 ± 0.06	1.95 ± 0.13	2.23 ± 0.05	2.11 ± 0.06	2.10 ± 0.10	0.50	0.24	0.15
Change from 26 wk pregnancy to 5 wk postpartum	0.02 ± 0.10	0.21 ± 0.11	0.05 ± 0.26	0.14 ± 0.10	0.07 ± 0.12	−0.03 ± 0.18	0.80	0.74	0.49
Change from 5–20 wk postpartum	−0.10 ± 0.09	0.04 ± 0.10	−0.15 ± 0.21	0.14 ± 0.09	−0.02 ± 0.10	0.07 ± 0.17	0.36	0.71	0.43

LSD, least squares difference; SNP, single nucleotide polymorphism
Values are mean ± SE. *P* values were obtained by analysis of variance. For 25(OH)D, season was entered as a covariate. Different superscript letters (a, b) in the same row indicate significant differences between the subgroups ($P \leq 0.05$, LSD post hoc test).
Bold values indicate statistical significance ($P \leq 0.05$).

concentrations than those carrying 1521 GC/1012 AG genotypes ($P = 0.03$, post hoc test; Table 4). No effect of polymorphisms, supplementation, or the interaction between them was observed on serum Ca concentrations.

Discussion

The present study investigated the effect of the interaction between Ca/D supplementation and polymorphisms in the promoter region of the *VDR* gene on the changes in maternal bone mass during the first 20 wk postpartum. The results reported herein suggest that Brazilian adolescent mothers with 1521 GG/1012 AA genotypes are prone to higher bone loss during lactation. However, they are also those who have bone mass benefits due to Ca/D supplementation during pregnancy, particularly in the femoral neck region.

As previously demonstrated [9], adolescent mothers receiving Ca/D supplementation (600 mg/d + 200 IU/d) during the last trimester of pregnancy had higher bone mass (BMC, BA, BMD, and BMD *z* score) in the lumbar spine at 20 wk postpartum, and a reduced rate of BMD loss at the femoral neck during the first 20 wk postpartum than those receiving placebo.

In the present study, we observed that the adolescent mothers with 1521 GG/1012 AA genotypes seem to be more susceptible to bone loss at the femoral neck from 5 to 20 wk postpartum. In the entire group of mothers (those receiving placebo and supplement) with 1521 GG/1012 AA genotypes, a greater decrease in the femoral neck BMC from 5 to 20 wk postpartum was observed in comparison to mothers with 1521 GC/1012 AG genotypes. However, mothers with 1521 GG/1012 AA genotypes were also those who tended to have greater femoral neck BMC at 20 wk postpartum. The tendency toward a higher femoral neck bone mass at 20 wk postpartum (despite of the higher rate of postpartum bone loss) in the adolescent mothers with 1521 GG/1012 AA genotypes possibly reflects a higher bone mass (at this specific site) before gestation, that was not investigated in the present study.

The higher bone loss at the femoral neck region from 5 to 20 wk in mothers with 1521 GG/1012 AA appeared to be attenuated by the increase in Ca and vitamin D intake during pregnancy in the supplemented group. While in the placebo group, higher losses in the femoral neck BMC and BMD were observed in mothers with 1521 GG/1012 AA genotypes; in the supplemented group, postpartum decrease in these bone parameters were similar in both 1521 GG/1012 AA and 1521 GC/1012 AG genotypes, suggesting a beneficial effect of the supplementation for those carrying the 1521 GG/1012 AA genotypes. Consistently, femoral neck BMD loss between 5 and 20 wk postpartum was lower in the mothers with 1521 GG/1012 AA genotypes and receiving supplementation than that in the mothers with the same genotypes but receiving placebo during pregnancy. Additionally, 1521 GG/1012 AA genotypes were associated with subtle loss at total hip bone area in the placebo group, whereas in the supplemented group it was associated with an increment in the same bone parameter.

We previously observed that the adolescents supplemented with Ca and vitamin D during pregnancy tended to have higher serum 25(OH)D concentrations at 5 and 20 wk postpartum than the group receiving placebo [9]. In the present study, it was observed that the changes in serum 25(OH)D concentrations from pregnancy to postpartum period (both at 5 and 20 wk) was significantly different between the two groups only for those mothers carrying the 1521 GG/1012 AA genotypes. As a result of the interaction between the genotypes and supplementation, it appears that, in terms of 25(OH)D, mothers with 1521 GG/1012 AA genotypes were more responsive to the supplementation, which in turn may have reflected in the observed bone responses.

Serum PTH seems to increase from pregnancy to lactation in adult mothers who were accustomed to a low-Ca diet [8,24] and also in adolescent mothers [25]. It was previously observed that in these Brazilian adolescent mothers, regardless of the supplementation, serum PTH increased from midpregnancy to lactation [9]. The present results suggested that such increases in serum PTH from pregnancy to lactation also occur regardless of the genotypes (when placebo and supplemented groups are combined). However, genotypes interacted with supplementation at 20 wk postpartum. A tendency of higher serum PTH was observed in mothers with 1521 GG/1012 AA genotypes and receiving supplementation than that in the mothers with the same genotypes but receiving placebo. Therefore, lower bone loss at the femoral neck region that was observed in the mothers with 1521 GG/1012 AA genotypes and receiving supplementation does not seem to be dependent on the serum PTH during the postpartum period.

Several studies have investigated the interaction between *VDR* gene polymorphisms and calcium and/or vitamin D intake from diet or supplements on bone mass and serum concentrations of vitamin D in adolescents [14,26–28]; however, only one focused on the SNP 1012 pb [18]. In European postmenarcheal adolescents with the 1012 AA genotype, lumbar spine BMC and BMD accrual were not influenced by milk intake or serum concentration of 25(OH)D. However, it was observed that milk intake >260 mL/d was required to ensure optimal bone accrual in girls bearing one or two G alleles at SNP1012 pb [18].

The SNPs 1521 pb (rs7139166) and 1012 pb (rs4516035) located at the VDR promoter region influence *VDR* gene transcriptional activity, which is higher in 1521 G/1012 A alleles [17,29]. In our study, variations at the promoter region of the *VDR* gene appeared to contribute to different bone mass responses to supplementation, with some indication of higher responsiveness to intervention in those carrying 1521 GG/1012 AA genotypes. However, we do not know whether similar interactions between genotype and supplementation on bone responses would occur if different conditions of intervention were applied.

The frequency of 1521 GG/1012 AA genotypes in Brazilian adolescent mothers was relatively high (50%), which may partially explain the previously described beneficial effect of Ca/D supplementation on bones [9]. The frequency of these genotypes can be up to 98% in sub-Saharan African populations (HapMap, The International HapMap Consortium [30]). Therefore, one could expect an even higher effect of Ca supplementation on bones in African populations. Interestingly, in Gambian adult mothers habitually consuming low-Ca diets (300 mg/d), Ca supplementation during pregnancy (1500 mg/d) resulted in lower BMC and BMD at the hip, and also in higher bone mobilization of the lumbar spine and distal radius during lactation [8]. These results may be indicative of a higher responsiveness of the 1521 GG/1012 AA genotypes; however, the direction of bone response might have been determined by the amount and composition of supplement. It has been speculated that the high Ca dose disrupted the process of metabolic adaptation to a low Ca intake [8].

The concept that a gene variant may be advantageous in one environmental or lifestyle context and disadvantageous in another has been proposed [31]. It may be that different results from Ca supplementation studies are a consequence of the complex interaction between the different intervention strategies adopted

and the different genetic background of the populations studied rather than a result of intervention or genetics alone.

The strengths of this study included the possibility of monitoring adolescent mothers for the long period from pregnancy to 20 wk postpartum, the important information available to be analyzed in conjunction with *VDR* gene polymorphisms, and the good adhesion to intervention. We recognize that the sample size, which was calculated to respond the primary outcomes of this randomized controlled trial, may have limited our conclusions. We also recognize that bone mass evaluation before supplementation would have considerably contributed to the interpretation of results. However, as DXA scanning is not usually performed during pregnancy to avoid unnecessary exposure of the fetus to ionizing radiation, the ideal baseline would be bone measurements before pregnancy, but this presents a challenge as adolescents usually do not plan to get pregnant.

Conclusion

Calcium plus vitamin D supplementation during pregnancy interacted with polymorphisms in the promoter region of the *VDR* gene, affecting maternal bone loss from 5 to 20 wk postpartum in Brazilian adolescents. Adolescent mothers with 1521 GG/1012 AA genotypes appeared to be more susceptible to bone mass loss at the femoral neck region during lactation. In these adolescents, an increased supply of Ca and vitamin D during pregnancy appeared to minimize the femoral neck bone loss during the first 20 wk postpartum.

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