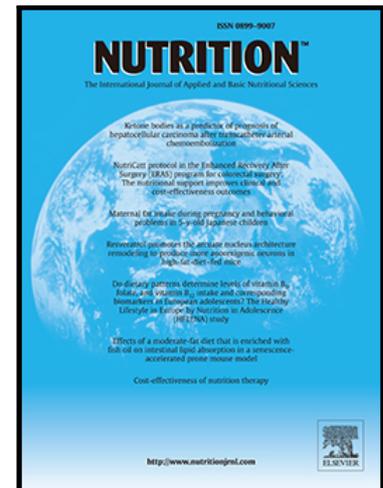# Journal Pre-proof

Variants in the gene encoding for vitamin D binding protein were associated with leukocyte telomere length: the Pró-Saúde Study

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Highlights

- Genetic predisposition to lower vitamin D status may contribute to shorter telomeres
- No significant association was observed between LTL and serum 25(OH)D concentration
- Variations in the vitamin D binding protein gene (GC - rs2282679) were associated to LTL
- Individuals carrying CC genotype (*GC* - rs2282679) had shorter LTL

Variants in the gene encoding for vitamin D binding protein were associated with leukocyte telomere length: the Pró-Saúde Study

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Declarations of interest: none.

List of abbreviations: 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hidroxyvitamin; BMI, Body mass index; DBP, vitamin D binding protein; GWAS, genome-wide association studies; LSD, least significant difference post hoc test; LTL, leukocyte telomere length; qPCR, quantitative Real Time polymerase chain reaction; ROS, reactive oxygen species; *RXRA*, Retinoid X Receptor Alpha; SEI, sun exposure index; SNP, single nucleotide polymorphism; T/S ratio, telomere to single copy gene ratio; VAT, visceral adipose tissue.

Introduction

Epidemiological and experimental evidence have shown the importance of vitamin D for the adequate function of many biological systems and its protective role on a number of extra skeletal complications (1). Vitamin D can be synthesized in the skin via exposure to ultraviolet B (UVB) radiation or obtained from restricted foods. Regardless of origin, vitamin D is converted to 25-hidroxyvitamin D [25(OH)D] in the liver and subsequently to its active metabolite 1,25-dihydroxyvitamin D [1,25(OH)$_2$D], that exerts its function mainly through the interaction with nuclear vitamin D receptor (VDR) which is largely distributed in human organism (2).

Given that 1,25(OH)$_2$D could down-regulate the production of reactive oxygen species (ROS) and the expression of several pro-inflammatory cytokines (3), vitamin D can prevent DNA damage, including the telomeric regions (4). Telomeres are repetitive protein-bound DNA sequences at the end of chromosomes that regulate cellular replicative capacity and protect chromosomes from fusing during mitosis (5). During the process of cell division, telomeric sequences are gradually lost; therefore, telomere shortening is a continuous process throughout life (6). Also, the high content of guanine makes telomeres more susceptible to damages caused by ROS and inflammatory processes (7,8). As these damages are less efficiently repaired compared to other regions of genome, both oxidative stress and inflammation can accelerate natural telomere shortening (7,8).

Some authors have explored associations between leukocyte telomere length (LTL) and serum concentrations of 25(OH)D, with inconclusive results (9-16). A direct association between serum concentrations of 25(OH)D and LTL was observed in women (9,10), but not in men (11). Larger population studies with adults have also resulted in both no association between serum concentrations of 25(OH)D and LTL (12, 16), as well as a direct association between them, albeit restricted to middle-aged adults (13). The mixed findings on the research on vitamin D and LTL may be due to the dynamic fluctuation that serum 25(OH)D is usually subjected to, whereas LTL measurement in a given moment is a result of a continuous process initiated in the beginning of life.

Some single nucleotide polymorphisms (SNPs) have been identified as genetic determinants of 25(OH)D serum concentrations. Due to their invariant nature, they may be useful in exploring the relationship between vitamin D and LTL (11). SNPs in the genes encoding vitamin D binding protein (GC gene), NAD synthetase 1 (NADSYN1), vitamin D 25-hydroxylase (CYP2R1), and vitamin D 24-hydroxylase (CYP24A1) have been identified as strongly associated with 25(OH)D concentrations in genome-wide association studies (GWAS) (17-21). Therefore, a genetic predisposition to lower vitamin D status may contribute to shorter telomeres.

The aim of the present study was to investigate the association between SNPs in vitamin D metabolic pathway genes (*NADSYN1*, *CYP2R1*, *CYP24A1* and *GC)*, serum 25(OH)D concentration, and LTL in Brazilian adults.

Material and methods

Overview

The Pró-Saúde Study is a prospective cohort study of civil servants at university *campi* in Rio de Janeiro, Brazil. Descriptions of the cohort and design details have been previously published (22). In brief, detailed self-administered questionnaires were administered to cohort members (n=3253) during four waves of data collection - 1999, 2001-02, 2006-07, and 2012-13 - to investigate social determinants of health and health-related behaviors (23). The Pró-Saúde Study has been periodically approved by the Ethics in Research Committee of the Social Medicine Institute, at the Rio de Janeiro State University (for wave four: CAAE: 04452412.0.0000.5260). All participants provided written informed consent.

Study population

This is a cross-sectional study conducted in a subsample carried out in parallel with the Pró-Saúde Study fourth wave (2012-13) of data collection. Participants (n=520) from the baseline cohort population, were randomly selected within strata of sex, age (less than 50 years vs. 50 years or more) and educational level (less than high school vs. high school or more), and invited to complete an additional interview that included, socioeconomic, nutritional and body composition data, together with a blood collection for biochemical and genetic analyses. Data collection occurred between July 2012 and October 2013.

Participants who had missing data on socioeconomic or behavioral issues (n=26) were excluded from the analyses, as well as few participants who self-reported as yellow (n=2) or indigenous (n=7). Additionally, participants were also excluded if they did not provide a whole blood sample (n=8), had inconsistent LTL values based on their age (n=7, outliers based on confidence intervals), had insufficient serum sample for 25(OH)D analysis (n=4) or had insufficient DNA sample for genotyping (n=2). Thus, a total of 464 participants were included in the current analyses.

Outcome assessment

DNA samples were isolated from whole blood using a commercial kit (Puregene Blood Kit – Qiagen, Hilden, Germany). The quality and concentration of DNA samples were evaluated by spectrophotometry (BioDrop DUO, BioDrop, Cambridge, England) and were stored at -80ºC, until the determination of LTL and genotyping.

LTL was measured by quantitative Real Time polymerase chain reaction (qPCR) based on the method described by Cawthon with modifications (24-26). Reactions were conducted in triplicate and included: genomic DNA (1.6 ng), 2x Rotor-Gene SYBR Green, PCR Master Mix (Qiagen, Hilden, Germany), primers Tel Forward (300 nM - CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT) and Tel Reverse (300 nM - GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT) or primer single gene Hbg1 Forward (300nM - GCTTCTGACACAACTGTGTTCACTAGC) and single gene Hbg2 Reverse (700nM - CACCAACTTCATCCACGTTCACC) (Integrated DNA Technologies,

Iowa, Coralville, U.S.A.), in a 24 μL reaction. Amplification was conducted in a Rotor-Gene Q Real-Time PCR cycler (Qiagen, Hilden, Germany) as follows: 95°C by 5 min, 25 cycles (telomere reaction) or 35 cycles (single gene reaction) at 98°C by 7 seconds and 60°C by 10 seconds. The telomere length for each sample was determined using the telomere to single copy gene ratio (T/S ratio) by the ΔCt method [Ct(telomere)/Ct (single gene)]. The T/S ratio for each sample (x) was normalized to the mean a T/S ratio of reference sample (r) [2 – (ΔCtx - ΔCtr) = 2-ΔΔCt], which was also used for the standard curve, both as a reference sample and as an inter-assay validation sample. All sample data included had reference samples with an inter-assay variation (CV) of less than 9%.

For qPCR validation purposes, the LTL was evaluated in a random sample (10%), using the Southern Blot method of Terminal Restriction Fragment (TRF), as described by Gutierrez-Rodrigues and coworkers (27). The analysis was conducted using a commercial kit (Telo TAGGG Telomere Length Assay – Roche Applied Science, Indianapolis, Indiana, U.S.A).

Exposure assessment

The SNPs rs12785878, rs10741657, rs6013897 and rs2282679 of the *NADSYN1, CYP2R1, CYP24A1* and *GC* genes, respectively, were analyzed. Real-time PCR allelic discrimination by TaqMan assay (Thermo Scientific Inc., Foster City, CA, U.S.A) was used for genotyping analysis. Probes and primers were designed by Thermo Scientific Inc. Real-time PCR was performed on a Step One Plus Real-Time PCR System (Thermo Scientific Inc). Results were analyzed using the SDS 2.3 software (Thermo Scientific Inc).

Serum 25(OH)D concentration was determined by a semi-automated chemiluminescent enzyme-labeled immunometric assay (Liaison, Diasorin, MN, U.S.A). Assays were conducted in duplicate and intra- and inter-assay coefficients of variation were 4.9% and 5.8%, respectively. Vitamin D status was categorized according to IOM (2011) (28) and the Endocrine Society (2011) (29): < 30 nmol/L, $30 - 50$ nmol/L, 51-75 nmol/L and ≥ 75 nmol/L.

Covariates assessment

Socioeconomic, demographic, health and behavioral markers identified from the literature that could affect LTL were included as covariates (8, 30-33). During the interview, participants self-reported their age, sex, marital status (single, married, separated/divorced and widower), race/skin color (white, brown, black, yellow, and indigenous, based on Brazilian official criteria) (34), educational attainment (high school or less and college education or higher) and household income. Equivalent *per capita* household income was calculated using the OECD equivalence scale (35) and categorized into <3, 3-6 and >6 Brazilian minimum wages *per capita*. Smoking status was categorized as "never smoker" or "current or former smoker", leisure-time physical activity in the previous two weeks (classified as "yes" or "no") and history of medical diagnoses of chronic conditions (diabetes, hypertension, hypercholesterolemia, heart attack, angina and/or pulmonary emphysema) were also self-reported. Body mass index (BMI, kg/m^2) was calculated using standing height (m) and body weight (kg) and categorized into three BMI strata based on the WHO classification (36) as underweight or normal, overweight and obesity I, II or III. High-sensitive C-reactive protein (hsCRP) was measured by

immunoturbidimetric latex assay using an automatic analyzer (A25 BioSystems, Barcelona, Spain). Month of blood collection, visceral adipose tissue (VAT) estimated by DXA and sun exposure index (SEI) were previously identified as determinants of 25(OH)D serum concentrations in this population (37) and therefore included as covariates in models of serum vitamin D as the primary exposure.

Statistical analyses

Descriptive statistics were utilized to characterize the study participants. Continuous variables were expressed by means ± SD and categorical variables by absolute and relative frequencies. Genotype frequencies for each SNP were assessed for Hardy-Weinberg equilibrium using the χ^2 test. All genotypes distribution followed the Hardy-Weinberg equilibrium ($P > 0.05$), except for rs6013897 (CYP24A1, $P = 0.04$), which was subsequently excluded from the analyses. Serum hsCRP correlations with 25(OH)D and LTL were evaluated by Pearson's correlation analysis. Mean LTL differences by categories of genotypes and serum 25(OH)D concentrations were evaluated with adjustment for age and sex by analysis of covariance (ANCOVA), followed by a least significant difference (LSD) post hoc test. To examine the associations between LTL and genotypes, multiple linear regression models were performed using LTL as the dependent variable and genotypes (mutually adjusted for one another) as independent variables. The first model was adjusted for age, sex, race/skin color and genotypes. The second model was additionally adjusted for educational attainment, marital status, and equivalent *per capita* household income. Model 3 was additionally adjusted for smoking status, leisure-time

physical activity, BMI and history of chronic disease diagnoses. Linear multiple regression models were also used to investigate associations between LTL and serum 25(OH)D concentrations as the independent variable. Model 1 was adjusted for age, sex, educational attainment, marital status, and equivalent *per capita* household income. Model 2 was additionally adjusted for smoking status, leisure-time physical activity and history of chronic disease diagnoses. Model 3 was additionally adjusted for skin type, month of blood collection, sun exposure index, visceral fat mass (as potential determinants of vitamin D) and hsCRP. Statistical analyzes were performed using SPSS version 23.0 software (SPSS Inc., Chicago, IL, U.S.A) and P values < 0.05 were considered statistically significant).

Results

The general characteristics of the study population are presented in **Table 1**. Participants were, on average, 51.4 years, predominantly white (49.4%), married (65.7%) and had college education or higher (56.9%), with an equivalent *per capita* household income less than 3 minimum wages (45.0%). Also, the majority of participants did not engage in any physical activity during the previous two weeks (59.5%), never smoked (63.8%), were overweight (40.9%) or obese (31.9%) and reported history of chronic disease diagnoses (58.6%). Serum hsCRP concentrations were on average 2.0±6.3 mg/L. The average LTL (T/S ratio) was 0.57±0.15, and was significantly higher in women compared to men (0.59±0.15 and 0.55±0.14, respectively, P = 0.02). In addition, age was inversely correlated with LTL (r = -0.168, P < 0.01). No statistically

significant correlation was observed between LTL and hsCRP serum concentrations (r = -0.42; P = 0.36).

Age- and sex- adjusted LTL mean (T/S ratio) was significantly lower in individuals carrying CC genotype (0.51±0.03) for rs2282679 (*GC* gene) compared with those carrying AC or AA genotypes (0.58±0.01 and 0.57±0.01, respectively, $P < 0.05$, LSD post hoc test, **Table 2**). No statistically significant differences were observed in LTL means between the three possible genotypes for rs12785878 (*NADSYN1* gene) and rs10741657 (*CYP2R1* gene) ($P > 0.05$). Results from multiple regression analysis showed that LTL of individuals carrying CC genotype (*GC* - rs2282679) was 0.061 T/S ratio units lower (95% CI: -0.120, -0.001) than those carrying AA genotype (Table 2). Similar results were observed evaluating the genetic recessive model, in which individuals carrying CC genotype had shorter LTL than those carrying AA combined to AC genotypes (β = -0.065, 95% CI: -0.123, -0.006). No statistically significant association with LTL was observed for *NADSYN1* - rs12785878 and *CYP2R1* - rs10741657.

Serum 25(OH)D concentrations were on average 48.3±20.4 nmol/L, and 55.6% of individuals showed vitamin D insufficiency [25(OH)D $\leq$ 50 nmol/L, IOM, 2011] (**Table 3**). No statistically significant differences in age- and sex-adjusted LTL means by categories of serum 25(OH)D were observed (P = 0.13, Table 2). Multiple regression models resulted in no statistically significant associations between categories of 25(OH)D concentrations and LTL (Table 3).

Discussion

The present study investigated the association between LTL and SNPs in genes codifying enzymes or transport proteins involved in vitamin D metabolism, as well as serum 25(OH)D concentrations. The results reported herein suggest that a SNP in vitamin D binding protein gene (*GC* - rs2282679) is associated with telomere length, with individuals carrying CC genotype having shorter LTL.

Biological plausibility of a possible implication of vitamin D on LTL shortening relies on its effects on the inflammatory process and/or the rate of cell proliferation (3, 38, 39). An association between hsCRP and LTL was not observed in the present study, similarly to previous reports (40, 41). Nevertheless, it has been frequently suggested that the process of telomere shortening can result from oxidative stress and inflammation (42), with evidences of an inverse association between LTL and serum CRP (33, 43) and inflammatory cytokines (44, 45) concentrations. It was demonstrated that active metabolite of vitamin D [1,25(OH)$_2$D] down-regulates the expression and production of several pro-inflammatory cytokines including TNF- α, IL-1β, IL-6, and IL-8 in monocytes (3, 46, 47). Moreover, it is also known that vitamin D has functions in the regulation of cell proliferation and differentiation (37, 38), which could affect the turnover rate of LTL.

Based on these experimental results, some studies investigated the association between LTL and serum concentrations of 25(OH)D, the major circulating metabolite and the best marker of vitamin D status. However, conflicting results were observed. A direct association between serum 25(OH)D concentrations and LTL has been reported in women (9,10). Vitamin D

deficiency (serum concentrations of 25(OH)D < 30nmol/L) was also significantly associated with shorter LTL only in white men and women, with no association with the other racial groups studied (14). The same study reported no significant association with LTL, when serum 25(OH)D was treated as a continuous variable (14). In a nationally representative sample of U.S.A. adults (National Health and Nutrition Examination Survey –NHANES), a subtle direct association between serum concentrations of 25(OH)D and LTL was observed only in middle-aged adults (13), with no evidence of association without age stratification (15). Similarly, serum concentrations of 25(OH)D were not associated with LTL after adjustment in adults from Northern Finland Birth Cohort 1966 (12) and ESTHER cohort study (16). Also, neither 25(OH)D nor 1,25(OH)$_2$D concentrations were associated with LTL in American men (11). In the present study, after adjustment for covariates, no significant association was observed between LTL and serum 25(OH)D concentrations.

At least in part, these mixed findings may be a consequence of evaluating serum concentrations of 25(OH)D cross-sectionally, which may not reflect vitamin D status throughout life. However, the measurement of LTL in a given moment is a result of a continuous shortening process since birth. Therefore, we hypothesized that SNPs in vitamin D metabolic pathway genes that could affect 25(OH)D serum concentrations throughout life would be promising markers to explore long-term effect of vitamin D in LTL. In the present study, one SNP was associated with LTL: *GC* - rs2282679. For this SNP, individuals carrying CC genotype had 12% lower T/S ratio than those carrying AA genotype.

Only two studies evaluated the association of vitamin D-related

polymorphisms and LTL (10,11). Studying women from Nurses' Health Study (US), Liu and colleagues (10) explored twelve SNPs (other than those explored in the present study) in vitamin D-related genes and found no association with LTL. In the study of Julin and coworkers (11), one SNP (rs41400444) in the *RXRA* (Retinoid X Receptor Alpha) gene was associated with longer LTL in American men. However, the authors emphasize that despite *RXRA* forms a heterodimer with $1,25(OH)_2$D-VDR in the cell nucleus before attaching to target genes, *RXRA* also has other roles in the cell that are independent of the vitamin D pathway (11). It is noteworthy that some of the main SNPs usually associated with vitamin D status were not included in those previous studies on LTL.

Several genome-wide association studies (GWAS) have identified SNPs associated with 25(OH)D serum concentrations (17, 19, 21, 48). Particularly, it has been proposed that SNPs in vitamin D metabolic pathway genes as *GC*, *NADSYN1*/*DHCR7*, *CYP2R1* and *CYP24A1* could identify individuals genetically predisposed to vitamin D insufficiency (17, 19, 21, 48).

The *GC* gene encodes the vitamin D binding protein (DBP) that stores and transports both 25(OH)D and $1,25(OH)_2$D (49). Thus, it is possible that SNPs in *GC* gene may affect DBP synthesis and, consequently, circulating 25(OH)D concentrations. Studies in different populations observed a strong association between *GC* - rs2282679 and vitamin D status (50), with lower concentrations of 25(OH)D in individuals carrying CC genotype (51-53). Similarly, *GC* - rs2282679 was also one of the determinants of serum 25(OH)D concentrations in the participants of the present study, with CC genotype associated with lower concentrations of 25(OH)D (54). The fact that individuals carrying CC genotype showed lower LTL compared to individuals carrying AA

genotype, supports the hypothesis of a long-lasting impact of vitamin D status on telomere shortening.

Limitations of our study include its cross-sectional nature. LTL and 25(OH)D were measured once, limiting causal inferences on telomere shortening process by vitamin D status. We believe that, at least in part, using SNPs in vitamin D metabolic pathway as additional markers overcame this limitation. However, the evaluation of genotype combination effect on LTL was limited by the sample size.

Conclusions

To our knowledge, this is the first study reporting a statistically significant association between LTL and a SNP in vitamin D binding protein gene (*GC* - rs2282679), recognized by GWAS as a determinant of 25(OH)D serum concentrations. The use of 25(OH)D measured at a single time appears to be little useful, when exploring the potential effect of vitamin D on LTL.

Our results suggest that SNPs related to vitamin D status, especially in *GC* gene, could be more relevant, due to its invariant nature, to explain the potential continuous influence of vitamin D status on telomere length maintenance throughout life.

Declaration of interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. General Characteristics of the Study Population: Pró-Saúde Study–Rio de Janeiro, Brazil, 2012-2013 (n=464).

	Frequencies	
	n	%
Age (years)		
≤ 44	90	19.4
45-59	297	64.0
≥ 60	77	16.6
Sex		
Female	239	51.5
Male	225	48.5
Race/skin color		
Black	83	17.9
Brown	152	32.8
White	229	49.4
Educational attainment		
High school or less	200	43.1
College education or higher	264	56.9
Equivalent household income (*per capita*, minimum wages)		
< 3 minimum wages	209	45.0
3-6 minimum wages	185	39.9
> 6 minimum wages	70	15.1
Marital Status		
Single	67	14.4
Married	305	65.7
Separated/divorced	76	16.4
Widower	16	3.4
Physical activity practice (in the last two weeks)		
Yes	188	40.5
No	276	59.5
Smoking status		
Never smoker	296	63.8
Current or former smoker	168	36.2
Body mass index (BMI, kg/m^2)		
Underweight or normal	126	27.2
Overweight	190	40.9
Obesity I, II or III	148	31.9
Diagnostic of chronic disease		
No	192	41.4
Yes	272	58.6

Table 2. Association between SNPs in vitamin D pathway genes and LTL: Pró-Saúde Study – Rio de Janeiro, Brazil,2012-2013.

	N (%)	LTL (T/S ratio)[*]	Model 1 β (95% CI)	Model 2 β (95% CI)	Model 3 β (95% CI)
NADSYN1 - rs12785878 (G>T)					
GG	124 (26.7)	0.57±0.01	Ref	Ref	Ref
GT	249 (53.7)	0.56±0.01	-0.015 (-0.047, 0.017)	-0.015 (-0.047, 0.017)	-0.015 (-0.047, 0.017)
TT	91 (19.6)	0.59±0.02	0.022 (-0.019, 0.062)	0.019 (-0.022, 0.060)	0.020 (-0.021, 0.061)
Dominance					
GG	124 (26.7)	0.57±0.01	Ref	Ref	Ref
GT + TT	340 (73.3)	0.57±0.01	-0.003 (-0.034, 0.028)	-0.004(-0.034, 0.027)	-0.003 (-0.034, 0.028)
Recessive					
GG + GT	373 (80.4)	0.56±0.01	Ref	Ref	Ref
TT	91 (19.6)	0.59±0.02	0.031(-0.003, 0.065)	0.028(-0.006, 0.062)	0.029 (-0.006, 0.063)
Overdominance					
GG + TT	215 (46.3)	0.58±0.01	Ref	Ref	Ref
GT	249 (53.7)	0.56±0.01	-0.024(-0.051, 0.003)	-0.022(-0.049, 0.005)	-0.023(-0.050, 0.005)
CYP2R1 - rs10741657 (A>G)					
AA	41 (8.8)	0.56±0.02	Ref	Ref	Ref
GA	188 (40.5)	0.56±0.01	-0.004 (-0.054, 0.047)	-0.002 (-0.052, 0.048)	-0.002 (-0.053, 0.049)
GG	235 (50.6)	0.58±0.01	0.014 (-0.036, 0.063)	0.014 (-0.035, 0.064)	0.015 (-0.035, 0.064)
Dominance:					
GG	235 (50.6)	0.56±0.01	Ref	Ref	Ref

GA + AA	229 (49.4)	0.58±0.01	-0.018(-0.045, 0.010)	-0.016(-0.044, 0.011)	-0.017(-0.044, 0.011)
Recessive:					
GG + GA	423 (91.2)	0.57±0.01	Ref	Ref	Ref
AA	41 (8.8)	0.56 ±0.02	-0.006(-0.053, 0.042)	-0.007(-0.054, 0.041)	-0.007(-0.056, 0.041)
Overdominance					
AA + GG	276 (59.5)	0.58±0.01	Ref	Ref	Ref
GA	188 (40.5)	0.56 ±0.01	-0.016 (-0.044, 0.011)	-0.015(-0.043, 0.012)	-0.016 (-0.043, 0.012)
GC - rs2282679 (A>C)					
AA	282 (60.8)	0.57±0.01[#]	Ref	Ref	Ref
AC	156 (33.6)	0.58±0.01[#]	0.012 (-0.017, 0.041)	0.012 (-0.017, 0.041)	0.012 (-0.017, 0.041)
CC	26 (5.6)	0.51±0.03[‡]	**-0.062 (-0.121, -0.003)**	**-0.061 (-0.120, -0.002)**	**-0.061 (-0.120, -0.001)**
Dominance:					
AA	282 (60.8)	0.57 ±0.01	Ref	Ref	Ref
AC + CC	182 (39.2)	0.57 ±0.01	-0.001 (-0.028, 0.027)	0.000 (-0.028, 0.028)	0.000 (-0.028, 0.028)
Recessive					
AA + AC	438 (94.4)	0.57±0.01[#]	Ref	Ref	Ref
CC	26 (5.6)	0.50 ±0.03[‡]	**-0.066 (-0.125, -0.008)**	**-0.065 (-0.123, -0.007)**	**-0.065 (-0.123, -0.006)**
Overdominance					
AA + CC	308 (66.4)	0.56 ±0.01	Ref	Ref	Ref
AC	156 (33.6)	0.58 ±0.01	0.017 (-0.011, 0.046)	0.017 (-0.011, 0.046)	0.018 (-0.011, 0.046)

[*]LTL values are age and sex adjusted mean ± SE.

[#,‡]Different superscript symbols in the same column indicate significant differences on

LTL (P<0.05; LSD post hoc test) by age and sex adjusted variance analysis.

Multiple regression models used to investigate associations between LTL and SNPs in vitamin D pathway genes: Model 1: adjusted by age, sex and race/skin color; Model 2: adjusted by variables in model 1 plus marital status, equivalent *per capita* household income, and educational attainment; Model 3: adjusted by variables in model 2 plus BMI, physical activity practice in the last two weeks, smoking status, and diagnostic of chronic disease.

Table 3. Association between 25(OH)D serum concentrations and LTL: Pró-Saúde Study – Rio de Janeiro, Brazil,2012-2013.

	N (%)	LTL (T/S ratio)[*]	Model 1 β (95% CI)	Model 2 β (95% CI)	Model 3 β (95% CI)
Serum 25(OH)D					
<30 nmol/L	86 (18.5)	0.59±0.02	Ref	Ref	Ref
30 - 50 nmol/L	172 (37.1)	0.58±0.01	-0.004 (-0.041, 0.034)	-0.004 (-0.042, 0.034)	0.000 (-0.039, 0.038)
51 - 75 nmol/L	168 (36.2)	0.56±0.01	-0.019 (-0.058, 0.020)	-0.021 (-0.060, 0.018)	-0.019 (-0.060, 0.022)
>75 nmol/L	38 (8.2)	0.53±0.02	-0.034 (-0.092, 0.025)	-0.035 (-0.094, 0.023)	-0.028 (-0.091, 0.036)

[*] LTL values are age and sex adjusted mean ± SE. Differences on LTL by serum 25(OH)D categories were tested by variance analysis (P<0.05; LSD post hoc test).

Multiple regression models used to investigate associations between LTL and 25(OH)D serum concentrations: Model 1: adjusted by age, sex, marital status, equivalent *per capita* household income and educational attainment; Model 2: adjusted by variables in model 1 plus physical activity practice in the last two weeks, smoking status, and diagnostic of chronic disease; Model 3: adjusted by variables in model 2 plus skin type, month of blood collection, sun exposure index, visceral fat mass, and hsCRP.