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Relationship between BsmI polymorphism and *VDR* gene methylation profile, gender, metabolic profile, oxidative stress, and inflammation in adolescents

Relación entre polimorfismo BsmI y perfil de metilación del gen VDR, *género, perfil metabólico, estrés oxidativo e inflamación en adolescentes*

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Abstract

Background: the biological activity of vitamin D depends on the activity of its receptor or VDR. On the other hand, the activity of this receptor is influenced by its state of methylation. The objective of this study was to verify if the BsmI polymorphism of the *VDR* gene influences its methylation profile in adolescents. Secondly, it was to verify if the status of some metabolic factors (oxidative stress, inflammation, lipid profile, and glycemia) in the serum, and gender-adjusted vitamin D levels are independent factors with an influence on the *VDR* methylation profile.

Methods and results: the study included 198 adolescents of both sexes, aged 15-19 years, who underwent testing for *VDR* gene methylation polymorphisms, serum vitamin D levels, and metabolic, oxidative stress, and systemic inflammation markers. It was observed that the BB genotype was less methylated than the other groups (26.1 % versus 30.3 %, and 29.3 % for Bb and bb, respectively), although without statistical differences between them. The odds ratio indicated a protection of 13 % (partially methylated) for vitamin D status, while alpha glycols increased the risk ratio (of being partially methylated) by 3 %. MDA was protective at a 28 % chance of risk that adolescents with higher levels of lipid peroxidation would be hypomethylated.

Conclusion: we conclude that the methylation profile of the *VDR* gene is not influenced by the different BsmI polymorphism genotypes, and that serum vitamin D and serum markers of oxidative stress and inflammation can modulate this profile.

Keywords:

Polymorphisms.
DNA methylation.
Vitamin D. *VDR* gene.

Resumen

Antecedentes: la actividad biológica de la vitamina D depende de la actividad de su receptor, el VDR. Por otro lado, la actividad de este receptor está influenciada por su estado de metilación. El objetivo de este estudio es verificar si el polimorfismo BsmI del gen VDR influye en el perfil de metilación del mismo en los adolescentes. En segundo lugar, verificar si los factores metabólicos (estrés oxidativo, inflamación, perfil lipídico y glucemia) del suero y la vitamina D ajustada por sexo actúan independientemente de los polimorfismos sobre el perfil de metilación del VDR.

Métodos y resultados: el estudio incluyó a 198 adolescentes de ambos sexos, de 15 a 19 años de edad, que se sometieron a análisis de polimorfismos de metilación del gen VDR, niveles de vitamina D, marcadores metabólicos, estrés oxidativo e inflamación sistémica. Se observó que el genotipo BB estaba menos metilado que los otros grupos (26,1 % contra 30,3 % y 29,3 % para Bb y bb respectivamente), aunque sin diferencias estadísticas entre ellos. El *odds ratio* indicó una protección del 13 % (parcialmente metilado) para el estado de la vitamina D, mientras que los alfa glicoles aumentaron el índice de riesgo (de estar parcialmente metilado) en un 3 %. La MDA fue protectora con un 28 % de probabilidad de riesgo de que los adolescentes con niveles más altos de peroxidación lipídica fueran hipometilados.

Conclusión: concluimos que el perfil de metilación del gen VDR no está influenciado por los diferentes genotipos del polimorfismo BsmI y que la vitamina D y los marcadores de estrés oxidativo e inflamación en el suero pueden modular este perfil.

Palabras clave:

Polimorfismos.
Metilación del ADN.
Vitamina D. Gen *VDR*.

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INTRODUCTION

Research in recent years has shown that the role of vitamin D transcends bone metabolism, and includes 3 % regulation of the human genome (1), inflammation control (2-4), oxidative stress (5), blood pressure regulation (6), immunoregulation, embryogenesis, and tumorigenesis (7-9). Even in adolescents it has been noted that vitamin D insufficiency is associated with abdominal adiposity, hypercholesterolemia, insulin resistance, and hypertension (10), as well as increases in malondialdehyde and interleukin 6 levels (11).

The vitamin D receptor (VDR) is a member of the family of nuclear steroid receptors, which are transcriptional regulators and responsible for the functionality of calcitriol, the active form of vitamin D (12). VDR is present in tissues and organs such as prostate, breast, colon, pancreas, and immune system cells (13), confirming the multi-systemic action of vitamin D.

DNA methylation is widely studied as an epigenetic marker, and the main gene silencing phenomenon (14). In humans, methylation takes place at the cytosine-guanine binding sites, where the regions of DNA that are enriched with these clusters form the CpG islands (15). These CpG islands are found in many promoter regions and, when methylated, transcription is interrupted (16). In addition, DNA methylation is considered a key to different biological processes, regulating reactions such as the cell cycle, cell differentiation, genomic imprinting, and inactivation of the X chromosome in women (17).

Given the importance of VDR, the study of its activity represents an important goal in the investigations of the dosing and metabolic effects of vitamin D. Considering that the response to serum vitamin D levels is directly related to its receptor (18), control of its gene expression is an influencing factor in the activity of this vitamin (19). The methylation profile is one of the determining aspects of gene expression, but so far only Beckett et al. (20) report the influence of VDR gene polymorphisms on its degree of methylation in relation to light exposure. Due to this scarcity of studies on the relationship between genetics and epigenetics, in this paper we assessed the methylation profile of the studied population and related it to genetic, metabolic, and demographic factors, and even to the concentration of vitamin D in the serum.

On the other hand, previous studies have shown that people genotyped with any of the *VDR* gene polymorphisms had lower serum vitamin D activity in terms of both concentration (21-23) and response to supplementation (24,25), as well as oxidative stress (26), inflammatory markers (27) and glycemia (28), when these variables were analyzed for vitamin D status. However, these studies did not clarify whether *VDR* gene functionality is involved in these responses in the elderly and adults, nor in children or adolescents.

It is also known that environmental factors (29), smoking (30), physical activity level (31), and dietary aspects are considered potential modulators of genetic activity, especially regarding DNA methylation, both globally and site-specifically (32,33). But these factors have also not been investigated regarding the control of *VDR* gene expression.

Given the above, the present work aims to verify whether the BsmI polymorphism of the *VDR* gene influences its methylation profile in a population of adolescents of both sexes. Secondly, it also aims to verify whether metabolic factors (oxidative stress, inflammation, lipid profile, and glycemia) serum vitamin D status, and demographics (gender) act in isolation as factors influencing polymorphisms in the *VDR* methylation profile.

MATERIALS AND METHODS

PARTICIPANTS

The data for the present study were collected from the population of a previous study designed to determine the prevalence of vitamin D insufficiency/deficiency in adolescents from the city of João Pessoa, PB, Brazil. It was conducted in 225 adolescents aged 15-19 years (34). All adolescents provided genetic material for the present study, but analysis losses occurred so that 196 adolescents (77 boys and 119 girls) remained for the purposes of this study.

The adolescents had already reached the postpubertal period (menarche for girls and pubic hair growth for boys), and had their cognitive status preserved. Exclusion criteria were: pregnant or lactating adolescents, use of vitamin D-containing dietary supplements, use of anticonvulsant drugs or of drugs for treating HIV/AIDS, adolescents diagnosed with type-I diabetes *mellitus*, nephrotic syndrome, acute or chronic kidney failure, liver diseases, hypothyroidism, or hyperthyroidism, alcohol drinkers and chronic smokers.

The study was submitted and approved by the Ethics and Research Committee of the Health Sciences Center (CCS) in accordance with Resolution 466 of the National Health Council (CAAE 43097115.2.0000.5188). All participants over the age of 18 and the parents or guardians of younger subjects were asked to freely sign an informed consent form. Children under 18 signed a consent form while their parents or guardians signed an informed consent form.

The adolescents were genotyped in relation to the *VDR* gene BsmI polymorphism, and the methylation profile of this gene was evaluated. In addition, blood samples were taken for analysis of serum vitamin D, PTH, glycemia, lipid profile, oxidative stress (MDA and TAC), inflammatory processes (hsPCR and A1GPA), and anthropometric data such as weight and height were also collected.

DNA ISOLATION

Chromosomal DNA was obtained from leukocytes. Leukocyte DNA was isolated, quantified, and transformed with sodium bisulfite according to the conditions described in a previously published study (adapted from (35)).

ANALYSIS OF THE BsmI POLYMORPHISM (rs1544410)

Genotypes were determined by restriction size polymerase chain reaction (PCR-RFLP). For variant rs1544410 the primers:

5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3 '(sense) and 5'-AACCAGCGGGAA GTCAAGGG-3' (antisense); temperatures of 94 °C (10 minutes), 58 °C (1 minute) and 72 °C (5 minutes) in 30 cycles, with an extra 10-minute extension step. The 825 bp product was digested to generate two fragments (650 bp and 175 bp) while the ancestral allele B would remain at 825 bp.

METHYLATION LEVELS

Leukocyte DNA was isolated, quantified, and transformed with sodium bisulfite according to the conditions described in a previously published study (36). The analysis of genomic DNA methylation levels from the blood was performed by the high-resolution, real-time PCR (HRM) method on an Applied Biosystems 7500 Fast Real-Time PCR System. PCR was performed in a total volume of 20 mL containing: 1 × buffer, 4 mM Mg^{2+}, 200 mM from each dNTPs (Qiagen), 250 nM from each primer, 5 mM SYTO® (Invitrogen), 1 U HotstarTaq DNA Polymerase (Qiagen), and 1 µl of bisulfite-modified DNA.

The primers for the *VDR* gene were designed from the genome sequence deposited in the UCSC genome browser: http://genome-euro.ucsc.edu/(chr8: 37,962,991-37,966,965) using sequence F: 5'-AGTTTTGGTTTGGTTAGTTTAGGTG-3 'Start size 111, Tm 25, GC% 68.00,' C's 8 for left primer, and sequence R: 51-TAAAATCAACCACCCTATAAACCAC ', Start size 372, Tm 25, GC% 52.00, 'C's 4 for the right primer.

The PCR program consisted of an initial enzymatic activation at 95 °C for 10 min, followed by 50 cycles of 45 sec at 95 °C, 45 sec at 60 °C, and 45 sec at 72 °C, with the final extension at 72 °C for 10 min. Fusion curves were normalized by calculating the "best-fit line" between two normalization regions before and after decreasing main fluorescence, representing the fusion of the PCR product using the software provided with the HRM v2.0 Software provided by the 7500 Fast PCR system.

BIOCHEMICAL ANALYSES

All participants were instructed to fast for 12 hours for blood collection and subsequent analysis of vitamin D, parathyroid hormone (PTH), lipid profile, glycemia, inflammatory markers such as ultra-sensitive C-reactive protein (hsCRP), and alpha-1-acid glycoprotein (AGPA), oxidative stress markers such as malondialdehyde (MDA) and total antioxidant capacity (TAC)), oxidizing and antioxidant markers, respectively.

Serum concentrations of 25(OH)D and PTH were measured by chemiluminescence immunoassay (UniCel Dxl 800, Beckman Coulter). The classification of vitamin D levels was performed based on the reference values used by the Endocrine Society, 2011, which considers: deficient serum 25(OH)D levels, below 20 ng/mL; insufficient levels between 21 and 29 ng/mL; and sufficient levels between 30 and 100 ng/mL (8). For PTH, 15-65 pg/mL were accepted as reference values (8).

MDA was quantified by reaction of thiobarbituric acid (TBARS) with the hydroperoxide decomposition products, according to the method described by Ohkawa, Ohishi, and Yagi (37). To this end, 250 µL of plasma sample were added to potassium chloride (KCl), and were incubated in a water bath at 37 °C for 60 minutes. The mixture was then precipitated with 35 % AA perchloric acid and centrifuged at 1400 rpm for 20 minutes at 4 °C. The supernatant was transferred to new microtubes, and 400 µL of 0.6 % thiobarbituric acid were added and incubated at 100 °C for 60 minutes. After cooling, the material was read in an ultraviolet spectrophotometer (Biospectro, model SP-220, Brazil) at a wavelength of 532 nm at room temperature.

Plasma TAC was assessed by the DPPH method. The procedure was based on the method described by Brand-Williams, Cuvelier, and Berset (38), in which an aliquot of 1.25 mg of DPPH was diluted in 100 mL of ethanol, and kept refrigerated and protected from light (with aluminum foil or amber glass). In appropriate centrifuge tubes 3.9 mL of the DPPH solution were added, and then 100 µL of plasma were added. The tubes were vortexed and allowed to stand for 30 minutes. They were then centrifuged at 10,000 rpm at 20 °C for 15 minutes, and the supernatant was used for spectrophotometer reading at 515 nm. Results were expressed as antioxidant activity (%), where:

$$AOA = 100 - [DPPH \bullet R] t / [DPPH \bullet R] B 100)$$

Where [DPPH • R] t and [DPPH • R] B are the remaining DPPH • concentration after 30 minutes, evaluated in the sample (t) and blank (B) prepared with distilled water.

HsCRP was analyzed using the immunonephelometric method, and reference values for CRP between 1 and 3 mg/l were considered based on the VI Brazilian Guideline on dyslipidemia and prevention of atherosclerosis by the Brazilian Society of Cardiology (39), which considers as risk for cardiovascular disease a high-sensitivity C-reactive protein > 3 mg/l (in the absence of nonsclerotic etiology). Finally, for the analysis of AGPA the immunonephelometric method was used and adopted as a reference for normal values of 40 to 150 mg/dL (40).

The lipid and glycemic profile analyses were performed on serum samples using Labtest commercial kits (Minas Gerais, Brazil), following the manufacturer's recommendations, and on a Labmax 240 premium automated analyzer (Lagoa Santa, MG, Brazil).

Total cholesterol was determined by the enzymatic method proposed by Trinder (41) at 500 nm. HDL-c was quantified by the manual method. For this procedure, a volume of 0.25 mL of precipitating substance was added to 0.25 mL of serum sample contained in microtubes, and mixed vigorously for 30 seconds. It was then centrifuged at 3,500 rpm for 15 minutes. The supernatant was removed and put into containers of 1 mL of Reagent 1 from the Cholesterol Liquiform Kit, and placed in a water bath for 10 minutes. Finally, the ultraviolet spectrophotometer (Biospectro, model SP-220, Brazil) was read at 500 nm.

Triglyceride values were determined using the enzymatic model proposed by Trinder (41), and the absorbance was obtained at a wavelength of 505 nm. Low-density lipoprotein (LDL-c) values were obtained by Friedwald's equation: LDL-c = (CT-HDL-c) - (TG / 5) (Friedewald; Levy; Fredrickson, 1972).

Blood glucose concentrations were determined using the glucose-oxidase colorimetric enzymatic method proposed by Trinder (41). Absorbance was obtained at a wavelength of 505 nm. They were used for reference values for the variables of the lipid and glycemic profile of adolescents as established by the Brazilian Society of Cardiology (39).

STATISTICAL ANALYSIS

The data were initially tested for normality and homogeneity by the Kolmogorov-Smirnov test. A one-way analysis of variance was used to compare the characteristics of adolescents categorized by the three potential genotypes. To verify the association between genotype and methylation profile the chi-square test was used. To verify the influence of influential variables on this association a logistic regression was used. All statistical treatments were performed using the SPSS, version 24 software, adopting $p < 0.050$.

RESULTS

The study population consisted of 196 adolescents from public schools, 61.1 % girls and 38.9 % boys between the ages of 15 and 19 years. When categorized by BB, Bb and bb genotypes, it was observed that the BB group was less methylated than the other groups, 26.1 % versus 30.3 % and 29.3 % for Bb and bb, respectively; however, without statistical differences between the three groups (Table I). In this same table it can be noted that the three genotypes were similar for the analyzed metabolic variables and demographic factors. Table I shows the values described for the variables studied.

Considering the criterion adopted that subjects would be considered unmethylated when their methylation level was below 25 %, partially methylated between 26 % and 75 %, and methylated above 75 % (42), it was observed that there were no methylated adolescents in the group. Meanwhile, only partially methylated subjects were 53 % and unmethylated subjects were 47 %. When the chi-square test was applied, considering the three genotypes, it was observed that there was no significant difference in the distribution of partially methylated and unmethylated subjects between categories BB, Bb and bb, as shown in table II.

To verify the influence of metabolic and demographic factors on the relationship of the BsmI polymorphism on the methylation profile of the VDR gene, a logistic regression analysis was performed, where the methylation profile (partially methylated and unmethylated) was considered the dependent variable, and the

Table I. The sample's anthropometric characteristics, methylation level, oxidative stress, inflammatory process, and metabolic profile (n = 196)

Variable	BB	Bb	Bb	p
Age	17.2 ± 1.2	16.8 ± 0.93	17.0 ± 1.1	0.190
BMI	23.4 ± 4.6	22.8 ± 4.7	22.9 ± 4.4	0.820
Methylation level	26.1 ± 12.4	30.3 ± 14.2	29.4 ± 13.0	0.243
Vitamin D (ng/dL)	31.0 ± 9.5	29.3 ± 8.1	28.5 ± 7.7	0.266
PTH	29.1 ± 14.2	31.1 ± 20.2	27.9 ± 11.3	0.411
TAC (unit)	30.3 ± 8.4	31.5 ± 7.6	31.9 ± 8.5	0.551
MDA (unit)	3.5 ± 1.1	3.6 ± 1.1	3.4 ± 1.1	0.804
HsCRP (mg/dL)	1.8 ± 2.0	1.9 ± 2.4	1.7 ± 2.5	0.888
A1GPA	89.0 ± 23.6	87.6 ± 23.2	84.6 ± 19.9	0.511
Triglycerides (mg/dL)	80.4 ± 43.9	79.4 ± 42.5	80.6 ± 33.7	0.980
Total cholesterol (mg/dL)	151.1 ± 29.1	156.4 ± 28.8	160.0 ± 25.4	0.225
HDL cholesterol (mg/dL)	44.9 ± 9.5	45.6 ± 9.4	47.3 ± 10.3	0.351
LDL cholesterol (mg/dL)	90.1 ± 23.6	94.8 ± 25.4	96.5 ± 21.7	0.337
Blood glucose (mg/dL)	86.6 ± 9.9	85.6 ± 9.5	83.2 ± 8.3	0.103

Data are expressed as mean and standard deviation (SD); 25(OH)D: 25-hydroxyvitamin D; HsCRP: C-reactive protein, ultrasensitive; AGPA: alpha 1 acid glycoprotein; MDA: malondialdehyde; TAC: total antioxidant capacity. ANOVA one way for $p < 0.050$.

Table II. *VDR* gene BsmI polymorphism with methylation profile

	BB	Bb	Bb	p-value
Hypomethylated	26 (59.09 %)	30 (43.47 %)	37 (43.52 %)	0.189
Partially methylated	18 (49.91 %)	39 (56.52 %)	48 (56.47 %)	
Total	44	69	85	

Data are absolute and relative frequencies (in parentheses) of adolescents in each genotype category. Chi-square test for $p < 0.050$.

demographic (age), anthropometric (BMI) and metabolic factors (serum vitamin D and PTH levels, oxidative stress, inflammation, lipid profile and glycemia) as independent variables.

When only the genotype was considered, a p-value of 0.186 was found, with a likelihood log of 267.611, confirming that the polymorphism as a predictor does not improve the regression adjustment. When considering the covariates, the best model found was the presence of the variables age, BMI, gender, vitamin D, PTH, glycemia, triglyceride, HDL cholesterol, CRP, A1GPA, MDA and TAC, which resulted in a Hosmer and Lemeshow test equal to 0.055, indicating the suitability of this model. As a result of this, the variables that showed to influence the methylation profile were vitamin D, alpha glycol and MDA. The odds ratio indicated that it increases the chance of adolescents with higher serum vitamin D values having a protection ratio of 13 % (partially methylated), while alpha glycosis increases the risk ratio (of being partially methylated) by 3 %. MDA was protective at a risk ratio of 28 % chance that adolescents with higher levels of lipid peroxidation would be hypomethylated. Table III shows all these values for the risk ratio, in addition to the other variables.

Table III. Logistic regression/number of observations = 196

Model 1 (VDR methylation profile)			
	RR	**Confidence interval**	**p-value**
Genotype 1, BB; 2, Bb; 3, bb			0.150
Genotype 1, BB; 2, Bb; 3, bb (1)	0.429	0.174-1.054	0.065
Genotype 1, BB; 2, Bb; 3, bb (2)	0.937	0.441-1.990	0.865
Age	1.142	0.831-1.568	0.414
Sex	0.845	0.389-1.839	0.672
BMI	0.951	0.878-1.029	0.209
Vitamin D (ng/dL)	*0.871*	*0.820-0.925*	*0.000**
PTH (pg/mL)	0.997	0.975-1.019	0.786
Blood glucose (mg/dL)	1.034	0.996-1.074	0.083
Triglycerides (mg/dL)	0.998	0.989-1.007	0.692
HDL cholesterol (mg/dL)	0.972	0.930-1.016	0.090
hsPCR (mg/dL)	0.852	0.707-1.027	0.097
A1GPA (mg/dl)	*1.030*	*1.008-1.053*	*0.008**
MDA	*0.718*	*0.519-0.993*	*0.045**
TAC	0.999	0.959-1.040	0.963

Data are relative ratio, considering the partially methylated state as reference, and confidence interval. 25(OH)D: 25-hydroxyvitamin D; hsCRP: C-reactive protein, ultrasensitive; AGPA: alpha-1 acid glycoprotein; MDA: malondialdehyde; TAC: total antioxidant capacity. Logistic regression test for $p < 0.050$.

DISCUSSION

Proper functioning of vitamin D in its active form, calcitriol, is known to depend on its VDR receptor, a member of the nucleus steroid receptor family (18). The presence of the *VDR* gene BsmI polymorphism has been associated with lower 25-hydroxyvitamin D levels, suggesting that this polymorphism may be linked to increased susceptibility to vitamin D deficiency (43,44). In addition, this polymorphism was associated with type-2 diabetes *mellitus*, reduced levels of insulin secretion (45), and metabolic syndrome (46), thus altering the metabolic action of vitamin D.

The association between methylation and the VDR gene has been studied. The authors (47) state that promoter methylation was the cause of the silencing of VDR gene expression in HIV-infected T-cells in humans, which did not happen in normal T-cells. These findings corroborate those (48) who stated that T-cells previously infected with HIV in humans increased promoter methylation in the VDR gene by 45-70 %, thus leading to a decreased expression of this gene. This evidence (49) suggests an inverse correlation between vitamin D status and infection, although some tests do not show the protective effect of vitamin D, still bringing inconclusive data.

As DNA methylation is widely studied as an epigenetic marker, being the main gene-silencing phenomenon, and because of the importance of VDR in the metabolism of vitamin D and the genetic influence on its activity, in this study we proposed to verify whether also epigenetic factors could influence the activity of this receptor, especially methylation of the *VDR* gene. As it is methylated, the actions necessary for the expression of the protein that activates this receptor would be impaired, since this gene follows the classical model of gene expression that is linked to promoter hypomethylation, which nullifies its expression (50).

Vitamin D insufficiency/deficiency has been a concern since adolescence, as this condition in early life can cause metabolic changes and is related to the inflammatory process and oxidative stress, thus increasing susceptibility to various pathologies. In addition, it is known that methylation of the VDR gene can modulate vitamin D activity, but it remains to be seen whether these organic changes are in fact influenced by methylation of the vitamin D receptor gene, and whether healthy adolescents have different methylation of VDR profiles in relation to the BsmI polymorphism since, separately, there is already evidence for genetic and epigenetic activity.

In the present study it was found that the genotypes BB, Bb, bb of the BsmI polymorphism of the *VDR* gene did not influence the methylation profile of this gene. However, after considering the metabolic and demographic variables as possible influences in multivariate regression, we found that the status of serum vitamin D, MDA, and A1GPA were able to modify the *VDR* methylation profile of adolescents.

Meyer and Bornaman (51) demonstrated this by observing that the expression of the *VDR* gene is influenced by its plasma vitamin D levels and its methylation. However, this study has a methodological design quite different from ours, since these authors studied adults with the objective of correlating a specific transcription factor of immune cells, CDX-2, with *VDR* gene methylation, without analyzing correlations with polymorphisms in this gene.

While on the one hand the methylation profile influences VDR activity, improving the release of 25(OH)D3 (51), few studies have verified whether any polymorphic genotype would also be a factor influencing methylation of the *VDR* gene. Only two studies (52,20) had verified the influence of the Bb genotype of the BsmI polymorphism on the methylation profile of the *VDR* gene, but the context of this study was very different from ours. They studied elderly subjects over 65 years of age to verify the degree of methylation of the *VDR* gene in relation to light exposure, and the influence of all polymorphisms, and found a positive relationship with mutant BsmI alleles increasing methylation of the *VDR* gene, whereas the mutant alleles TaqI and FokI decreased methylation.

It is noteworthy that we did not find any hypermethylated adolescents in our sample, only hypomethylated and unmethylated adolescents, with the VDR of these being suitable for transcription. In addition, other factors act to control gene expression such as histone modification, transcription factors, and micro RNA (50), factors that were not analyzed in this study. These findings lead to the need for further studies with this type of population, or even with adults/elderly, to provide an outcome relating the BsmI polymorphism of the *VDR* gene with its methylation.

Although our primary hypothesis that the BsmI genotype would influence the *VDR* methylation profile has not been confirmed, environmental factors analyzed by us (serum vitamin D levels, inflammatory markers and oxidative stress) were intervening variables for the modulation of the methylation profile of this gene, this being the main finding of our study. Therefore, we demonstrated that environmental factors, rather than genetic ones, were better able to control *VDR* expression, and that lifestyle was an important factor for the health of this population.

Our study also showed that inflammation reduces VDR activity, indicating that the inflammatory process influences *VDR* gene expression. This implies that the inflammatory process hinders the action of the vitamin D receptor. However, this data should be viewed with caution since, if an association was seen with AIGPA, the risk factor was only 3 %. In addition, we found no relationship of the methylation profile of the *VDR* gene to CRP, which is an inflammatory marker similar to AIGPA, so further studies are needed to categorically confirm this statement. Cytokine analyses are suggested, as these markers are more directly indicative of systemic inflammation.

Regarding oxidative stress status (53), it negatively regulates the expression of *VDR* in endothelial cells. Similarly, eight weeks of vitamin D supplementation increased total antioxidant activity and glutathione peroxidase, thus demonstrating beneficial effects on oxidative stress (54). However, our data is in the opposite direction of the previous literature, since adolescents with higher serum vitamin D levels had higher lipid peroxidation. This data would indicate vitamin D as a risk factor for oxidative stress, contrary to expectations that vitamin D reduces oxidative stress. One possible explanation is the fact that in the present study we analyzed only one indicator of oxidative stress, so that the effect found may have been a random statistical result.

The clinical interpretation of our findings demonstrates that greater care is needed with habits that stimulate vitamin D synthesis, because even in healthy adolescents the literature shows a high prevalence of hypovitaminosis D in those from Northeastern Brazil (34), Southern Brazil (23), Southeast Brazil (10), and also worldwide (55,56). In addition, there is a relationship between vitamin D deficiency and future onset of fertility-related diseases, especially in the female population, such as endometriosis, breast cancer, and polycystic ovary syndrome (57). However, there are no reports that adolescents exhibit immediate damage from vitamin D deficiency, as is the case with adults and the elderly (58,59).

Therefore, genotype did not appear to be an influencing factor on the methylation profile of the *VDR* gene, but environmental factors such as serum vitamin D levels, inflammatory markers and oxidative stress should be considered. Thus, even in adolescents, the physiological lifestyle and profile should already be taken into account when dealing with the problem of hypovitaminosis D.

CONCLUSION

Given the above, we conclude that the methylation profile of the *VDR* gene is not influenced by the different BsmI polymorphism genotypes in a population of adolescents, but serum vitamin D, and markers of oxidative stress (MDA) and inflammation (A1GPA) were able to modulate this profile when analyzed as influencing factors.

The limitations of our study include the fact that we did not analyze any other markers of the inflammatory process, such as cytokines, as well as of oxidative stress, such as glutathione peroxicity. For this reason, further studies are needed to verify the relationship between genetics and epigenetics, and their possible influences on vitamin D cell receptor activity.

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