Association Between Methylation Levels of LPL, ADRB3 and MTHFR Genes in Leukocytes from Individuals with Isolated or Associated Morbidities and Inflammation: A Cross-Sectional Study

Thais Costa Matte (✉ thaiscostamatte@gmail.com)

Federal University of Paraiba: Universidade Federal da Paraiba Centro de Ciencias da Saude

<https://orcid.org/0000-0002-4553-9304>

Raquel Patrícia Ataíde Lima

Federal University of Paraiba: Universidade Federal da Paraiba Centro de Ciencias da Saude

Rafaella Cristhine Pordeus Luna

Federal University of Paraiba: Universidade Federal da Paraiba Centro de Ciencias da Saude

Maria da Conceição Rodrigues Gonçalves

Federal University of Paraiba: Universidade Federal da Paraiba Centro de Ciencias da Saude

Flávia Emília Leite de Lima Ferreira

Federal University of Paraiba: Universidade Federal da Paraiba Centro de Ciencias da Saude

Sônia Cristina Pereira de Oliveira Ramalho Diniz

Federal University of Paraiba: Universidade Federal da Paraiba Centro de Ciencias da Saude

Roberto Teixeira de Lima

Federal University of Paraiba: Universidade Federal da Paraiba Centro de Ciencias da Saude

Aléssio Tony Cavalcanti de Almeida

Paraiba Federal University: Universidade Federal da Paraiba

Alexandre Sérgio Silva

Paraiba Federal University: Universidade Federal da Paraiba

Alcides da Silva Diniz

Federal University of Pernambuco: Universidade Federal de Pernambuco

Naila Francis Paulo de Oliveira

Paraiba Federal University: Universidade Federal da Paraiba

Darlene Camati Persuhn

Paraiba Federal University: Universidade Federal da Paraiba

Maria José de Carvalho Costa

Federal University of Paraiba: Universidade Federal da Paraiba Centro de Ciencias da Saude

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Association between methylation levels of *LPL*, *ADRB3* and *MTHFR* genes in leukocytes from individuals with isolated or associated morbidities and inflammation: A cross-sectional study

Thais Costa Matte^{1*}, Raquel Patrícia Ataíde Lima¹, Rafaella Cristhine Pordeus Luna¹, Maria da Conceição Rodrigues Gonçalves¹, Flávia Emília Leite de Lima Ferreira¹, Sônia Cristina Pereira de Oliveira Ramalho Diniz¹, Roberto Teixeira de Lima¹, Aléssio Tony Cavalcanti de Almeida², Alexandre Sérgio Silva¹, Alcides da Silva Diniz³, Naila Francis Paulo de Oliveira⁴, Darlene Camati Persuhn⁴, Maria José de Carvalho Costa¹

* thaiscostamatte@gmail.com

Abstract

Epigenetic alterations such as DNA methylation have been associated with the etiology of inflammation-related diseases. The present study evaluated the association between the methylation levels of *LPL*, *ADRB3* and *MTHFR* genes and the stage of inflammation in individuals with isolated or associated morbidities, as well as in individuals without morbidities. This is a cross-sectional population-based study, in which 261 adults, between 20 and 59 years, individuals of both sex were selected. Inflammatory parameters were evaluated in blood, and the evaluation of methylation levels in the promoter of *LPL*, *ADRB3* and *MTHFR* genes was performed in peripheral blood leukocytes. For statistical treatment, normality analysis was performed using the Lilliefors test, multiple linear regression, in addition to odds ratio. For all tests, the significance level adopted was 5%. In individuals with isolated morbidities, a positive association was observed between CRP values (2.49mg/L±3.7) and *LPL* gene methylation levels (35%±18) (p=0.003) and

with associated morbidities, females had higher levels of *LPL* gene methylation ($40\% \pm 20$) ($p = 0.041$) and for *MTHFR* gene methylation levels ($35\% \pm 18$) a positive association was found with MDA values ($3.02\mu\text{mol/L} \pm 0.8$) ($p=0.032$). In addition, the use of medications did not influence the level of methylation for any of the three genes analyzed. Among individuals with isolated or associated morbidities, there was an association between the methylation levels of the *LPL* gene with the CRP values and females, and furthermore with the *MTHFR* gene and MDA. This study could help to understand the etiology and treatment of different morbidities, enabling the discovery of new combats resources.

Keywords: Epigenetics. DNA methylation. Inflammatory Markers. Chronic Non-Communicable Diseases.

Background

The presence of diabetes mellitus, dyslipidemia, hypertension and/or obesity is considered an important indicator of cardiovascular risk, according to the “Strategic Action Plan for Confronting Chronic Non-Communicable Diseases” (CNCDs) of the Ministry of Health of Brazil, with these CNCDs being responsible for the increase in mortality [1].

The epigenetic profile has major potential to better characterize the phenotype of CNCDs, helping to identify individuals at higher risk of developing metabolic complications [2, 3]. In cardiovascular diseases, gene methylation plays an important role in its pathogenesis [4], and in diabetes mellitus type 1 and 2, epigenetic modifications have been identified as a mechanism by which the environment interacts with the genome and modifies its risk [5].

51 The presence of chronic inflammation is a biological mechanism, related to the
52 development of CNCDs [6]. Inflammation mediates epigenetic changes, resulting in
53 multiple signaling pathways that culminate in the activation of gene transcription. In
54 contrast, gene transcription factors and other proteins form transcription complexes that
55 regulate the expression of some genes involved in the inflammatory process [7, 8].

56 The lipoprotein lipase (*LPL*) gene is responsible for encoding a lipoprotein with
57 the same name and is located on chromosome 8q22 [9]. The LPL enzyme has a central
58 role in lipid metabolism, as it works by hydrolyzing lipoproteins rich in triglycerides that
59 are available for energy needs and for storage in the adipose tissue [10, 11]. The increase
60 in *LPL* methylation levels in leukocytes was correlated with dyslipidemic changes, such
61 as lower levels of high-density lipoprotein (HDL) and higher levels of low-density
62 lipoprotein (LDL) [12], increasing the risk of cardiovascular disease, obesity and insulin
63 resistance [13, 14].

64 The β 3 adrenergic receptor gene (*ADRB3*) is located on chromosome 8 [15] and
65 encodes a receptor protein that is a member of the adrenergic receptor family [16] that is
66 mainly expressed in brown, beige and white adipocytes. Activation of the enzyme acts by
67 stimulating brown adipocytes and accelerating the utilization of beige adipocytes, as well
68 as stimulating glucose uptake [17, 18]. Methylation in the *ADRB3* gene in leukocytes has
69 been related to dyslipidemia [19], likewise being a causal factor in the alteration of the
70 waist-hip ratio and alterations in blood pressure in obese individuals [20].

71 The methylenetetrahydrofolate reductase (*MTHFR*) gene exerts an important
72 influence on the DNA methylation process and is located on chromosome 1 (1p26.3) [21].
73 Its role is due to its participation in the production of folate. The folate and methionine
74 cycles together participate in the so-called “one carbon metabolism” which consists of a
75 set of interconnected pathways that provide methyl groups to make methyl available for

methylation [21, 22]. Changes in their methylation levels analyzed in leukocytes are related to the pathophysiology of diabetes and its chronic complications [23, 24], likewise cardiovascular disease and cancer [24, 25].

Studies regarding the influence of more than one gene with inflammatory variables on different associated morbidities presence in the same individual are still scarce, even though this is the most common form of disease prevalence in populational studies. Therefore, this study could help to elucidate the genes roles and mechanisms played on CNCs, along with discovering innovative methods to overcome them, through the evaluation of relationships among methylation levels of the LPL, ADRB3 and MTHFR genes in a human-based population with different levels of morbidities and inflammation.

Methods

Study design

Cross-sectional population-based study linked to a population-based survey entitled "II Cycle of Diagnosis and Intervention of the Most Prevalent Food, Nutritional and Non-Communicable Diseases of the Population in the Municipality of João Pessoa/PB" (II Ciclo de Diagnóstico e Intervenção da Situação Alimentar, Nutricional e das Doenças não Transmissíveis mais Prevalentes da População do Município de João Pessoa/PB – II DISANDNT/Paraíba)", carried out from May 2015 to May 2016. It was funded by FAPESQ/MS/CNPQ [26].

The research protocol was approved by the Research Ethics Committee at the Health Sciences Center (Centro de Ciências da Saúde – CCS) of the Federal University of Paraíba (Universidade Federal da Paraíba, UFPB), under the protocol number

0559/2013. Research participants signed the informed consent form (ICF), in accordance with the ethical standards of research involving human beings.

Population and Sampling

Males and females adults aged between 20 and 59 years old from different socioeconomic conditions, residing in the East and West zones of the city of João Pessoa were selected to participate in the survey. Individuals previously diagnosed with any neuropsychiatric disorders; users of anorectics or anabolic steroids; pregnant women or children were prevented to take part in the project.

The sample was calculated based on previous similar studies [27, 28] along with a single sampling procedure, consisting of several levels according to the data available from the City Council, such as the city map, number of blocks per neighborhood, and data from the Brazilian Institute of Geography and Statistics (Instituto Brasileiro de Geografia e Estatística, IBGE) [29].

At a first level, a stratified sampling over the blocks [30] was used, given the heterogeneity of the variable income and the association between income, disease prevalence, and nutrition. Neighborhoods were classified by income class into 4 strata, based on the map of city blocks. After stratification, the number of representative blocks per zone was calculated [30]. A formula was used to obtain the weight of each stratum and another to calculate the sample per zone [31].

Therefore, the total number of neighborhoods visited, corresponding to the East and West zones of the municipality of João Pessoa was 24, with an estimate of visits carried out in 105 blocks. After defining the number of blocks per neighborhood, they were drawn by lot. The selected block served as a reference for the selection of visited

households. Thus, the number of samples in this study totaled 261 individuals. The significance utilized was 0.05.

Data collection

The home visits and the survey were carried out by undergraduate students of the Nutrition Program and Master's and PhD candidates of the Postgraduate Program in Nutrition Sciences (Programa de Pós-graduação em Ciências da Nutrição, PPGCN) of the UFPB. All the selected households that were within the age group and wanted to participate in the research have participated of the data collection. Demographic and epidemiological characterization questionnaires were applied. Participants were asked about the diagnosis and use of medications used to treat diseases: hypertension, diabetes, obesity, cardiovascular disease, and dyslipidemia etc. After data collection, the population was divided into three categories: (1) group without morbidities (individuals without a diagnosis of morbidity, n=67); (2) group with isolated morbidities (individuals diagnosed with at least one morbidity, n=114); (3) group with associated morbidities (individuals diagnosed with at least two morbidities, n=80).

Nutritional assessment

Triplicate measurements of weight and height were done, whereupon the mean was utilized to classify the participants according to the Body Mass Index (BMI) charts, under the World Health Organization (WHO) parameters [32].

Sample collection

Households that had completed the third 24-hour recall were visited by an experienced nurse that performed peripheral blood sample collections. The participants

were required to do a 12-hours fasting before the procedure was done. Two sterile vacuum tubes (4ml) were used, one for serum analysis (containing clot activator) and the other for plasma analysis (with EDTA anticoagulant), including DNA methylation analysis. To ensure the effectiveness of the procedure, a follow-up was conducted, whereby the households were called by the team twice: to schedule the examination and guidance needs; and to confirm and reinforce the guidelines. The samples were kept at -20°C until the biochemical analysis and DNA methylation profile were performed.

DNA Methylation Level Analysis

The methylation levels in the promoter of the *ADRB3*, *MTHFR* and *LPL* genes were evaluated using the HRM (High Resolution Melting) technique with the aid of the Applied Biosystems 7500 Fast System Real Time Thermocycler. For that, the DNA previously extracted according to Oliveira et al. [27] was quantified by fluorescence (*Qubit™*), had its purity checked by a Nanodrop ® spectrophotometer (*Thermo Scientific, California, USA*) and kept at -20°C until the analysis. Afterwards, the samples were submitted to DNA conversion by bisulfite from the Cells-to-CpG [™] conversion kit (*Applied Biosystems, Life Technologies, California*), according to the manufacturer's instructions. The primers as well as the CRP conditions used were performed as previously described [33].

To calculate methylation levels, commercially available bisulfite-converted control DNAs were purchased as CpGenome positive (100% methylated) and negative control (0% methylated) (*Chemicon, Milipore, Billerica, MA, USA*). We performed the appropriate dilutions to obtain the following ratios: 0, 25, 50, 75 and 100%; all samples were run in triplicate to acquire the standard curves and compare with the melting profile of the samples.

Statistical analysis

All statistical analyzes were performed utilizing the STATA 13 software [34]. Initially, normality test was performed using the Lilliefors test. To identify the existence of a significant association between the different levels of methylation of *LPL*, *ADBR3* and *MTHFR* genes, amongst individuals without morbidities, with isolated or associated morbidities, a multiple linear regression model was performed. Finally, the odds ratio was evaluated, in order to assess the association of medicine intake and the methylation levels of the studied genes. For all tests, the significance level assumed was 5%.

Results

Amongst the 261 participants aged between 20 and 59 years, there was a predominance of females (66.8%). Descriptive data regarding age, sex, BMI, and medication use is demonstrated in Table 1.

Table 1 General characteristics of the adult individuals

	NO MORBIDITIES (n=67)	WITH ISOLATED MORBIDITIES (n=114)	WITH ASSOCIATED MORBIDITIES (n=80)
Age	32.4	37.4	37.4
Female	n = 46 (68.65%)	n = 69 (60.5%)	n = 60 (74.1%)
Male	n= 21 (31.34%)	n= 45 (39.5%)	n= 21 (25.9%)
BMI (kg/m ²)	21.67	27.14	28.83
Medicine			
No	n = 60 (89.55%)	n = 87 (76.32%)	n = 24 (30%)
Yes	n = 07 (10.45%)	n = 27 (23.68%)	n = 56 (70%)

Among individuals with isolated morbidities, stood out those who were overweight (overweight or obese) 68.42% and hypertension with 6.14%. In individuals with associated morbidities, 38.75% were overweight and had hypertension, 17.5% were overweight and had respiratory system diseases, 13.75% were overweight and had diabetes, 13.75% were overweight and had rheumatic diseases and 8.75% overweight and had dyslipidemia. Only 32.5% of individuals had 3 or more morbidities.

Regarding the methylation levels of the *LPL*, *ADRB3* and *MTHFR* genes, and the levels of inflammatory markers in the population studied, no statistically significant differences were found between the groups (Table 2). The mean methylation levels between groups ranged between 35-40% for the *LPL* gene, 40-44% for the *ADRB3* gene and 33-35% for the *MTHFR* gene ($p>0.05$). Regarding the inflammatory parameters, the means were within the reference values in all groups.

Table 2 Methylation levels of *LPL*, *ADRB3* and *MTHFR* genes, and inflammatory markers

	NO MORBIDITIES		WITH ISOLATED MORBIDITIES		WITH ASSOCIATED MORBIDITIES		
	MEAN	SD	MEAN	SD	MEAN	SD	P value
<i>LPL</i> GENE (%)	35	16	35	18	40	20	$p = 0.2081$
<i>ADRB3</i> GENE (%)	44	20	41	18	40	17	$p = 0.4311$
<i>MTHFR</i> GENE (%)	33	16	35	18	35	18	$p = 0.8664$
TAC (%)	40	0.13	42	0.15	40	0.14	$p = 0.6091$
MDA ($\mu\text{mol/L}$)	2.8	1.03	2.77	0.83	3.02	0.80	$p = 0.0509$
CRP (mg/L)	2.29	3.09	2.49	3.7	2.58	3.25	$p = 0.8742$
AGP (mg/dL)	63.25	22.31	65.99	17.59	65.16	17.7	$p = 0.6423$

SD: Standard deviation.

Table 3 presents a multiple regression analysis between methylation levels of the studied genes and inflammatory markers, when the model was adjusted for sex and age. We observed that in individuals without morbidities ($n=67$) no significant association was found between the methylation levels of selected genes and the inflammatory parameters. In individuals with isolated morbidities ($n=114$), we found that when CRP increases by 1 (mg/L) the methylation levels of the *LPL* gene increase by 0.0139, showing a statistical difference ($p=0.003$).

Table 3: Association between methylation levels of *LPL*, *ADRB3* and *MTHFR* genes and inflammatory markers

Individuals with isolated morbidities				
	Coefficient	95% CI	t statistics	P value
<i>LPL</i> Gene				
Female	-0.0171	-0.0957 - 0.0615	-0.43	0.667
Age	-0.0003	-0.0037 - 0.0032	-0.15	0.880
TAC	-0.2281	0.5811 - 0.0352	1.22	0.053
MDA	0.0381	-0.0061 - 0.0823	1.71	0.090
CRP	0.0139	0.0339 - 0.0029	1.75	0.003*
AGP	-0.0008	-0.0029 - 0.0012	-0.82	0.416
Gene <i>ADRB3</i>				
Sex	-0.0712	-0.1502 - 0.0077	-1.79	0.077
Age	-0.0003	-0.0037 - 0.0032	-0.17	0.868
TAC	0.1858	-0.0482 - 0.4198	1.58	0.118
MDA	0.0462	-0.0706 - 0.0482	1.27	0.089
CRP	-0.0053	-0.0153 - 0.0047	-1.05	0.297
AGP	0.0014	-0.0007 - 0.0035	1.32	0.189
<i>MTHFR</i> Gene				
Sex	0.0029	-0.0794 - 0.0852	0.07	0.944
Age	-0.0020	-0.0057 - 0.0015	-1.14	0.259
TAC	0.0222	-0.2216 - 0.2662	0.18	0.856
MDA	0.0028	-0.0435 - 0.0491	0.12	0.906
CRP	0.0023	-0.0082 - 0.0127	0.43	0.667
AGP	-0.0012	-0.0033 - 0.0010	-1.06	0.292
Individuals with associated morbidities				
	Coefficient	95% CI	t statistics	P value
Gene <i>LPL</i>				
Female	0.1154	0.0051 - 0.2257	2.09	0.041*
Age	-0.0002	-0.0047 - 0.0043	-0.08	0.935
TAC	0.2622	-0.0864 - 0.6108	1.50	0.138
MDA	0.0263	-0.0813 - 0.0520	0.73	0.456
CRP	0.0034	-0.0142 - 0.0211	0.39	0.701
AGP	-0.0016	-0.0049 - 0.0016	-0.99	0.324
Gene <i>ADRB3</i>				
Sex	-0.0272	-0.1218 - 0.0673	-0.57	0.567
Age	0.0007	-0.0032 - 0.0046	0.36	0.719
TAC	-0.0962	-0.3949 - 0.2024	-0.64	0.522
MDA	0.0372	-0.0157 - 0.0900	1.40	0.165
CRP	0.0031	-0.0172 - 0.0431	0.32	0.765
AGP	-0.0009	-0.0036 - 0.0019	-0.27	0.543
Gene <i>MTHFR</i>				
Sex	-0.0175	-0.1155 - 0.0806	-0.36	0.723
Age	-0.0015	-0.0056 - 0.0025	-0.77	0.444
TAC	-0.0012	-0.3109 - 0.3085	-0.01	0.994
MDA	0.0755	-0.1112 - 0.0086	1.05	0.032*
CRP	-0.0050	-0.0206 - 0.0107	-0.63	0.529
AGP	0.0005	-0.0023 - 0.0034	0.37	0.712

In this model, the following variables were included: lipid profile, homocysteine and folic acid, but no association was found with the methylation of the respective genes studied. Analyzes were also performed in individuals without morbidities, however, no significant results were found.

Among individuals with associated morbidities (n=80), females had higher levels of *LPL* gene methylation with an increase of 0.1154, with a statistical difference (p=0.041). In this same population for the *MTHFR* gene, we found that as the MDA

increases by 1 $\mu\text{mol/L}$, the gene methylation levels increase by 0.0755, with a significant statistical difference ($p=0.032$).

In table 4, a simple linear regression was performed analyzing the odds ratio for the association of use of medications with the levels of methylation of the genes studied. No significant differences were found in any of the groups when subdivided by the number of presented morbidities, and it was observed that the use of medications did not influence the level of methylation for any of the three genes analyzed in individuals without morbidities and with isolated or associated morbidities.

Table 4 Medication usage and methylation levels of *LPL*, *ADRB3* and *MTHFR* genes

Medication usage	Individuals without morbidities			
	Odds Ratio	95% IC	Std. Err.	P value
Gene <i>ADRB3</i>	0.6077	0.1112 – 3.3209	0.5266	0.565
Gene <i>MTHFR</i>	1.1904	0.2137 – 6.6330	1.0433	0.842
Medication usage	Individuals with isolated morbidities			
	Odds Ratio	95% IC	Std. Err.	P value
<i>LPL</i> Gene	2.0794	0.8218 – 5.2616	0.9849	0.122
<i>ADRB3</i> Gene	0.9865	0.3953 – 1.4619	0.4603	0.977
<i>MTHFR</i> Gene	0.8695	0.3499 – 2.1606	0.4038	0.763
Medication usage	Individuals with associated morbidities			
	Odds Ratio	95% IC	Std. Err.	P value
<i>LPL</i> Gene	0.8543	0.3243	0.4222	0.750
<i>ADRB3</i> Gene	0.7315	0.2808	0.3574	0.522
<i>MTHFR</i> Gene	0.7623	0.2910	0.3745	0.581

Discussion

The *LPL*, *ADRB3* and *MTHFR* genes that encode lipase, beta-adrenergic receptor and methylenetetrahydrofolate reductase proteins, respectively, are genes that are associated with the state of inflammation characteristic of morbidities [13, 14, 19, 21], and for this reason they were selected for the present study, which aimed to analyze their methylation profile, alongside inflammatory parameters in individuals without morbidities and with isolated or associated morbidities, and to evaluate their associations.

It was observed that the methylation levels of the three genes studied were similar between the groups, indicating that there are no alterations in the methylation profile in the CpG sites studied when considering individuals with or without morbidities. Likewise, inflammatory and antioxidant parameters were similar between groups.

One of the possible justifications for these results is that, as it is data from individuals who are not in a hospital environment (being compensated for the evolution of the disease due to the use of medications or change in lifestyle), they did not present changes in the methylation profile.

In a study evaluating the epigenome of DNA samples from whole blood in critically ill patients, 668 differentially methylated regions were found between septic and non-septic patients. There was a predominance of hypermethylated sites (61%), compared to hypomethylated sites (39%) in septic patients. However, none of the genes mentioned in the study coincide with those worked in this research [35]. When analyzing the methylation in the *ABO* and *AGTRI* genes in hospitalized patients, positive associations were found between their hypermethylation and cardiovascular disease, however, this association with *AGTRI* was only found in men [36, 37].

In addition, despite being within the reference value, CRP was in the upper tertile (>2mg/dL), which may indicate an inflammatory change in the body [38]. CRP is an acute-phase inflammatory protein extensively used worldwide as a biomarker of inflammation, infection and/or cardiovascular events [39]. Evidence suggests that it is not only a marker of inflammation but also plays an active role in the inflammatory process [40]. However, lifestyle factors including smoking, sex, age, and BMI, in addition to changes in lipid levels and blood pressure can also act by changing their levels in the body [41].

According to the results shown in Table 3, adults who were not diagnosed with morbidity did not show any association between the lipid profile, the measured inflammatory markers nor the methylation levels of the *LPL*, *ADRB3* and *MTHFR* genes.

Nevertheless, the association between *LPL* gene methylation levels in individuals with isolated morbidities and CRP, has shown a significant association ($p=0.003$), indicating that when CRP values were higher, *LPL* gene methylation levels increased by individuals with isolated morbidities. It suggests that the increase in *LPL* methylation levels could lead to a decrease in lipoprotein lipase glycoprotein synthesis. An association was found between increased levels of methylation in promoters of this gene in subcutaneous adipose tissue and a reduction in *LPL* expression [42]. Although the interpretation of gene methylation studies is difficult to interpret, given the multiple differences in methylation patterns at CpG sites in different cells and tissues [43]. The *LPL* enzyme is related to the transport pathways of lipid metabolism [10, 11]. Because it works by hydrolyzing triglyceride-rich lipoproteins, the reduction in its functioning increases the risk of developing some diseases such as metabolic syndrome [44], insulin resistance [14, 11], dyslipidemia and obesity [13].

It was observed that the CRP level was associated with hypermethylation or hypomethylation in a variety of genes such as genes encoding inflammatory markers such as *IL-6*, *TGFA*, *CXCL3*, *CXCL11*, *CXCL23*, etc. [45]. However, it is still unknown whether changes in the methylation profile act as a causal factor or consequence of the inflammatory state. In a study using the GWAS and EWAS techniques, no associations were found between PCR values for DNA methylation. However, when performing the causal effect test of DNA methylation on PCR, the increase in methylation of *TMEM49*, *BCL3* and *MIR21* genes showed associations with PCR levels. These results suggested that the association of methylation profile with PCR is more likely to be influenced by

causal factors of DNA methylation rather than the opposite [46]. No studies were found that made the association between the levels of methylation of the *LPL* gene with the serum levels of CRP, and the present study is innovative in bringing this association.

In individuals with associated morbidities, females were significantly associated with the level of methylation of the *LPL* gene. That is, when the individual is female, the methylation level of the *LPL* gene increases by 0.11%. Scholars point out that women may have higher levels of methylation in their genomes compared to men, indicating a possible difference in methylation between the sex [47].

In a study analyzing the sex association in the methylation of CpG sites, 12,177 CpG sites showed significant differences in methylation between women and men. In autosomal chromosomes, the differences in methylation between the sex were 46.1%, with greater methylation in women compared to men, a pattern also observed when analyzing the methylation profiles of the X chromosome [48]. These data are consistent with the systematic review study in which 31 genetic loci were associated with sex differences in cardiometabolic diseases and men in general had lower levels of methylation in their genome [49].

A factor that enables so many divergences in this profile is due to hormonal changes that exist between sex, especially in sex hormones. These, in turn, can directly regulate the DNA, in addition to modulating the epigenetic profile differently in each of the sex [50]. Sex-specific associations were observed in CpG sites, in a study in which methylation in the *LPL* gene had negative associations with glucose only in women [51].

In individuals with associated morbidities, MDA also showed a significant association ($p=0.032$), but with the *MTHFR* gene, indicating that when the MDA increases, the level of methylation of the *MTHFR* gene also increases and, consequently, leading to a reduction in expression of the *MTHFR* gene [52]. Unlike the present study,

other authors did not find significant associations between the *MTHFR* gene methylation profile in leukocytes and serum MDA levels [24]. However, studies associating *MTHFR* and MDA methylation remain scarce.

Studies involving other genes have shown significant results in this association, the increase in *MLH* and *IFNAR* methylation levels were positively related to MDA marker levels [53, 54]. Higher levels of MDA are related to increased levels of lipid peroxidation in cells [55] and because it is generated during oxidative stress, it can be detected in plasma and different tissues of patients suffering from a wide range of diseases [55, 56, 57].

Long-term oxidative stress is known to lead to chronic alterations in enzyme, transcriptional, epigenetic and genomic regulation. The formation of reactive oxygen species promotes cell proliferation by changing metabolic and cell control mechanisms, causing DNA damage, genomic instability, and inflammation. Thus, the increase in MDA is also involved in cell signaling and the formation of DNA adducts, which have the ability to affect chromatin modifications [53, 51]. Furthermore, important effects have been identified in different types of immune system cells indicating that MDA may be biologically active as a mediator not only of oxidative stress, but also of inflammation [58].

Genetic variations of genes involved in the metabolism of a carbon can lead to dysregulation of DNA methylation patterns, for example, the *MTR* and *MTHFR* genes [6]. Furthermore, an abnormal performance of *MTHFR* can lead to metabolic disturbances through changes in homocysteine levels, for example, *MTHFR* deficiency can result in hyperhomocysteinemia [59]. Associations between *MTHFR* methylation and increased HCY levels are frequently observed, and studies point to HCY as a potent risk

factor for oxidative stress, in addition to several other mechanisms that lead to the development of cardiovascular diseases [21].

Additionally, there is possibly a compensation when HCY is elevated, leading to demethylation of *MTHFR* in the blood in order to increase the expression of the MTHF enzyme and increase the conversion of homocysteine into methionine [60]. In that mentioned study, this picture was evident in patients with clinical diabetic nephropathy, who presented high levels of homocysteine, together with demethylated gene profiles.

In the present study, although there was no difference between the methylation levels of the *MTHFR* gene and the mean values of homocysteine in the groups, it was found that approximately one third of the sample per group (no morbidities 29.85%, with isolated morbidities 36, 84% and with associated morbidities 29.6%) had moderately high levels of homocysteine (results not demonstrated). Probably these high results in homocysteine did not influence the levels of methylation of the gene as they were homogeneously distributed in the three groups. One of the possible justifications for the group without morbidities to also present an increase in serum homocysteine levels, would be due to the use of birth control medications, the contraceptives [61, 62].

The *ADRB3* gene was not significantly associated with any of the studied variables. Furthermore, no significant changes were found regarding its methylation profile. More methylated profiles of *ADRB3* are commonly found in obese individuals, due to their participation in the regulation of body weight [15]. However, the present study had a greater number of eutrophic individuals (43.5%), when compared to obese individuals (20.2%), which may have influenced the results found regarding the methylation profile of this gene.

Alterations in the *ADRB3* gene have an influence on metabolic and lipid disorders [63]. *ADRB3* hypermethylation in adipose tissue of obese individuals has been linked to

a greater susceptibility to visceral obesity and alteration in body fat distribution [64] and methylation in the promoter of the *ADRB3* gene in visceral adipose tissue has been linked as a causal factor in the alteration waist-hip ratio, as well as the change in blood pressure in obese individuals [20].

Table 4 shows that the use of medication was not considered a factor influencing the methylation profile for any of the three genes analyzed in individuals with isolated or associated morbidities. It is necessary to highlight that among the 67 individuals without diagnosed diseases, 14 (20.9%) were using medication and/or supplements, with nutritional supplements rich in proteins or contraceptives being the most used. Because of this, the multiple regression analysis at first was performed by inserting the group without morbidities using these substances and at the second moment this group was not compared to the others, keeping the result described previously.

Based on the literature, it was expected that individuals with isolated or associated morbidities using medications would present alterations in the levels of methylation in leukocytes, when compared to individuals without morbidities. It is believed that medications have an influence on epigenetic processes and that this influence may justify the beneficial and/or deleterious effects attributed to the use of medications [65].

Among the main medications used in the treatment of diabetes, metformin stands out. This medication has been shown to act in multiple actions in epigenetic regulation and its effects on DNA methylation include increasing and decreasing the methylation profile in promoters of different genes [66, 67]. One of the possible mechanisms by which metformin influences DNA methylation includes modulation of the activity of DNA methyltransferases (DNMT). It is known that while AMPK directly inhibits DNMT activity, metformin can indirectly reduce the endogenous activity of DNMT inhibitors.

391 However, the general effect of metformin on DNA methylation and gene expression is
392 not fully understood [67].

393 To control plasma cholesterol levels, statin is one of the most prescribed
394 medications. However, it has been observed to contribute to the increased risk of diabetes
395 and the mechanism that involves this process remains unclear [68]. The *ABCG1*, *ABCA1*
396 and *ACSL3* genes were inversely associated with the use of statins, methylation in CpG
397 islands and diabetic markers under study. These genes stood out for being involved in
398 cholesterol metabolism and glycemic pathways [68]. There are also reports of epigenetic
399 effects of angiotensin receptor blocking medications such as Enalapril, Lisinopril and
400 Captopril. Furthermore, Losartan was shown to be able to reverse the hypomethylation
401 of the AT1 promoter, assuming that the effects of Losartan on blood pressure and
402 proteinuria probably represent epigenetic processes [69].

403 No studies on the absence of changes in DNA methylation levels related to the
404 use of medication were found in the consulted literature. Possibly, the absence of changes
405 in the levels of DNA methylation related to the use of medications found in the present
406 study may have been found because it is a population-based study, in which different
407 medications were used in different pathologies.

408 In addition, these results may have been influenced by the responses of the
409 participants, as 76.31% of the individuals with a medical diagnosis of one morbidity and
410 30% with a medical diagnosis of two or more morbidities were not taking medications.
411 On the other hand, individuals who had no medical diagnosis of any morbidity, 10.45%
412 were already using medications (data presented in table 1).

413 It is observed that there are few articles that associate PCR and MDA markers
414 with the studied genes. Therefore, the present study presents itself as a pioneer in better
415 understanding the metabolic processes involving these markers and the *LPL*, *ADRB3* and

MTHFR genes. The understanding of these processes is of fundamental importance both in the treatment of diseases, as well as in discovering new combat strategies, and the results should be analyzed together with the results of other epigenetic and genetic analyses.

As for the limitations and strengths of the study, the absence of studies on the association between methylation levels of *LPL*, *ADRB3* and *MTHFR* genes with isolated and associated morbidities and inflammation are considered strong points. As limitations of the study, the lack of gene expression analysis is pointed out.

Conclusion

In individuals without morbidities, no associations were observed between the methylation levels of selected genes and inflammatory parameters. In individuals with isolated morbidities, a positive association was observed between CRP values and *LPL* gene methylation levels, and in individuals with associated morbidities, female individuals had higher levels of *LPL* gene methylation. In this same population, for the methylation levels of the *MTHFR* gene, a positive association was found with the MDA values and finally it was observed that the use of drugs was not considered a factor influencing the methylation levels of the three genes analyzed in individuals with morbidities.

Abbreviations

ADRB3: β_3 adrenergic receptor; AGP: Alpha-1-acid glycoprotein; TAC: Total antioxidant capacity; CCS: Health Sciences Center; CNCD: Chronic Noncommunicable Diseases; DNMT: DNA methyltransferases; HCY: Homocysteine; HDL: High Density Lipoprotein; HRM: High Resolution Melting; IBGE: Brazilian Institute of Geography and Statistics; II DISANDNT/PB: II Cycle of Diagnosis and Intervention of the Food,

Nutritional and Non-Communicable Diseases Most Prevalent in the Population of João Pessoa/PB; BMI: Body Mass Index; LDL: Low Density Lipoprotein; LEFTADS: Laboratory for the Study of Physical Training Applied to Performance and Health; *LPL*: Lipoprotein lipase; MDA: Malondealdehyde; *MTHFR*: Metylenetetrahydrofolate reductase; CRP: C-reactive protein; TBARS: Thiobarbituric Acid; FICF: Free and Informed Consent Term; UFPB: Federal University of Paraíba.

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of the CCS/UFPB, under the Protocol Number 0569/15.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

467 Conceptualization: Thais Costa Matte, Raquel Patrícia Ataíde Lima, Naila Francis
 468 Paulo de Oliveira, Maria da Conceição Rodrigues Goncalves, Sônia Cristina Pereira de
 469 Oliveira Ramalho Diniz, Darlene Camati Persuhn, Maria José de Carvalho Costa. Project
 470 Administration: Rafaella Cristhine Pordeus Luna, Alcides da Silva Diniz, Darlene Camati
 471 Persuhn, Maria José de Carvalho Costa. Methodology: Thais Costa Matte, Raquel Patrícia
 472 Ataíde Lima, Rafaella Cristhine Pordeus Luna, Alcides da Silva Diniz, Aléssio Tony
 473 Cavalcanti de Almeida, Flávia Emília Leite de Lima Ferreira. Formal Analysis: Thais
 474 Costa Matte, Raquel Patrícia Ataíde Lima, Aléssio Tony Cavalcanti de Almeida, Roberto
 475 Teixeira de Lima, Alexandre Sergio Silva, Maria José de Carvalho Costa. Software:
 476 Flávia Emília Leite de Lima Ferreira, Alexandre Sergio Silva.

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482

483 **Authors details**

484 1 Department of Nutrition, Health Sciences Center, Federal University of Paraíba
 485 (UFPB), João Pessoa, Paraíba, 58000-000, Brazil. 2 Department of Economics, Center
 486 for Applied Social Sciences, Federal University of Paraíba (UFPB), João Pessoa, Paraíba,
 487 58000-000, Brazil. 3 Department of Nutrition, Health Sciences Center, Federal University
 488 of Pernambuco (UFPE), Recife, Várzea, 50000-000, Brazil. 4 Department of Molecular
 489 Biology, Center for Exact Sciences and Natural Sciences, Federal University of Paraíba
 490 (UFPB), João Pessoa, Paraíba, 58000-000, Brazil.

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