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Association between Homocysteine and Polymorphisms in MTHFR in Brazilian Obese Women

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Abstract

Background: Brazilian national surveys have indicated a rise in obesity and cardiovascular disease in women.
Objective: To determine the frequency of 677C>T and 1298A>C polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene in obese Brazilian women and to assess the potential association of these polymorphisms with serum concentrations of homocysteine (Hcy), folate and cobalamin after fortification of wheat and corn flour with folic acid in Brazil.
Methods: A cross-sectional study was conducted from 2008 to 2009 with 133 obese women. Commercial kits were employed to perform laboratory analyses including measurement of lipids and glucose using enzymatic methods, total Hcy and serum folate using a competitive immunoassay and cobalamin based on chemiluminescence. Genotyping was performed by PCR, followed by restriction fragment length polymorphism analysis.
Results: The average age of participants was 39.0±4.4 years and mean body mass index was 32.5±2.1 kg/m². The distributions of the genotypes were CC (47%), CT (44%), and TT (9%) for the position MTHFR 677 and AA (60%), AC (35%), and CC (5%) for the position 1298. Hcy levels correlated negatively with serum folate in the group displaying the 677CT, 1298AC, or 1298CC genotypes ($r=-0.554$, $p<0.01$).
Conclusion: Our findings suggest that obese Brazilian women with genotypes 677TT have higher Hcy concentrations than those carrying the genotypes 677CT and 677CC. Additionally, genotypes 1298CC are associated with higher Hcy concentrations than genotypes 1298AC and 1298AA.

Keywords: Hyperhomocysteinemia; Obesity; Folic acid; Methylenetetrahydrofolate reductase (NADPH2)

Introduction

In recent decades, the increasing prevalence of obesity has become a major public health problem worldwide[1]. Subsequently, in recent years, other factors such as hyperhomocysteinemia (HHcy) were identified as additional risks that lead to cardiovascular diseases (CVD).

Homocysteine (Hcy) is a sulfur-containing amino acid formed by demethylation of the essential amino acid methionine[2]. The metabolism of Hcy involves two main reactions: transsulfuration and remethylation[3]. In negative balance conditions of methionine, Hcy is remethylated in a reaction catalyzed by the enzyme methionine synthase, which is a cofactor of cobalamin. This reaction converts the 5-methylenotetra-tetrahydrofolate (MTHF) to tetrahydrofolate which then performs its function within the cells. Methylenotetra-tetrahydrofolate reductase (MTHFR) is considered a key enzyme in this reaction, since it catalyzes the reduction of folic acid 5,10-methylenotetra-tetrahydrofolate (MTHF) to

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5-MTHF[3,4]. Thus, participation of MTHFR in the methionine and Hcy metabolism can influence the concentrations of Hcy, making Hcy a new and relevant independent risk marker for early occlusive vascular diseases[5,6]. In this context, the polymorphisms of MTHFR may increase the genetic predisposition to such events[7].

Obese women are a growing population in Brazil and this group has an increased risk for CVD[8]. The hypothesis of this study is that the polymorphisms 677C> T and 1298A>C in the MTHFR gene are frequent in this population and are associated with high concentrations of Hcy, making these women more susceptible to CVD.

Studies of these polymorphisms in the population of obese women are scarce and controversial. This is the first study that investigated obese Brazilian women, a mixed population, in the period of wheat flour and corn flour post-fortification with folic acid.

The objective of this study was to determine the frequency of polymorphisms 677C>T and 1298A>C in the MTHFR gene in obese Brazilian women and to evaluate the possible association of these polymorphisms with serum concentrations of Hcy after fortification of wheat and corn flours with folic acid in Brazil.

Methods

Cross-sectional study conducted from 2008 to 2009 with 133 obese women treated at the Centro de Pesquisa em Nutrição Clínica do Hospital Universitário Clementino Fraga Filho, of Universidade Federal do Rio de Janeiro, RJ, Brazil, which assist obese patients with cardiovascular risk factors.

The study protocol was approved by the Ethics Committee of Hospital Universitário Clementino Fraga Filho of Universidade Federal do Rio de Janeiro under no. 017/08. All women signed an Informed Consent Form.

The following inclusion criteria were adopted: nutritional diagnosis of overweight, obesity grade 1 and 2 (body mass index (BMI) 25.0—39.9 kg/m^2)[8]; non-smokers and non-drinkers (average alcohol consumption <15 g per day), justified by the fact that smoking and alcohol consumption in large quantities increase Hcy concentrations[9,10]; without any restriction as to ethnicity, since Brazil is known for presenting a mixed population; age ranging from 30 to 45 years, age group representing the population with the highest incidence of obesity among women[11].

Those with clinical diagnosis of diabetes mellitus, chronic use of vitamin supplements, renal, liver and heart failure, pregnant women, nursing mothers, in postmenopausal period, using medications such as methotrexate, trimethoprim, colesterolamine and cyclosporine due to influence on Hcy concentrations.

ABBREVIATIONS AND ACRONYMS

- *CVD – cardiovascular diseases*
- *Hcy – homocysteine*
- *HHcy – hyperhomocysteinemia*
- *BMI – body mass index*
- *MTHFR – methylenetetrahydrofolate reductase*

Information on age, socioeconomic status, family history, medical history and use of supplements and medications were obtained through a structured questionnaire prepared by the researchers.

Clinical and anthropometric evaluation

Body mass (kg) and height (m) were measured using digital platform scale Filizola® (São Paulo, Brazil) and a vertical stadiometer, respectively[12]. BMI was calculated using the following formula: weight divided by squared height (kg/m^2)[8]. Waist circumference was obtained at the medium point between the last rib and the iliac crest with an inelastic tape[13]. Blood pressure levels were measured with a calibrated mercury sphygmomanometer[14].

Biochemical evaluation

Blood samples were collected after an overnight fast of 12 hours in tubes containing or not tetra-acetic ethylenediaminetetraacetic anticoagulant acid (Vacutainer®, Becton Dickinson, NJ, USA). The rates of serum and plasma were obtained by centrifugation at 4000 rpm for 15 minutes (Excelsa Baby I centrifuge; Fanem, São Paulo, Brazil), and stored at -20C until analysis.

Serum glucose, triglycerides, HDL-cholesterol and total cholesterol were determined by the enzymatic method as instructed by the manufacturer (kits CELM and katal; Katal Biotechnologica Ind. Com. Ltda, Minas Gerais, Brasil; and CELM-Cia. Equipadora de Laboratórios Moderneros - São Paulo, Brasil). The following were considered normal values: glucose <100 mg/dL, triglycerides <150 mg/dL; HDL-c ≥50 mg/dL, total cholesterol <200 mg/dL, since these were the normal values supplied by the manufacturers. The low-density lipoprotein (LDL-c) was calculated by the Friedewald[15] formula, the ideal reference value of which was <100 mg/dL.

Plasma concentrations of cobalamin were determined by chemiluminescence, automated method, with IMMULITE 2000® kit (São Paulo, Brazil). Normal values were considered > 120 pmol/L[16]. Plasma concentrations of folate and Hcy were determined by competitive immunoassay using the same kit. Suitable values were considered those ≥7 nmol/L and <10 mol/L, respectively[16,17].

Analysis of C677T and A1 298C polymorphisms of the MTHFR gene

The genomic DNA was extracted from peripheral blood using a commercial kit (MASTERPURE™ DNA Purification kit for blood - Epicentre, Wisconsin, USA). Genotyping was performed by the reaction technique in polymerase chain PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism).

The PCR reactions for evaluating the MTHFR 677C> T polymorphism were performed according to Chen et al.[18]. A fragment of 198 base pairs (bp) was obtained, and 8 μl of the PCR product was exposed over 4 hours at 37 °C with the restriction enzyme (HinfI) (BioLabs, New England, USA); then, electrophoresis of the digested fragments was conducted through polyacrylamide gels at 8% stained with silver. Homozygous individuals (CC) for the MTHFR, normal allele, resulted in a fragment of 198 bp; heterozygous individuals (CT), a pattern of two fragments (198 bp and 175 bp) and homozygous individuals (TT) for mutation resulted in a fragment of 175 bp.

To assess the MTHFR 1298A>C polymorphism, PCR was performed using the protocol described by van der Put et al.[19] A fragment of 163 bp was obtained and 7 μl of the PCR product was exposed over 4 hours at 37 °C with 0,67U of the restriction enzyme (MBOII) (BioLabs, New England, USA); then electrophoresis of digested fragments was conducted through polyacrylamide gels at 8% stained with silver. Homozygous individuals (AA) for the MTHFR normal allele produced two fragments of 56 bp; while a standard of two fragments (84 bp and 56 bp) was seen by heterozygotes (AC). Homozygous individuals for the polymorphism CC resulted in two fragments of 84 pb.

Statistical analysis

Allele and genotype frequencies were estimated by direct counting. The Hardy-Weinberg equilibrium was determined by analysis of the chi-square test for all genotypes. Six groups were defined according to the following genotypes: 677CC and 1298AA; 677CC and 1298AC; 677CC and 1298CC; 677CT and 1298AA; 677TT and 1298AA; 677CT and 1298AC or 1298CC.

All dependent variables, particularly Hcy, were expressed as means and standard deviation. The groups were compared using one-way analysis of variance (one-way ANOVA) with post hoc LSD test. The Pearson correlation between Hcy and biomarkers in different groups was calculated. For the statistical analyses, the software Statistical Package for the Social Sciences for Windows, version 17.0 (SPSS Inc Chicago, Ill, USA) was used. Differences were considered significant when $p < 0.05$.

Results

The study included 133 obese women. The distribution of genotypes for the position 677 of gene MTHFR was the following: CC 47% (n=62); CT, 44% (n=59); TT 9% (n=12). The frequencies of alleles C and T were 69% and 31%, respectively. The distribution of genotypes for the MTHFR 1298 gene position was: AA, 60% (n=80); AC, 35% (n=46); CC, 5% (n=7). The frequencies of alleles A and C were 77% and 23%, respectively. The distribution of both MTHFR genotypes was consistent with the Hardy-Weinberg equilibrium.

The average age of participants was 39.0±4.4 years and BMI ranged from 25.2 kg/m² to 38.0 kg/m², with an average value of 32.5±2.1 kg/m². The general characteristics of the study population are found in Table 1.

The 677TT/1298AA group (n=12) presented significantly Hcy higher serum concentrations than the following groups: 677CC/1298AA (n=34) $p<0.01$; 677CC/1298AC (n=23) $p<0.01$; 677CT/1298AA (n=34) $p=0.016$; 677CT/1298AC/1298CC (n=25) $p<0.01$.

Table 2 shows the Pearson correlations between plasma Hcy and the biomarkers studied in the different groups. In group 677CT/1298AC/1298CC (n=25) Hcy correlated negatively with serum folate concentrations. In groups 677CC/1298AA (n=34) and 677CC/1298AC (n=23) Hcy correlated positively with BMI.

When the main variables of this study are compared by genotypes (Table 3), women with genotype TT, for position 677, had significantly higher Hcy concentrations than women with genotypes CC and CT, but they had clinically lower plasma folate and cobalamin concentrations. Besides this, women with genotype CC for position 1298 had significantly higher Hcy concentrations than women with genotype AC.

The distribution of serum Hcy in the six groups studied is shown in Figure 1. There was a statistically significant difference ($p<0.01$) between the group 677TT/1298AA, which presented higher serum concentrations of Hcy

compared to the following groups: 677CC/1298AA, 677CC/1298AC, 677CT/1298AA, 677CT/1298AC/1298CC (n=25). There was no significant correlation between Hcy concentrations and cobalamin.

There was a statistically significant difference between the group 677TT/1298AA compared to the others, except for the group 677CC/1298CC.

The Pearson correlation between the homocysteine and plasma folate in the different groups is presented in Figure 2. In group 677CT/1298AC/1298CC, there was a negative correlation, statistically significant with $p<0.01$. In the other groups, although negative, the correlations were not significant, except for the group 677TT/1298AA, which presented a non-significant positive correlation.

Table 1
General characteristics of the study population

Genotypes	Total		677CC/ 1298AA		677CC/ 1298AC		677CC/ 1298CC		677CT/ 1298AA		677TT/ 1298AA		677CT/ 1298AC+ 1298CC	
	n=133		n=34		n=23		n=5		n=34		n=12		n=25	
Characteristics	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	SD	**Mean**	**SD**	**Mean**	**SD**
Age (years)	39.0	4.4	39.1	4.8	39.4	4.1	40.2	4.5	37.2	4.5	39.5	4.0	40.2	3.9
BMI (kg/m²)	32.5	2.1	32.6	2.0	32.6	2.1	33.6	0.9	32.6	1.6	32.3	2.1	32.3	2.6
Waist circumference (cm)	99.7	7.1	99.5	6.8	99.5	5.9	106.8	4.1	98.9	6.9	97.1	8.5	101.1	7.8
SBP (mmHg)	121.5	13.9	116.5	9.2	121.6	11.9	128.0	4.5	123.2	11.9	120.8	13.1	125.6	22.3
DBP (mmHg)	80.2	10.9	76.8	9.9	80.0	11.8	80.0	10.0	81.5	10.4	82.5	12.1	82.3	11.5
Total cholesterol (mg/dL)	185.5	37.3	185.1	35.8	193.0	38.9	193,4	26.7	173.3	33.4	196.9	45.4	188.7	39.4
HDL-c (mg/dL)	52.7	11.3	51.9	11.2	50.8	12.8	46.0	9.9	54,8	11.4	53.1	8.1	53.7	11.5
LDL-c (mg/dL)	106.6	34.9	111.0	34.0	112.3	38.2	118.2	24.3	96,7	34.1	117.3	39.9	101.2	32.2
Triglycerides (mg/dL)	132.9	82.1	110.5	66.1	149.6	64.2	146.6	41.1	114,5	99.1	132.4	56.7	170.4	95.4
Serum glucose (mg/dL)	85.2	11.3	85.1	7.8	83.9	13.4	86.8	9.1	85.1	14.7	84.5	7.7	86.6	10.6
Plasma folate (nmol/L)	11.4	3.6	11.6	3.5	12.3	4.0	10.7	4.2	11.8	3.1	9.7	2.7	11.0	4.1
Cobalamin (pmol/L)	448.2	208.3	513.4	241.9	460.2	207.3	415.5	182.1	434.8	221.8	376.8	160.2	407.1	150.7
Plasma homocysteine (μmol/L)	6.4	3.3	6.0	2.4	6.0	2.6	8.1	5.8	6.6	3.9	9.1 *	4.0	5.2	1.9

Values expressed as mean ±standard deviation (compared by ANOVA).
* $p<0.05$, significant difference between the groups 677TT/1298AA compared with the others
BMI - body mass index; SBP - systolic blood pressure; DBP - diastolic blood pressure; SD - standard deviation
Among the individuals genotyped as 677TT, all of them presented genotype 1298AA

Table 2
Pearson correlations between homocysteine and biomarkers in the different groups studied

Genotypes	677CC/1298AA		677CC/1298AC		677CC/1298CC		677CT/1298AA		677TT/1298AA		677CT/1298AC + 1298CC	
	n=34		n=23		n=5		n=34		n=12		n=25	
Biomarkers	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Age (years)	0.212	0.228	0.128	0.561	0.539	0.349	0.043	0.810	0.453	0.139	0.009	0.964
BMI (kg/m²)	0.382	0.026*	0.422	0.045*	0.740	0.153	-0.002	0.989	-0.286	0.368	0.248	0.232
Waist circumference (cm)	0.336	0.052	0.209	0.339	0.342	0.573	0.221	0.209	-0.192	0.550	0.222	0.286
Total cholesterol (mg/dL)	0.100	0.574	-0.209	0.338	0.011	0.986	-0.207	0.240	0.256	0.422	-0.090	0.668
HDL-c (mg/dL)	0.136	0.442	0.231	0.289	-0.799	0.105	0.102	0.568	0.157	0.627	0.219	0.293
LDL-c (mg/dL)	0.046	0.797	-0.285	0.187	0.099	0.874	-0.230	0.191	0.150	0.641	-0.024	0.914
Triglycerides (mg/dL)	0.042	0.814	-0.018	0.936	0.687	0.200	-0.044	0.807	0.384	0.217	-0.160	0.446
Serum glucose (mg/dL)	0.098	0.582	-0.286	0.186	0.099	0.874	-0.035	0.845	-0.467	0.126	0.334	0.103
Cobalamin (pmol/L)	-0.128	0.470	0.058	0.794	-0.107	0.863	-0.135	0.447	-0.055	0.864	0.020	0.923
Plasma folate (nmol/L)	-0.226	0.198	-0.220	0.314	-0.205	0.741	-0.216	0.221	0.345	0.271	-0.554	0.004*

*p<0.05, significant difference between the groups
BMI - body mass index; HDL-c - high-density lipoprotein; LDL-c - low-density lipoprotein

Table 3
Main variables by genotype

Polimorfismo	Genotype	Homocysteine (mmol/L)		Plasma folate (nmol/L)		Cobalamin (pmol/L)	
		Mean	SD	Mean	SD	Mean	SD
MTHFR 677 C>T	CC (n=62)	6.2	2.9	11.8	3.7	485.8	224.6
	CT (n=59)	6.0	3.3	11.5	3.6	423.1	193.9
	TT (n=12)	9.2*	4.0	9.8	2.8	376.8	160.2
p-value		<0,01		=0.07		=0.10	
MTHFR 1298 A>C	AA (n=80)	6.7	3.5	11.4	3.3	459.6	226.1
	AC (n=46)	5.5	2.3	11.8	4.1	432.8	182.5
	CC (n=07)	8.1*	4.8	9.8	4.0	418.5	162.7
p-value		<0.05		=0.17		=0.49	

Values expressed as mean ±standard deviation (compared by ANOVA); MTHFR - methylenetetrahydrofolate reductase; SD - standard deviation
*p<0.05, significant difference between groups

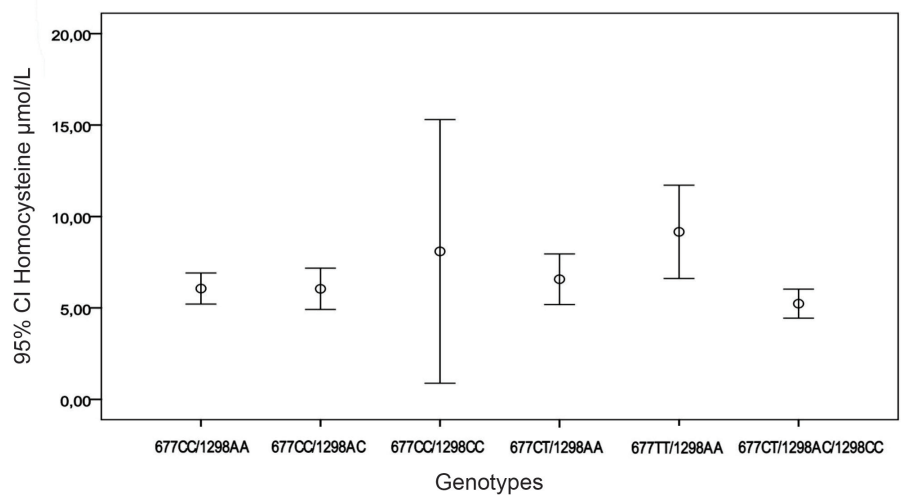

Figure 1
Plasma homocysteine distribution according to genotype in the population studied

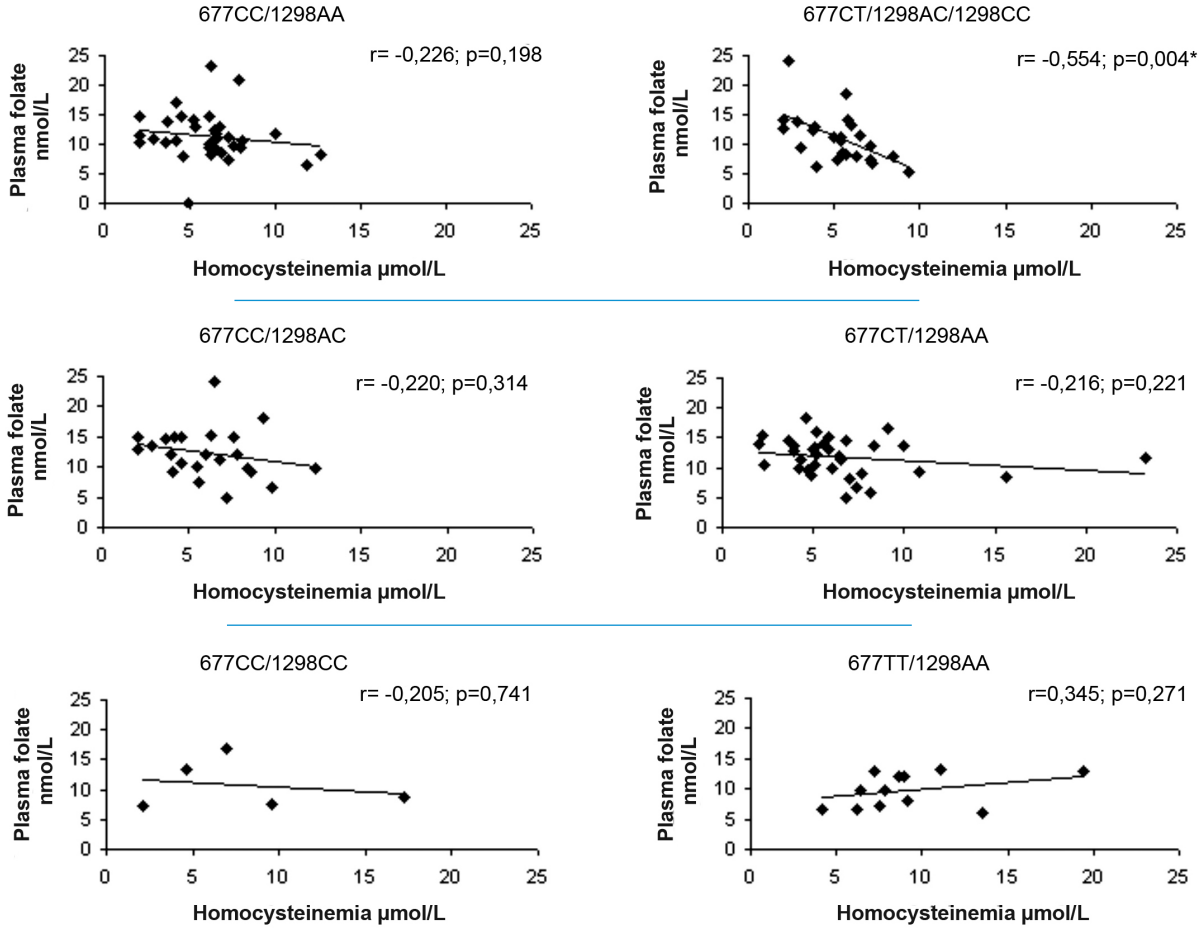

Figure 2
Correlation between serum concentrations of homocysteine and folate in both groups.
* p<0.05, significant correlation

Discussion

CVD is the leading cause of death in developed and developing countries[20]. Obesity is one of the classic risk factors for CVD, and has become an important public health problem[1]. In recent years, studies targeted at obtaining a better understanding of the disease identified risk factors for CVD, such as HHcy. Evidence of prospective studies shows that high concentrations of Hcy play an important role in the long-term risk of recurrent stroke and mortality[21]. Furthermore, HHcy has been associated with atherosclerosis and thrombosis, and is an independent risk marker for CVD. Its causes include both genetic and environmental factors[22].

The main factors that may influence Hcy concentrations include: increasing age, male gender, smokers, women in the postmenopausal period, folate deficiency, cobalamin deficiency, alcohol consumption, coffee consumption, use of folate antagonist drugs (methotrexate), diabetes, renal failure, and polymorphisms in the genes of folate and cobalamin metabolism[23,24]. In order to minimize the influence of the aforementioned factors and aiming to check the association of polymorphisms 677C>T and 1298A>C of the gene MTHFR with Hcy concentrations, this study included only women, non-smokers, non-alcoholic and excluded those who had factors known to trigger HHcy.

In a previous study conducted by this group of researchers, including obese Brazilian individuals (24 men, 39 women), the frequency of genotypes for position 677 of MTHFR was 64% CC, 32% CT, and 4% TT[25]. The genotypic frequency of individuals with the polymorphism 677C>T in the study were lower than those found in this study. The difference was possibly due to population disparities.

Data about the role of homocysteine in CVD favor the hypothesis that folic acid fortification contributes to the reduction in mortality from stroke, at least with regard to primary prevention[26]. In 1996, the U.S. Federal Drug Administration (FDA) required that all enriched grain products be fortified with folic acid from January 1998. As a result of this fortification, the intake of folic acid increased by an average of 190 μg/d[27].

The supplementation of folic acid reduces concentrations of Hcy[28], which may explain the Hcy concentrations found in this study, which was conducted after wheat flour and corn flour fortification with folic acid; and the negative correlation between homocysteine and plasma folate. This form of primary prevention can effectively reduce the risk of stroke[29].

The first findings on the association of both C677T and A1298C polymorphisms of MTHFR with HHcy showed that individuals with compound heterozygosity for both polymorphisms were more susceptible to the development of HHcy, despite normal serum levels of folic acid[30]. Another study showed that homozygous individuals for the 677TT mutation had significantly high levels of plasma Hcy, while in individuals 677TT, these concentrations were nearly two times higher than the values of individuals 677CT and 677CC[31]. Likewise, this study also demonstrated that women with 677TT polymorphism had significantly higher Hcy serum concentrations than those with polymorphisms 677CT and 677CC.

The 1298AC polymorphism associated with 677CT results in decreased MTHFR activity, which is higher in homozygous individuals (CC) than heterozygous individuals (AC) or normal individuals (AA). Another study involving 377 Jewish individuals, including 190 men and 186 women, aged 32-95, showed a significant effect of genotype 677TT in Hcy concentrations ($p<0.01$). Hcy plasma concentrations were significantly higher among homozygous individuals (TT) with the C677T mutation compared to individuals with genotype C677C, which is consistent with most previous reports[32].

These results are similar to those found in this study, in which homozygous individuals (TT) presented higher Hcy concentrations compared to other groups. Considering the low incidence of this genotype in the population and the fact that its effect is neutralized through adequate intake of folic acid, these results point to one of the reasons for controversy over the HHcy, and are similar to the findings of a previous report[9].

The Pearson correlation between Hcy and plasma folate in the different groups suggests the existence of a negative association between both. However, this study does not allow evaluating causality. There is no evidence against the higher intake of folic acid as a measure to reduce cardiovascular risk in the population studied, but this study does not reinforce this statement.

The results of this study suggest that Brazilian women with genotypes 677TT and 1298CC are more likely to develop HHcy. However, considering that this study was carried out after the start of addition of folic acid in flours, a procedure that became mandatory in 2004, it is believed that the susceptibility can be associated with lower plasma homocysteine concentrations. This is supported by a study of the same population of Brazilian women in which homocysteine concentrations were compared

before and after the fortification of wheat and corn flours[33].

The significant correlation between Hcy and BMI concentrations were similar to the results found in a previous study[9]. These results reinforce the need for specific studies targeted at overweight individuals, especially women, of whom, according to the Instituto Brasileiro de Geografia e Estatística[11], 16.9% are obese, compared to 12.5% of men. The high prevalence of obesity in the population and higher Hcy concentrations among women with genotype 677TT suggest that preventive measures for CVD should be implemented, ensuring adequate amounts of folic acid and encouraging weight reduction in order to minimize the deleterious effects of increased oxidative stress in obese individuals[34,35].

The main limitation of this study is the lack of a control group (non-obese individuals); and the cross-sectional design of the research study that does not support the evidence of causality, only hypotheses for the possible implications of the polymorphisms investigated in homocysteine; and the results that can only be applied to women aged 30-45 according to the characteristics described above. In addition, there is no evidence that the results were influenced by potential outliers.

In conclusion, obese women with genotypes 677TT/1298AA presented higher Hcy concentrations concerning genotypes 677CC/1298AA, 677CC/1298AC, 677CT/1298AA, 677CT/1298AC/1298CC; and genotypes 677CT/1298AC/1298CC presented a negative correlation between homocysteine and plasma folate.

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Potential Conflicts of Interest
No relevant conflicts of interest.

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