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MTRR rs326119 polymorphism is associated with plasma concentrations of homocysteine and cobalamin, but not with congenital heart disease or coronary atherosclerosis in Brazilian patients

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ABSTRACT

Background: Differences in the distribution of the *MTRR* rs326119 polymorphism (c.56 + 781 A>C) between patients with congenital heart disease (CHD) and controls have been described in Chinese individuals. The association is thought to be due to deregulation of homocysteine-cobalamin pathways. This has not been replicated in other populations. The primary objective of this study was to assess the influence of the *MTRR* rs326119 polymorphism on biochemical parameters of vitamin B12 metabolism, coronary lesions, and congenital heart disease in Brazilian subjects.
Methods: We selected 722 patients with CHD, 1432 patients who underwent coronary angiography, and 156 blood donors. Genotyping for the *MTRR* polymorphism was evaluated by high-resolution melting analysis, and biochemical tests of vitamin B12 metabolism were measured.
Results: Subjects carrying the AC or CC genotypes had higher homocysteine concentrations (9.7 ± 0.4 μmol/L and 10.1 ± 0.6 μmol/L) and lower cobalamin concentrations (260.5 ± 13.3 pmol/L and 275.6 ± 19.9 pmol/L) compared with the subjects carrying the AA genotype (8.7 ± 0.5 μmol/L and 304.8 ± 14.7 pmol/L), respectively. A multiple linear regression model also identified a significant association between the number of C variant alleles with the concentrations of homocysteine and cobalamin. Nonetheless, the allelic and genotypic distributions for *MTRR* rs326119 were not associated with CHD or coronary atherosclerosis in the studied samples.
Conclusion: Our findings indicate that the *MTRR* rs326119 variant might be a genetic marker associated with homocysteine and cobalamin concentrations, but not a strong risk factor for CHD or coronary atherosclerosis in the Brazilian population.

1. Introduction

Homocysteine is a molecule of the blood produced when methionine is broken down in an organism. This metabolism is dependent on vitamins B6 and B12 and folate. The main form of folate in plasma, 5-methylenetetrahydrofolate (5-MTHF), participates in the re-methylation pathway and is formed from the reduction of 5,10-methylenetetrahydrofolate by the enzyme methylenetetrahydrofolate reductase (MTHFR). In this process, homocysteine receives a methyl group by the activity of the enzyme methionine-synthase (MTR). Methionine synthase reductase (MTRR) is involved in reducing cob(II)alamin (B12r) to methylcobalamin (MeCbl), the cofactor form used in methionine synthase (MTR) [1–6].

Both MTHFR and MTRR are essential enzymes responsible for, among many other functions, keeping homocysteine at adequate levels. In fact, genetic variations in the genes that codify these enzymes have been shown to modulate homocysteine levels [7–9]. Furthermore, some studies have shown associations of hyperhomocysteinemia with the development of cardiovascular disease, and increased risks of atherosclerosis and thrombosis [9–11]. However, the relationship between genetic polymorphisms, homocysteine level, and cardiovascular phenotypes is still controversial and has been the focus of many studies [12–14].

A study, performed in a Chinese Han population, investigated polymorphisms in the *MTRR* gene. The study identified a significant difference in the distribution of the *MTRR* rs326119 polymorphism (c.56 + 781 A>C) between subjects with congenital heart disease (CHD) and controls. An association of the same polymorphism with

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homocysteine levels was also observed [15]. Authors then hypothesized that homocysteine metabolism could be the cause of an increased risk of developmental problems in individuals with the risk allele. This, however, has not been replicated in other populations. The main aim of this study was to assess the influence of the *MTRR* rs326119 polymorphism on biochemical parameters of B12 vitamin metabolism, and in cardiovascular phenotypes, namely, coronary atherosclerosis and CHD in Brazilian patients.

2. Subjects and methods

In this study, patients who underwent coronary angiography (n = 1432) plus blood donors (n = 156) were used as both a control group for CHD (n = 722), and as samples for testing the association between MTRR polymorphism and coronary atherosclerosis and homocysteine levels, respectively. The University of São Paulo ethics committee approved the protocol, and all participants signed an informed consent document.

2.1. Patients who underwent coronary angiography

For this study, 1432 consecutive patients who underwent diagnostic coronary angiography for coronary artery disease were selected at the Laboratory of Hemodynamics, Heart Institute (Incor), São Paulo, Brazil. All patients had a clinical diagnosis of angina pectoris and stable angina. No patient enrolled in this study was currently experiencing an acute coronary syndrome. Patients with previous acute ischemic events, heart failure classes III–IV, hepatic dysfunction, familiar hypercholesterolemia, previous heart or kidney transplantation, and in antiviral treatment were excluded [16].

2.2. Blood donors

Also included in the study were 156 subjects from the Blood Donation Center/University of São Paulo Medical School [17]. Inclusion and exclusion criteria for enrollment were the same as for those who donated blood.

2.3. Patients with CHD

Prospectively recruited to the study were 722 patients with CHD from the Pediatric Cardiology Outpatient Clinic – Heart Institute (Incor), São Paulo, Brazil. Patients were evaluated by history, review of the medical records, and physical examination and were classified according to their anatomical defect as previously described [18]. No patient enrolled in this study had clinical features of syndromic disease. An informed consent was obtained from progenitors or participants in accordance with protocols approved by the local IRB.

2.4. Demographic data and laboratory tests

A trained interviewer collected demographic and clinical information from all participants. Twelve-hour fasting blood samples were used for biochemical testing conducted according to standard techniques. Serum folate, serum cobalamin, and total homocysteine were determined by the ion capture method, enzyme immunoassay, and fluorescence polarization immunoassay, respectively.

2.5. Coronary artery disease scores

From the individuals who underwent coronary angiography, 20 coronary segments were scored: each vessel was divided into 3 segments (proximal, medial, and distal), except for the secondary branches of the right coronary artery (posterior ventricular and posterior descending), which were divided into proximal and distal segments. Stenosis greater than 50% in any coronary segment was granted 1 point, and the sum of points for all 20 segments constituted what is here referred to as an Extension Score. Lesion severity was calculated as follows: none and irregularities, 0 points; <50%, 0.3 points; 50–70%, 0.6 points; >70–90%, 0.8 points; and >90–100%, 0.95 points. The Severity Score was calculated through the sum of points for all 20 coronary segments [19].

2.6. Genotyping

Genomic DNA was extracted from peripheral blood following a standard salting-out procedure. *MTHFR* polymorphisms (c.677C>T, c.1317T>C, and c.1298A>C) were detected by polymerase chain reaction (PCR) followed by digestion by restriction enzyme. Genotyping for the *MTRR* rs326119 was detected by PCR followed by high-resolution melting (HRM) analysis with the Rotor Gene 6000 instrument (Qiagen, Courtaboeuf, France). The QIAgility (Qiagen, Courtaboeuf, France), an automated instrument, was used according to manufacturer's instructions to optimize the sample preparation step [20]. Amplification of the fragment for the *MTRR* rs326119 (c.56 + 781 A>C, intron 1) polymorphism was performed using the primer sense 5′-CGGTTCATTCACCGAAAGC-3′ and antisense 5′-AATTGGTGGGCTGTCAATTT-3′ (79 pairs base). A 40-cycle PCR was carried out with the following conditions: denaturation of the template DNA for first cycle at 94 °C for 120 s, denaturation at 94 °C for 20 s, annealing at 50.5 °C for 20 s, and extension at 72 °C for 22 s. PCR was performed with the addition of fluorescent DNA-intercalating SYTO9 (1.5 μM; Invitrogen, Carlsbad, USA). In the HRM phase, the Rotor Gene 6000 was used to measure the fluorescence in each 0.1 °C temperature increase in the range of 70–85 °C. Melting curves were generated by the decrease in fluorescence with the increase in the temperature; and for genotype calling, nucleotide changes resulted in different curve patterns. Samples of the observed curves were analyzed using bidirectional sequencing as a validation procedure (ABI Terminator Sequencing Kit and ABI 3500XL Sequencer - Applied Biosystems, Foster City, CA, USA) [21–23].

2.7. Statistical analysis

Categorical variables are presented as percentages, while continuous variables are presented as mean ± standard deviation. The chi-square test was performed for comparative analysis of general characteristics, frequencies of the *MTRR* genotype or variant allele, and for Hardy–Weinberg equilibrium. ANOVA test was performed for comparing the biochemical parameters of B12 vitamin metabolism, and angiographic data means according to *MTRR* polymorphism. When p value was significant in the ANOVA test, Tukey's post hoc test was performed to identify the different group. Biochemical data and angiographic data were adjusted for age, sex, and race. Biochemical parameters of the B12 vitamin metabolism were also adjusted for *MTHFR* polymorphisms (c.677C>T, c.1317T>C, and c.1298A>C). The following variables were included, as independent variables, in the multiple linear regressions for the biochemical parameters of the B12 vitamin metabolism: number of variant alleles for the *MTRR* rs326119 (0, 1 or 2 for AA, AC, or CC, respectively), age, sex, race, and *MTHFR* polymorphisms (c.677C>T, c.1317T>C, and c.1298A>C). Multivariate logistic regression analysis was performed to evaluate the odds ratio (OR) for coronary lesions. In this model, coronary lesion frequency was compared between normal coronary arteries versus 1-vessel, 2-vessel, and 3-vessel disease. Statistical analyses were carried out using SPSS software (version 16.0, IBM, New York, NY), with the level of significance set at $p \leq 0.05$.

3. Results

3.1. Allele and genotype distribution of the MTRR rs326119 polymorphism

For the CHD sample (n = 722), the frequency of the rs326119 C variant allele was 46.5% and the distribution of the genotypes was 19.3% for variant homozygous, 54.4% for heterozygous, and 26.3% for wild-type.

For the blood donors (n = 156), the frequency of the rs326119 C variant allele was 41.0%, and the distribution of the genotypes was 17.9% for variant homozygous, 46.2% for heterozygous, and 35.9% for wild-type. For the patients who underwent coronary angiography (n = 1432), the frequency of the rs326119 C variant allele was 45.9%, and the distribution of the genotypes was 19.6% for variant homozygous, 52.7% for heterozygous, and 27.7% for wild-type. The genotypic distribution for the *MTRR* rs326119 polymorphism was in accordance with the Hardy–Weinberg equilibrium (HWE) for all sample groups (HWE $p \geq 0.01$) ($X^2 = 6.38$, $p = 0.01$; $X^2 = 0.33$, $p = 0.56$; $X^2 = 5.43$, $p = 0.02$, respectively).

3.2. Biochemical parameters of vitamin B12 metabolism according to MTRR rs326119 polymorphism

Table 1 shows significant differences in the biochemical parameters of vitamin B12 metabolism among *MTRR* rs326119 genotypes. Subjects carrying AC or CC genotypes had higher homocysteine concentrations and lower cobalamin concentrations compared to individuals carrying the AA genotype, adjusted for age, sex, race, and *MTHFR* polymorphisms. We did not observe any difference in the folate concentration among studied genotypes (Table 1).

Table 2 shows a multiple linear regression model. We identified a significant association between the number of C variant alleles and the concentrations of homocysteine and cobalamin. Also, with the presence of 3 *MTHFR* polymorphisms, the *MTHFR* c.677C>T polymorphism was associated with concentrations of homocysteine, cobalamin, and folate (Table 2).

3.3. Angiographic data according to MTRR rs326119 polymorphism in patients who underwent coronary angiography

Table 3 shows no significant association between coronary atherosclerosis extension and MTRR genotypes (AA versus AC or CC) in a multivariate model (OR = 1.08, 95%CI = 0.85–1.37, p = 0.50). Also, no significant association was observed for 1 variant allele (OR = 1.01, 95%CI = 0.69–1.47, p = 0.96) or for 2 variant alleles (OR = 1.18, 95%CI = 0.84–1.66, p = 0.33). Regarding the derived scores of coronary atherosclerosis burden, no significant difference was found among *MTRR* genotypes AA, AC, and CC (Extension Score: 2.2 ± 1.7, 2.2 ± 1.6, and 2.3 ± 1.7; Severity Score: 1.6 ± 1.3, 1.6 ± 1.2, and 1.7 ± 1.3) (p = 0.58 and p = 0.45, respectively).

3.4. Comparison of frequencies of the MTRR genotypes or C variant allele among CHD patients and controls

Table 4 shows the distribution of the *MTRR* genotypes among sample groups. We did not observe an association between the *MTRR* polymorphism and CHD or ventricular septal defects (VSD), as previously reported in a Chinese population, in our samples. Genotypic and allelic frequencies for the *MTRR* polymorphism in patients with CHD and those with CHD and VSD were compared with frequencies of blood donors and of patients who underwent coronary angiography (we considered these 2 last sample groups as controls).

Table 1
Concentrations of homocysteine, cobalamin, and folate, according to *MTRR* rs326119 genotypes.

Biochemical parameter	*MTRR* rs326119 c.56+781 A>C			p value
	AA (n = 56)	AC (n = 72)	CC (n = 28)	
Homocysteine, μmol/L	8.7 ± 0.5[a]	9.7 ± 0.4[b]	10.1 ± 0.6[b]	0.04
Cobalamin, pmol/L	304.8 ± 14.7[a]	260.5 ± 13.3[b]	275.6 ± 19.9[b]	0.03
Folate, nmol/L	11.9 ± 0.6	12.3 ± 0.5	12.3 ± 0.8	0.92

Biochemical data were adjusted for age, sex, race, and *MTHFR* polymorphisms.
Values with different superscript letters are significantly different (Tukey's post hoc test).

Table 2
Variables of a multiple linear regression model for concentrations of homocysteine, cobalamin, and folate.

Variable	β coefficient (standard error)	p value
Homocysteine, μmol/L		
Number of variant alleles for the *MTRR* rs326119	1.1 (0.6)	0.02
Age	−0.01 (0.03)	0.98
Sex (male)	2.1 (0.6)	<0.001
Race (non-White)	−0.1 (0.4)	0.85
MTHFR c.677C>T	2.4 (0.5)	0.001
MTHFR c.1317T>C	0.5 (0.7)	0.40
MTHFR c.1298A>C	−0.4 (0.5)	0.42
Cobalamin, pmol/L		
Number of variant alleles for the *MTRR* rs326119	−33.0 (19.5)	0.03
Age	−0.4 (0.9)	0.69
Sex (male)	0.7 (18.8)	0.97
Race (non-White)	11.0 (14.4)	0.45
MTHFR c.677C>T	−36.8 (15.4)	0.02
MTHFR c.1317T>C	0.7 (22.1)	0.98
MTHFR c.1298A>C	−3.4 (16.8)	0.84
Folate, nmol/L		
Number of variant alleles for the *MTRR* rs326119	0.4 (0.7)	0.60
Age	0.04 (0.03)	0.30
Sex (male)	−1.8 (0.7)	0.02
Race (non-White)	−0.4 (0.6)	0.49
MTHFR c.677C>T	−1.6 (0.6)	0.01
MTHFR c.1317T>C	0.7 (0.9)	0.43
MTHFR c.1298A>C	−0.4 (0.7)	0.51

Number of variant alleles for *MTRR* rs326119: 0, 1 or 2 for AA, AC, or CC, respectively.

4. Discussion

Elevated levels of homocysteine have been associated with many diseases, such as neurodegenerative disorders, recurrent pregnancy loss, neural tube defects, ischemic heart disease and stroke, atherosclerosis, and congenital defects [24–34]. Our study shows that the *MTRR* rs326119 polymorphism is associated with levels of plasma homocysteine. This finding was the first replication of Zhao et al.'s and Cheng et al.'s studies, in a non-Chinese population [15,34]. However, our study did not identify an association of the *MTRR* polymorphism with CHD or coronary artery disease.

Subjects carrying AC or CC genotypes of *MTRR* rs326119 polymorphism had higher homocysteine and lower cobalamin concentrations, compared with subjects carrying the AA genotype. Our finding suggests that the *MTRR* rs326119 polymorphism might be a genetic marker for homocysteine and cobalamin concentrations. Some studies showed a negative correlation between homocysteine and B12 vitamin levels [15,35]. Regarding vitamin B12 metabolism, increased levels of homocysteine may reflect folate and B12 vitamin deficiency. These

Table 3
Logistic regression multivariate analysis of the coronary lesion odds ratio in the patients who underwent coronary angiography.

Variables	OR	95% IC	p value
Genotypes for the *MTRR* rs326119	1.08	0.85–1.37	0.50
Sex (male)	3.73	2.64–5.27	<0.001
Age	1.03	1.02–1.05	<0.001
Self-declared race			
White (reference)	1.00		
Intermediate	1.35	0.57–3.44	0.52
Black	0.90	0.35–2.66	0.57
Body mass index	0.95	0.92–0.98	0.003
Statin use	1.74	1.11–2.73	0.02
Smoking	1.22	0.85–1.76	0.27
Total cholesterol	1.02	1.01–1.03	0.001

Genotypes for the *MTRR* rs326119 were AA genotype versus AC or CC genotypes.
Coronary lesion frequency was compared between normal coronary arteries versus 1-vessel, 2-vessel, and 3-vessel disease.

Table 4
Frequencies of the *MTRR* genotypes or C variant allele, according to studied subjects.

	Patients with CHD (n = 722)	Patients with VSD (n = 213)	Blood donors (n = 156)	Patients who underwent coronary angiography (n = 1432)
Genotype comparison				
AA, n	190	52	56	397
AC, n	393	116	72	755
CC, n	139	45	28	280
p value[a]	–	0.77	0.07	0.73
p value[b]	0.77	–	0.08	0.62
Variant allele comparison				
C variant allele	46.5%	48.3%	41.0%	45.9%
p value[a]	–	0.53	0.09	0.75
p value[b]	0.53	–	0.06	0.37

[a] Comparison of frequencies of the *MTRR* genotypes or C variant allele of the congenital heart disease (CHD) group with other groups.
[b] Comparison of frequencies of the *MTRR* genotypes or C variant allele of the CHD patients with ventricular septal defects (VSD) with other groups.

associations may also be due to the effects of changed vitamin B12 metabolism on homocysteine remethylation and synthesis of DNA methylation, resulting in elevated plasma concentrations of homocysteine [34,36,37]. The functional variant of *MTRR rs326119* is likely to stimulate hyperhomocysteinemia and can further induce DNA hypomethylation. This variant can also result in low levels of methionine and hyperhomocysteinemia by dysfunction of MTR-catalyzed homocysteine recycling to methionine [34].

Zhao et al.'s interesting study found a genetic association by using biochemical data, but they also describe an association with CHD in patient samples from a Han Chinese population [15]. Zhao et al. demonstrated the decay of *MTRR* transcription activity using a functional assay. They examined 28 in vivo cardiovascular tissue samples and observed decreased *MTRR* RNAm levels. In vitro luciferase assays confirmed the influence of the functional c.56 + 781 A>C variant [15]. Concerning cardiovascular diseases, a possible pathway is that increased levels of homocysteine inhibit a nitric oxide (NO)-dependent mechanism regulating cardiac O_2 consumption and NO-dependent vasodilatation [38].

Our main hypothesis for the lack of a genetic association with CHD is that the different ethnicity of our patient samples might be involved. This could lead to different associations by linkage disequilibrium with other functional variants. It can also lead to a completely different gene × environment scenario and justify a different threshold for association in these populations. Further genetic association studies are warranted to check the association of this *MTRR* polymorphism with the complex phenotype of CHD among different ethnic groups. In the same way, there is an *MTRR* polymorphism located in a coding region (c.66A>G) that was associated with vitamin B12 biochemical parameters, heart disease, and higher risk for coronary artery disease in some populations [6,15,39–41]. However, many other studies were not able to find the same genetic association [42–45].

Our study has some limitations. First, we only evaluated 1 *MTRR* polymorphism. Second, CHD and coronary atherosclerosis are complex phenotypes that depend on multiple other genetic and environmental factors. Third, our sample size of individuals with biochemical tests available is relatively small. Despite these facts, we were able to identify significant differences among genotypes replicating data found by Zhao et al. and Cheng et al. [15,34].

In conclusion, our findings indicate that the *MTRR* rs326119 variant might be a genetic marker associated with concentrations of homocysteine and cobalamin, but not with CHD and coronary atherosclerosis in the Brazilian population.

Competing interests

The authors declare that they have no competing interests.

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