




Research Article
Human and Medical Genetics

Association between folate metabolism polymorphisms and breast cancer: a case-control study

Ana Paula D'Alarme Gimenez-Martins¹, Márcia Maria Urbanin Castanhole-Nunes^{1,3},
Carlos Henrique Viesi do Nascimento-Filho¹, Stéphanie Piacenti dos Santos¹,
Ana Livia Silva Galbiatti-Dias^{1,3}, Glaucia Maria de Mendonça Fernandes¹, Caroline Izak Cuzziol¹,
José Luis Esteves Francisco^{2,3}, Érika Cristina Pavarino^{1,3} and Eny Maria Goloni-Bertollo^{1,3} 

¹Faculdade de Medicina de São José do Rio Preto (FAMERP), Departamento de Biologia Molecular, Unidade de Pesquisa em Genética e Biologia Molecular (UPGEM), São José do Rio Preto, SP, Brazil.

²Faculdade de Medicina de São José do Rio Preto (FAMERP), Departamento de Ginecologia e Obstetrícia, São José do Rio Preto, SP, Brazil.

³Fundação da Faculdade Medicina de São José do Rio Preto (FUNFARME), São José do Rio Preto, SP, Brazil.

Abstract

We investigated the association between methylenetetrahydrofolate reductase (*MTHFR* C677T and A1298C), methionine synthetase (*MTR* A2756G), and methionine synthase reductase (*MTRR* A66G) polymorphisms involved in folate pathway and breast cancer risk, and the interaction between these polymorphisms and tobacco and alcohol consumption. Furthermore, we evaluated the association between these polymorphisms and clinicopathological variables. This case-control study included 606 Brazilian women, comprising 128 patients with breast cancer and 478 controls. *MTHFR* and *MTR* polymorphisms were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and *MTRR* polymorphisms using real-time PCR. Age ≥ 50 years (odds ratio [OR]: 2.65; 95% confidence interval [CI]: 1.65–4.26; $p < 0.001$) and alcohol consumption (OR: 1.76; 95% CI: 1.0–2.85; $p = 0.021$) were associated with an increased risk of breast cancer. For *MTHFR* A1298C, we observed a reduced risk of developing breast cancer in the codominant model (genotype CC–OR: 0.22; 95% CI: 0.06–0.74; $p = 0.014$), recessive model (OR: 0.22; 95% CI: 0.07–0.76 $p = 0.004$), and log-additive model (OR: 0.70; 95% CI: 0.49–0.98; $p = 0.035$). Women aged ≥ 50 years and those who are alcohol consumers had increased susceptibility to breast cancer, and *MTHFR* A1298C modulated the risk for this disease. This is the first study to evaluate the association between polymorphisms in folate metabolism and breast cancer in the northwest region of São Paulo State, Brazil.

Keywords: Breast carcinoma, genetic polymorphism, methylenetetrahydrofolate reductase (*MTHFR*), methionine synthetase (*MTR*), methionine synthase reductase (*MTRR*).

Received: January 06, 2021; Accepted: August 08, 2021.

Introduction

Breast cancer is the most common cancer and the second most common cause of mortality (INCA, 2019; Siegel *et al.*, 2021). Thus, the discovery of predictors of breast cancer plays a central role in monitoring women and early detection, which increases the chances of curative treatment. Factors related to developed countries linked to increased breast cancer cases include early menarche, older age upon first occasion of giving birth, nulliparity, obesity, alcohol consumption, breastfeeding, and physical activity (Key *et al.*, 2001; Arthur *et al.* 2020). Moreover, the identification of genetic markers related to breast cancer development is fundamental for guiding therapeutic approaches (Lee *et al.*, 2017). Using genetic risk profiles, personalized medicine can be achieved (Naushad *et al.*, 2012).

Send correspondence to Eny Maria Goloni-Bertollo. Faculdade de Medicina de São José do Rio Preto (FAMERP), Departamento de Biologia Molecular, Unidade de Pesquisa em Genética e Biologia Molecular (UPGEM), Avenida Brigadeiro Faria Lima, 5416, 15090-000, São José do Rio Preto, SP, Brazil. Email: eny.goloni@famerp.br.

There are potential biomarkers, such as polymorphisms of the folate pathway or one-carbon pathway. The latter pathway is responsible for the donation of the methyl group, which is further used in important processes such as DNA methylation, histone methylation, and protein methylation (Mentch and Locasale, 2016; Hiraoka and Kagawa, 2017). Folate acid and diet provide the methyl group, leading to the conversion of dihydrofolate to tetrahydrofolate. Subsequently, tetrahydrofolate is converted to 5,10-methylenetetrahydrofolate, which donates a methyl group for DNA synthesis and converges to 5-methyltetrahydrofolate, a reaction catalyzed by the methylenetetrahydrofolate reductase (*MTHFR*) enzyme (Hiraoka and Kagawa, 2017; Scaglione and Panzavolta, 2014). Under methionine synthetase (*MTR*) and methionine synthase reductase (*MTRR*) enzyme activity, 5-methyltetrahydrofolate is converted to tetrahydrofolate, and the donated methyl group is used in methylation circuits, including the conversion of homocysteine to methionine, and is pivotal to the activity of S-adenosyl-L-methionine (SAM) synthase and DNA methyltransferases (Galbiatti, *et al.*, 2012a; Mentch and Locasale, 2016; Hiraoka and Kagawa, 2017).

The function of this pathway is not only restricted to methylation events but also to DNA synthesis, thereby playing an important role in cancer development. Therefore, to better understand the activity of these enzymes, it is necessary to understand the effects of single nucleotide polymorphisms (SNPs) on cancer susceptibility. The *MTHFR* C677T polymorphism has been associated with susceptibility to developing breast cancer among Asian women, but no association was observed in Caucasians (Chen *et al.*, 2019), suggesting the relevance of ethnicity and region. Interestingly, SNPs in the one-carbon pathway were differentially methylated in breast tissues from healthy women (Song *et al.*, 2016), indicating the role of these SNPs in methylation circuits. Taken together, these findings remain ambiguous, and more case-control studies are required.

Here, we aimed to investigate the association between *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G (rs1805087), and *MTRR* A66G (rs1801394) polymorphisms and the risk of developing breast cancer in women from Northwest São Paulo State, Brazil. We further examined the association of risk factors, including age, smoking habits, alcohol consumption, number of pregnancies, body mass index, hormone therapy, and clinicopathological variables, with breast cancer development. Overall, we showed that the *MTHFR* A1298C polymorphism reduced the risk of developing breast cancer, whereas alcohol intake increased the risk.

Subjects and Methods

Study population

The present case-control study was approved by the National Ethics Committee (CAAE – 04069612.1.0000.5415), Sao Jose do Rio Preto Medical School (FAMERP), Sao Paulo, Brazil (process number: 84397). All individuals agreed with the informed consent form, according to Resolution 466/12 of the National Health Council (CONEP). This study is consistent with Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) (Gallo *et al.*, 2011).

We evaluated 606 eligible women, comprising 128 patients (case group) after total excision or biopsy were diagnosed and histologically confirmed to have breast cancer and 478 healthy women volunteers (control group); only women without any history of cancer or familial history of cancer aged >40 years were included. All women were admitted between January 2013 and January 2015 to the Hospital de Base, São Jose do Rio Preto Medical School, São José do Rio Preto, Brazil.

The control group was recruited from blood donors who met the government guidelines for blood donor selection (<http://www.hemonline.com.br/portarias/rdc153/indexframe.htm>). During recruitment of the control group, efforts were made to maintain a similarity in age and geographic region between the control and case groups. In individuals who were most recently recruited to the control group (n=219), we also performed a standardized questionnaire to obtain personal data, demographic and epidemiological factors such as lifestyle (smoker and alcohol drinker), reproductive history (number of live births, number of miscarriages, number of stillbirths, and use of contraceptives), and family history of cancer (breast

cancer and/or other cancers). Measurements of height and weight were used to calculate the body mass index (BMI).

Additional information about the diagnosis, histological type, tumor location, tumor size (T), regional lymph node involvement (N), presence of metastasis (M), progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor-type 2 (HER2) overexpression were obtained from the medical records and pathology reports. TNM classification of malignant tumors was classified according to the UICC/AJCC of 2010 (Sobin *et al.*, 2010) and grouped into stage I (T1N0), stage II (T1N1 and T2N0–1), stage III (T3N0–1 and T1–3N2), and stage IV (T4N0–3, T1–3N3, and T1–4N0–3M1) according to the AJCC Cancer Staging Manual (Fleming *et al.*, 1997). Tumor stages I and II were considered non-advanced, while stages III and IV were considered advanced. The clinicopathological characteristics of intrinsic breast cancer subtypes were defined according to Perou *et al.* (2000) and Goldhirsch *et al.* (2011): Luminal A: ER- and/or PgR-positive, HER2 negative and Ki-67 <14%; Luminal B (HER2-negative): ER- and/or PgR-positive, HER2-negative and Ki-67 high; Luminal B (HER2-positive): ER- and/or PgR-positive, any Ki-67, HER 2 overexpressed or amplified; HER2-positive: ER and PgR absent and HER2 overexpressed or amplified; and “basal-like” (triple-negative): ER and PgR absent and HER2-negative (Perou *et al.*, 2000; Goldhirsch *et al.*, 2011)

We considered smokers who consumed at least 100 cigarettes during their lifetime and alcohol drinkers as those who consumed more than one drink weekly (one drink corresponding to approximately 44 mL of liquor, or 118 mL of wine, or 350 mL of beer) (Carpenter and Cohen, 1990).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes according to a previously detailed method (Miller *et al.*, 1988) with modifications. *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), and *MTR* A2756G (rs1805087) polymorphisms were determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) with the following primers: *MTHFR* C677T, sense 5'-TGA AGG AGA AGG TGT CTG CGG GA-3', anti-sense 5'-AGG ACG GTG CGG TGA GAG TG-3'; and *MTR* A2756G, sense 5'-CCAGGG TGC CAG GTA TAC AG-3', anti-sense 5'-GCC TTT TAC ACT CCT CAA AAC-3'. Genotyping of the *MTHFR* C677T polymorphism was accomplished using the restriction enzyme, HinfI. The resulting fragments were 198 bp (C allele), 175 bp, and 23 bp (T allele). The *MTR* A2756G polymorphism was genotyped using the restriction enzyme, HaeIII. The resulting fragments were 413 and 85 bp (A allele) and 290, 123, and 85 bp (G allele) (Galbiatti *et al.*, 2010; Barbosa *et al.*, 2012; Zara-Lopes *et al.*, 2016). Genotyping of *MTRR* A66G (rs1801394) polymorphisms was performed using the Real Time PCR–SNP Genotyping Assay (Thermo Fisher Scientific, Carlsbad, CA) – Assay ID: C_3068176_10 (20X), in following the manufacturer's instructions. The reactions were performed using the StepOnePlus™ Real-Time PCR System (Galbiatti *et al.*, 2012b) (Table 1). To improve the reliability of the analysis, we performed genotyping confirmation in 10% of cases and

Table 1 – Primers sequence, restriction enzyme and fragments size.

Polymorphisms	Primers Sequence	Restriction Enzyme	Fragments Size
<i>MTHFR C677T</i>		<i>Hinf</i> I	
sense	5'- TGA AGG AGA AGG TGT CTG CGG GA 3'		C allele - 198 bp
anti-sense	5'- AGG ACG GTG CGG TGA GAG TG 3'		T allele – 175, 23 bp
<i>MTHFR A1298C</i>		<i>Mbo</i> II	
sense	5'- CAA GGA GGA GCT GCT GAA GA 3'		A allele – 72, 28 bp
anti-sense	5'- CAA CTC CAG CAT CAC TCA CT 3'		C allele - 100, 28 bp
<i>MTR A2756G</i>		<i>Hae</i> III	
sense	5'- CCA GGG TGC CAG GTA TAC AG 3'		A allele – 413, 85 bp
anti-sense	5'- GCC TTT TAC ACT CCT CAA AAC 3'		G allele-290, 123, 85 bp

MTHFR - Methylene tetrahydrofolate reductase; MTR - methionine synthase, bp- base-pair*

control samples randomly for each polymorphism. We found 100% concordance.

Statistical analysis

Multiple logistic regression was used to determine the effects of variables on breast cancer risk and clinicopathological characteristics using the Minitab/Windows program - Version 14.0. The model included the following variables: age (reference: <50 years old; median), smoking habits (reference: non-smoker), alcohol consumption (reference: no alcohol drinkers), number of pregnancies (reference: ≥ 3 pregnancies), BMI (reference: <25 kg/m²), hormone therapy (reference: not using hormone therapy), *MTHFR C677T* (reference: genotype CC), *MTHFR A1298C* (reference: genotype AA), *MTR A2756G* (reference: genotype AA), and *MTRR A66G* (reference: genotype AA). The clinicopathological characteristics were T classification (reference: T1 and T2), N classification (reference: no lymph nodes affected), and M classification (reference: no metastasis). Logistic regression was adjusted for age, sex, smoking habits, and alcohol consumption.

The Hardy–Weinberg equilibrium (HWE) was assessed using the chi-square test using the program SNPStats (available at http://bioinfo.iconcologia.net/SNPstats_web). The SNPStats online computer program was used to analyze the following models: 1) codominant (heterozygous vs. homozygous wild type and polymorphic homozygous vs. homozygous wild type), 2) dominant (heterozygous plus polymorphic homozygous vs. homozygous wild type), 3) recessive (polymorphic homozygous vs. homozygous wild type plus heterozygous), 4) overdominant (wild homozygous vs. heterozygous plus polymorphic homozygous), and 5) additive (weight polymorphic homozygote vs. heterozygote 2 plus homozygous wild-type) (Fernandes et al., 2016). The relationship between polymorphisms and clinicopathological features was also analyzed using SNPStats. All results are presented as odds ratios (OR) with a 95% confidence interval (95% CI); p-values <0.05 were considered statistically significant.

Results

In this study, we observed that women aged ≥ 50 years old (OR: 2.65; 95% CI: 1.65–4.26; $p < 0.001$) and who consumed alcohol (OR: 1.76; 95% CI: 1.09–2.85; $p = 0.021$) had an increased risk of breast cancer. Smoking habits, number of pregnancies, BMI ≥ 25 kg/m², and hormone therapy

showed no association with the risk of breast cancer (data not shown). Regarding clinicopathological characteristics, we found that 94.6% of all breast cancers were invasive ductal carcinomas, 4.6% had lobular carcinoma, and 0.8% mixed ductal and lobular carcinoma. Regarding TNM classification, 62.5% of cases were T1 or T2, 42.2% of cases had affected lymph nodes, and 34.4% of cases had metastasis. The frequency of breast cancer subtypes was as follows: 48.4% for luminal B, 26.6% for luminal A, 12.5% for triple-negative, and 10.1% for HER2 overexpression.

The analyses of *MTHFR C677T* (rs1801133), *MTHFR A1298C* (rs1801131), *MTR A2756G* (rs1805087), and *MTRR A66G* (rs1801394) polymorphisms; tumor size; lymph node involvement; and metastasis are shown in Table 2. We did not find a significant association between polymorphisms and clinical features. Regarding SNPs and breast cancer subtypes, we did not observe an association between luminal A, luminal B, HER2 overexpression, and triple-negative subtypes and polymorphisms (Table 3). We observed that the *MTHFR A1298C* (rs1801131) polymorphism reduced the risk of developing breast cancer in the codominant model (genotype CC – OR: 0.22; 95% CI: 0.06–0.74; $p = 0.014$), recessive model (OR: 0.22; 95% CI: 0.07–0.76; $p = 0.004$), and log-additive model (OR: 0.70; 95% CI: 0.49–0.98; $p = 0.035$). No significant association was found between the *MTR A2756G* (rs1805087), *MTRR A66G* (rs1801394), and *MTHFR C677T* (rs1801133) polymorphisms analyzed and models ($p > 0.05$). The genotype distributions of *MTHFR C677T* (rs1801133), *MTHFR A1298C* (rs1801131), *MTR A2756G* (rs1805087) and *MTRR A66G* (rs1801394) polymorphisms and breast cancer risk are presented in Table 4 (128 cases and 478 controls).

HWE analysis (Table 4) showed that the case and control groups were in equilibrium for *MTHFR C677T* (rs1801133) (case, $p = 1.00$; control, $p = 0.76$) and *MTR A2756G* (rs1805087) (case, $p = 0.07$; control, $p = 0.7$) polymorphisms. In the control group, *MTHFR A1298C* (rs1801131) and *MTRR A66G* (rs1801394) polymorphisms showed disequilibrium ($p = 0.01$ and $p = 0.03$). The frequencies of variant alleles for SNPs (Table 4) were: allele C, 60% case and 66% control for the *MTHFR C677T* (rs1801133) polymorphism; allele A, 80% case and 72% control for the *MTHFR A1298C* (rs1801131) polymorphism; allele A, 82% case and 79% control for the *MTR A2756G* (rs1805087) polymorphism; and allele A, 60% case and 57% control for the *MTRR A66G* (rs1801394) polymorphism.

Table 2 – Association between *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G polymorphisms and clinical tumor size (T1-T2 vs T3-T4), lymph nodes involvement and metastasis.

Clinical features	MTHFR C677T		MTHFR A1298C		MTR A2756G		MTRR A66G	
	CC	CT+TT	AA	AC+CC	AA	AG+GG	AA	AG+GG
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Tumor size								
T1-T2	29 (22.7)	51 (39.8)	49 (38.2)	31 (24.2)	50 (39.1)	30 (23.3)	25 (19.5)	55 (43.0)
T3-T4	16 (12.5)	31 (24.2)	29 (22.7)	18 (14.1)	32 (25.0)	15 (11.8)	22 (17.2)	25 (19.5)
Unknown	01 (0.8)	0 (0)	01 (0.8)	0 (0)	01 (0.8)	0 (0)	01 (0.8)	0 (0)
OR (95% CI)	1.00	1.31 (0.57-3.03)	1.00	1.18 (0.53-2.64)	1.00	0.77 (0.34-1.74)	1.00	0.50 (0.23-1.09)
<i>p</i> value		0.522		0.682		0.526		0.081
Lymph nodes								
No	27 (21.1)	43 (33.6)	41 (32.0)	29 (22.7)	44 (34.4)	26 (20.3)	25 (19.5)	45 (35.2)
Yes	17 (13.2)	37 (28.9)	36 (28.0)	18 (14.1)	37 (28.9)	17 (13.2)	20 (15.6)	34 (26.5)
Unknown	02 (1.6)	02 (1.6)	02 (1.6)	02 (1.6)	02 (1.6)	02 (1.6)	03 (2.4)	01 (0.8)
OR (95% CI)	1.00	1.98 (0.83-4.71)	1.00	0.80 (0.35-1.81)	1.00	0.62 (0.27-1.43)	1.00	0.87 (0.39-1.95)
<i>p</i> value		0.122		0.591		0.264		0.730
Metastasis								
No	29 (22.7)	51 (39.8)	44 (34.4)	36 (28.0)	48 (37.5)	32 (25.0)	30 (23.3)	50 (39.1)
Yes	16 (12.5)	28 (21.8)	32 (25.0)	12 (9.4)	31 (24.2)	13 (10.1)	15 (11.7)	29 (22.7)
Unknown	01 (0.8)	03 (2.4)	03 (2.4)	01 (0.8)	04 (3.2)	0 (0)	03 (2.4)	01 (0.8)
OR (95% CI)	1.00	1.19 (0.50-2.82)	1.00	0.48 (0.21-1.11)	1.00	0.51 (0.22-1.20)	1.00	1.06 (0.47-2.40)
<i>p</i> value		0.697		0.08		0.123		0.894

OR, odds Ratio; Adjusted for age, smoking habits, alcohol consumption, gestations, BMI (Body-mass index), hormone therapy and polymorphisms.

Table 3 – Association between *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G polymorphisms and breast cancer subtype.

Breast Cancer Subtype	MTHFR C677T		MTHFR A1298C		MTR A2756G		MTRR A66G	
	CC	CT+TT	AA	AC+CC	AA	AG+GG	AA	AG+GG
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Luminal A	12 (9.4)	22 (17.2)	19 (14.8)	15 (11.7)	22 (17.2)	12 (9.4)	15 (11.7)	19 (14.8)
OR (95% CI)	1.00	1.01 (0.42-2.45)	1.00	1.64 (0.70-3.85)	1.00	1.07 (0.44-2.58)	1.00	0.62 (0.27-1.45)
<i>p</i> value		0.975		0.253		0.879		0.272
Luminal B	22 (17.2)	40 (31.2)	39 (30.5)	23 (17.9)	38 (29.7)	24 (18.7)	18 (14.1)	44 (34.4)
OR (95% CI)	1.00	0.77 (0.34-1.71)	1.00	0.91 (0.42-1.98)	1.00	1.39 (0.63-3.04)	1.00	2.16 (0.98-4.76)
<i>p</i> value		0.514		0.817		0.415		0.056
HER2 Overexpression	04 (3.2)	09 (7.0)	08 (6.2)	05 (3.9)	10 (7.8)	03 (2.4)	06 (4.6)	07 (5.5)
OR (95% CI)	1.00	1.31 (0.33-5.29)	1.00	1.04 (0.29-3.73)	1.00	0.35 (0.08-1.50)	1.00	0.58 (0.16-2.08)
<i>p</i> value		0.704		0.948		0.156		0.405
Triple Negative	05 (3.9)	11 (8.5)	12 (9.4)	04 (3.2)	10 (7.8)	06 (4.6)	07 (5.5)	09 (7.0)
OR (95% CI)	1.00	1.36 (0.40-4.57)	1.00	0.50 (0.14-1.75)	1.00	0.94 (0.29-3.09)	1.00	0.63 (0.20-1.95)
<i>p</i> value		0.622		0.276		0.923		0.425
Unknown	03 (2.4)	0 (0)	01 (0.8)	02 (1.6)	03 (2.4)	0 (0)	02 (1.6)	01 (0.8)

OR, odds Ratio; Adjusted for age, smoking habits, alcohol consumption, gestations, BMI (Body-mass index), hormone therapy and polymorphisms.

Table 4 – Analysis of MTHFR C677T, MTHFR A1298C, MTR A2756G and MTRR A66G polymorphisms with breast cancer.

Model	Genotype	Case N (%)	Control N (%)	OR (95%CI)	p	Genotype	Case N (%)	Control N (%)	OR (95%CI)	p	
<i>MTHFR C677T</i>						<i>MTHFR A1298C</i>					
Codominant	CC	46 (35.9)	207 (43.3)	1.00 1.21 (0.77-1.89) 1.71 (0.91-3.22)	0.25	AA	79 (61.7)	261 (54.6)	1.00 0.90 (0.59-1.39) 0.22 (0.06-0.74)	0.01*	
	CT	61 (47.7)	218 (45.6)			AC	46 (35.9)	170 (35.6)			
	TT	21 (16.4)	53 (11.1)			CC	3 (2.3)	47 (9.8)			
	C allele	153 (60)	632 (66)			A allele	204 (80)	692 (72)			
	T allele	103 (40)	324 (34)			C allele	52 (20)	264 (28)			
	HWE	p=1.0	p=0.76			HWE	p=0.28	p=0.016			
Dominant	CC	46 (35.9)	207 (43.3)	1.00	0.21	AA	79 (61.7)	261 (54.6)	1.00	0.20	
	CT+TT	82 (64.1)	271 (56.7)	1.31 (0.86-2.00)		AC+CC	49 (38.3)	217 (45.4)	0.76 (0.50-1.16)		
Recessive	CC+CT	107 (83.6)	425 (88.9)	1.00	0.15	AA+AC	125 (97.7)	431 (90.2)	1.00	0.004*	
	TT	53 (11.1)	53 (11.1)	1.55 (0.87-2.75)		CC	3 (2.3)	47 (9.8)	0.22 (0.07-0.76)		
Overdominant	CC+TT	67 (52.3)	260 (54.4)	1.00	0.82	AA+CC	82 (64.1)	308 (64.4)	1.00	0.92	
	CT	61 (47.7)	218 (45.6)	1.05 (0.70-1.58)		AC	46 (35.9)	170 (35.6)	1.02 (0.67-1.57)		
Log-additive	—	—	—	1.28 (0.95-1.74)	0.1	—	—	—	0.70 (0.49-0.98)	0.03*	
<i>MTR A2756G</i>						<i>MTRR A66G</i>					
Codominant	AA	83 (64.8)	300 (62.8)	1.00 1.22 (0.79-1.89) 0.23 (0.03-1.77)	0.12	AA	48 (37.5)	168 (35.1)	1.00 0.99 (0.63-1.56) 0.76 (0.42-1.35)	0.59	
	AG	44 (34.4)	156 (32.6)			AG	57 (44.5)	211 (44.1)			
	GG	1 (0.8)	22 (4.6)			GG	23 (18.0)	99 (20.7)			
	A allele	210 (82)	756 (79)			A allele	153 (60)	547 (57)			
	G allele	46 (18)	200 (21)			G allele	103 (40)	409 (43)			
	HWE	p=0.07	p=0.78			HWE	p=0.46	p=0.03			
Dominant	AA	83 (64.8)	300 (62.8)	1.00	0.63	AA	48 (37.5)	168 (35.1)	1.00	0.66	
	AG+GG	45 (35.2)	178 (37.2)	1.11 (0.721-1.71)		AG+GG	80 (62.5)	310 (64.8)	0.91 (0.60-1.39)		
Recessive	AA+AG	127 (99.2)	456 (95.4)	1.00	0.06	AA+AG	105 (82)	379 (79.3)	1.00	0.30	
	GG	1 (0.8)	22 (4.6)	0.21 (0.03-1.64)		GG	23 (18)	99 (20.7)	0.76 (0.45-1.29)		
Overdominant	AA+GG	84 (65.6)	322 (67.4)	1.00	0.27	AA+GG	71 (55.5)	267 (55.9)	1.00	0.68	
	AG	44 (34.4)	156 (32.6)	1.28 (0.83-1.98)		AG	57 (44.5)	211 (44.1)	1.09 (0.72-1.65)		
Log-additive	—	—	—	0.98 (0.67-1.43)	0.91	—	—	—	0.89 (0.67-1.17)	0.40	

**p* – values significant. OR, odds Ratio; Adjusted for age, smoking habits, alcohol consumption, gestations, BMI (Body-mass index), hormone therapy and polymorphisms.

Discussion

Our results showed that age ≥ 50 years ($p < 0.001$) and alcohol consumption ($p = 0.021$) were risk factors for breast cancer in women. Women aged ≥ 50 years have been associated with an increased risk of breast cancer in other studies (Sangrajrang *et al.*, 2010; Akilzhanova *et al.*, 2013; He *et al.*, 2014; Jiang-Hua *et al.*, 2014; Gong *et al.*, 2015). Age is also associated with menopause, body-mass index, and hormone therapy due to the influence of hormones during women's lives (Hvidtfeldt *et al.*, 2015). In a pooled cohort study, these authors found that overweight women (median age, 56 ± 5.0 years) under hormone therapy had an increase in the absolute risk of breast cancer (p interaction = 0.003) (Hvidtfeldt *et al.*, 2015). Also, the risk of breast cancer increases with the levels of endogenous estradiol (Key *et al.*, 2002). In their case-control study (313 cases and 626 controls), Ericson and colleagues confirmed the association between menopausal hormone therapy and breast cancer risk ($p < 0.001$) in women in Malmo (Ericson *et al.*, 2009). The present study did not evaluate the effects of menopausal hormone therapy. However, we did evaluate hormone use during women's life, and found no statistically significant relationship between hormone use and breast cancer risk, as well as between $BMI \geq 25$ kg/m², smoking habits, and number of pregnancies, and breast cancer risk/development. In contrast, we observed an association between alcohol consumption and breast cancer risk. Consistent with

our findings, Ma *et al.* (2009) and Ericson *et al.* (2009) also found a statistically significant association between alcohol intake and breast cancer. Alcohol could be an important factor for inducing carcinogenesis because it may induce epigenetic alterations, aberrant DNA methylation, and reduced folate absorption (Varela-Rey *et al.*, 2013).

Regarding polymorphisms, we found disequilibrium in the control group for *MTHFR* A1298C (rs1801131) and *MTRR* A66G (rs1801394) polymorphisms. HWE represents the non-evolution and homogeneity of a population. However, the diversity of this population, models, and complexity of the diseases can lead to an imbalance between sample groups; therefore, a control group is more likely to be in disequilibrium (Wittke-Thompson *et al.*, 2005).

In the present study, we did not find a significant association between *MTHFR* C677T, *MTR* A2756G, and *MTRR* A66G polymorphisms and breast cancer risk. Surprisingly, the *MTHFR* A1298C polymorphism showed a protective effect on breast cancer development, which opens the door for new investigations. The role of this polymorphism in cancer susceptibility is controversial, especially when considering patient ethnicity (Huang *et al.*, 2007; Li *et al.*, 2014). From this perspective, the current investigation in a Latino population is an important piece of the puzzle. From a biochemical perspective, the *MTHFR* A1298C polymorphism shows decreased enzymatic activity, although not as low as that of

the C677T polymorphism and, therefore, might dysregulate the balance of DNA synthesis/methylation mechanisms (Weisberg *et al.*, 1998). Altogether, the role of these polymorphisms in cancer development is uncertain, which points toward additional effects to be uncovered. Moreover, the balance of DNA synthesis/methylation circuits might be more complex than anticipated (Huang *et al.*, 2007; Li *et al.*, 2014).

Studies of C677T and A1298C polymorphisms in the *MTHFR* gene have shown that these SNPs decrease enzyme activity and alter folate levels, which leads to carcinogenesis (Suzuki *et al.*, 2008; Ericson *et al.*, 2009; Carvalho *et al.*, 2012; Barbosa *et al.*, 2012; Babyshkina *et al.*, 2013; Jiang-Hua *et al.*, 2014). A meta-analysis by Li *et al.*, involving 57 case-control studies, suggested that C677T polymorphisms in the *MTHFR* gene may contribute to breast cancer development, while the A1298C polymorphism was not associated with disease risk (Li *et al.*, 2014). However, another study in Thai women found no association between C677T and A1298C, partially corroborating our findings (Sangrajrang *et al.*, 2010). Sharp and colleagues reported that the *MTHFR* A1298C SNP in a case-control study (62 women and 64 controls) reduced breast cancer risk in women with the 1298CC genotype compared to that of the AA genotype in the Scotland population (Sharp *et al.*, 2002). Moreover, a study with Kazakhstan women found a 1.2-fold decrease in breast cancer risk in a dominant model (Akilzhanova *et al.*, 2013). These findings are consistent with our results in which we observed that *MTHFR* A1298C polymorphism reduced breast cancer risk in a codominant model, recessive model, and log-additive model.

In this study, we did not observe an association between *MTR* A2756G and *MTRR* A66G polymorphisms and breast cancer development. A meta-analysis involving 12 case-control studies for *MTR* A2756G SNP, seven studies for *MTRR* A66G polymorphisms (Weiner *et al.*, 2012), and two studies involving Chinese women also found no association between *MTR* A2756G polymorphism and breast cancer (He *et al.*, 2014; Jiang-Hua *et al.*, 2014). Consistent with our findings, other studies, and the study by Naushad *et al.*, which included 244 case-control pairs of Indian women (Naushad *et al.*, 2012) also found no significant association between the *MTRR* A66G polymorphism and breast cancer risk (Kotsopoulos *et al.*, 2008; Sangrajrang *et al.*, 2010).

Regarding breast cancer subtype, our study observed no association between the polymorphisms studied and luminal A, luminal B, HER2 overexpression, and triple-negative subtypes. Naushad *et al.* (2012) showed that the *MTHFR* C677T polymorphism is associated with a risk for the luminal B subtype, and the *MTR* A2756G polymorphism was associated with an increased risk for the luminal A subtype. For the *MTRR* A66G polymorphism, they also did not observe an association between any breast cancer subtypes, as in this study (Naushad *et al.*, 2012). Batschauer *et al.* (2011) found no association between the *MTHFR* C677T polymorphism and HER2 receptor or ER and PR. Babyshkina *et al.* (2013) found that patients with at least one polymorphic allele for the *MTHFR* C677T polymorphism were significantly associated with a positive ER. For *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G polymorphisms, Babyshkina *et al.* (2013) did not observe an association with breast cancer subtype. Tumors negative for hormone receptors and positive for HER2 have a worse

prognosis in breast cancer (Lang *et al.*, 2009; Batschauer *et al.*, 2011); however, they may not be associated with *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G polymorphisms in folate metabolism.

Our study is the first to show the profile of the female population from Northwestern São Paulo State, Brazil. Since few studies have been performed analyzing folate metabolism polymorphisms and clinical and histopathological features of breast cancer. Overall, this study states that age ≥ 50 years and alcohol consumption increases the risk of breast cancer in women. We did not find a significant association between *MTHFR* C677T, *MTR* A2756G, and *MTRR* A66G polymorphisms and breast cancer development. However, women with the 1298CC polymorphic genotype of the *MTHFR* gene had a lower risk of breast cancer development. Therefore, further studies are warranted to confirm the role of these SNPs in breast cancer development.

Acknowledgments

We thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Financial Code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq for the financial support (Process CNPq universal – 470833/2012-2, Productivity grant- 310987/2018-0). The Faculty of Medicine of São José do Rio Preto – FAMERP and Faculty of Medicine of São José do Rio Preto Foundation – FUNFARME for institutional support. Gynecologic and Obstetric Services of Hospital de Base, Sao Jose do Rio Preto and physician members, Dr. Newton Antônio Bordin Júnior, Ms. Sílvia Aparecida Perea, Ms. José Luis Esteves and Alexandre Mansur Biscaro and Pathology Service, and Physical Dalisio de Santi Neto. We would like to thank Dr. Alexandre Lins Werneck and Editage (www.editage.com) for English language editing.

Conflict of Interest

The authors declare that there are no conflicts of interest

Author Contributions

APDG-M, ECP and EMG-B conceived and the study; JLEF provide samples; APDG-M, CHVNF, SPS, MMUC-N and ALSG-D conducted the experiments; APDG-M, MMUC-N, CHVNF and ALSG-D analyzed the data; APDG-M and MMUC-N wrote the manuscript; CHVNF, ALSG-D, GMMF, MMUC-N, CIC, JLEF, ECP and EMG-B critically read the manuscript, provided intellectual insights, and critically discussed the project. All authors read and approved the final version.

References

- Akilzhanova A, Nurkina Z, Momynaliev K, Ramanculov E, Zhumadilov Z, Rakhypbekov T, Hayashida N, Nakashima M and Takamura N (2013) Genetic profile and determinants of homocysteine levels in Kazakhstan patients with breast cancer. *Anticancer Res* 33:4049-59.
- Arthur RS, Wang T, Xue X, Kamensky V and Rohan TE (2020) Genetic factors, adherence to healthy lifestyle behavior, and risk of invasive breast cancer among women in the UK Biobank. *J Natl Cancer Inst* 112:893-901.

- Babyskhina N, Malinovskaya E, Nazarenko M, Koval M, Gervas P, Potapova O, Slonimskaya E and Cherdyntseva N (2013) The effect of folate-related SNPs on clinicopathological features, response to neoadjuvant treatment and survival in pre- and postmenopausal breast cancer patients. *Gene* 518:397-404.
- Barbosa RCC, Da Costa DM, Cordeiro DE, Vieira AP and Rabenhorst SH (2012) Interaction of MTHFR C677T and A1298C, and MTR A2756G gene polymorphisms in breast cancer risk in a population in Northeast Brazil. *Anticancer Res* 32:4805-11.
- Batschauer AP, Cruz NG, Oliveira VC, Coelho FF, Santos IR, Alves MT, Fernandes AP, Carvalho MG and Gomes KB (2011) HFE, MTHFR, and FGFR4 genes polymorphisms and breast cancer in Brazilian women. *Mol Cell Biochem* 357:247-53.
- Carpenter G and Cohen S (1990) Epidermal growth factor. *J Biol Chem* 265:7709-12.
- Carvalho Barbosa REC, Menezes DC, Freire TF, Sales DC, Alencar VH and Rabenhorst SH (2012) Associations of polymorphisms of folate cycle enzymes and risk of breast cancer in a Brazilian population are age dependent. *Mol Biol Rep* 39:4899-907.
- Chen X, Ahmada H, Zhang T, Bai Z and Wang C (2019) Association of folate intake and related gene polymorphisms with breast cancer. *J Nutr Sci Vitaminol (Tokyo)* 65:459-69.
- Ericson UC, Ivarsson MI, Sonested E, Gullberg B, Carlson J, Olsson H and Wirfält E (2009) Increased breast cancer risk at high plasma folate concentrations among women with the MTHFR 677T allele. *Am J Clin Nutr* 90:1380-9.
- Fernandes GM, Russo A, Proença MA, Gazola NF, Rodrigues GH, Biselli-Chicote M, Silva AE, Netinho JG, Pavarino É and Goloni-Bertollo EM (2016) CYP1A1, CYP2E1 and EPHX1 polymorphisms in sporadic colorectal neoplasms. *World J Gastroenterol* 22:9974-83.
- Fleming I, Cooper J, Hanson D (1997) AJCC Cancer Staging Manual. Lippincott Raven Publishers, Philadelphia, PA.
- Galbiatti AL, Ruiz MT, Maniglia JV, Raposo LS, Pavarino-Bertelli EC and Goloni-Bertollo EM (2012a) Head and neck cancer: genetic polymorphisms and folate metabolism. *Braz J Otorhinolaryngol* 78:132-9.
- Galbiatti AL, Da Silva LM, Ruiz-Cintra MT, Raposo LS, Maniglia JV, Pavarino EC and Goloni-Bertollo EM (2012b) Association between 11 genetic polymorphisms in folate-metabolising genes and head and neck cancer risk. *Eur J Cance*, 48:1525-31.
- Galbiatti AL, Ruiz MT, Biselli-Chicote PM, Chicote-Biselli PM, Raposo LS, Maniglia JV, Pavarino-Bertelli EC and Goloni-Bertollo EM (2010) 5-Methyltetrahydrofolate-homocysteine methyltransferase gene polymorphism (MTR) and risk of head and neck cancer. *Braz J Med Biol Res* 43:445-50.
- Gallo V, Egger M, McCormack V, Farmer PB, Ioannidis JP, Kirsch-Volders M, Matullo G, Phillips DH, Schoket B, Stromberg U *et al.* (2011) Strengthening the reporting of observational studies in epidemiology - molecular epidemiology (STROBE-ME): An extension of the STROBE statement. *Prev Med* 53:377-87.
- Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B, Senn HJ and Members P (2011) Strategies for subtypes--dealing with the diversity of breast cancer: Highlights of the St. Gallen international expert consensus on the primary therapy of early breast cancer (2011). *Ann Oncol* 22:1736-47.
- Gong Z, Yao S, Zirpoli G, David Cheng TY, Roberts M, Khoury T, Ciupak G, Davis W, Pawlish K, Jandorf L *et al.* (2015) Genetic variants in one-carbon metabolism genes and breast cancer risk in European American and African American women. *Int J Cancer* 137:666-77.
- He JM, Pu YD, Wu YJ, Qin R, Zhang QJ, Sun YS, Zheng WW and Chen LP (2014) Association between dietary intake of folate and MTHFR and MTR genotype with risk of breast cancer. *Genet Mol Res* 13:8925-31.
- Hiraoka M and Kagawa Y (2017) Genetic polymorphisms and folate status. *Congenit Anom (Kyoto)* 57:142-9.
- Huang Y, Han S, Li Y, Mao Y and Xie Y (2007) Different roles of MTHFR C677T and A1298C polymorphisms in colorectal adenoma and colorectal cancer: a meta-analysis. *J Hum Genet* 52:73-85.
- Hvidtfeldt UA, Tjønneland A, Keiding N, Lange T, Andersen I, Sørensen TI, Prescott E, Hansen Å, Grønbæk M, Bojesen SE *et al.* (2015) Risk of breast cancer in relation to combined effects of hormone therapy, body mass index, and alcohol use, by hormone-receptor status. *Epidemiology* 26:353-61.
- Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA) – Coordenação de Prevenção e Vigilância – INCA (2019). Estimativa 2020: incidência de câncer no Brasil. Ministério da Saúde, Rio de Janeiro. 177 pp.
- Jiang-Hua Q, De-Chuang J, Zhen-Duo L, Shu-De C and Zhenzhen L (2014) Association of methylenetetrahydrofolate reductase and methionine synthase polymorphisms with breast cancer risk and interaction with folate, vitamin B6, and vitamin B12 intakes. *Tumour Biol* 35:11895-901.
- Key T, Appleby P, Barnes I, Reeves G and Group ehabcc (2002) Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. *J Natl Cancer Inst* 94:606-16.
- Key TJ, Verkasalo PK and Banks E (2001) Epidemiology of breast cancer. *Lancet Oncol* 2:133-40.
- Kotsopoulos J, Zhang WW, Zhang S, McCready D, Trudeau M, Zhang P, Sun P and Narod SA (2008) Polymorphisms in folate metabolizing enzymes and transport proteins and the risk of breast cancer. *Breast Cancer Res Treat* 112:585-93.
- Lang JE, Mosalpuria K, Cristofanilli M, Krishnamurthy S, Reuben J, Singh B, Bedrosian I, Meric-Bernstam F and Lucci A (2009) HER2 status predicts the presence of circulating tumor cells in patients with operable breast cancer. *Breast Cancer Res Treat* 113:501-7.
- Lee YJ, Lim T, Han MS, Lee SH, Baek SH, Nan HY and Lee C (2017) Anticancer effect of luteolin is mediated by downregulation of TAM receptor tyrosine kinases, but not interleukin-8, in non-small cell lung cancer cells. *Oncol Rep* 37:1219-26.
- Li K, Li W and Dong X (2014) Association of 677 C>T (rs1801133) and 1298 A>C (rs1801131) polymorphisms in the MTHFR gene and breast cancer susceptibility: a meta-analysis based on 57 individual studies. *PLoS One* 9:e71290.
- Ma E, Iwasaki M, Junko I, Hamada GS, Nishimoto IN, Carvalho SM, Motola Jr J, Laginha FM and Tsugane S (2009). Dietary intake of folate, vitamin B6, and vitamin B12, genetic polymorphism of related enzymes, and risk of breast cancer: A case-control study in Brazilian women. *BMC Cancer* 9:122.
- Mentch SJ and Locasale JW (2016) One-carbon metabolism and epigenetics: understanding the specificity. *Ann N Y Acad Sci* 1363:91-8.
- Miller SA, Dykes DD and Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215.
- Naushad SM, Pavani A, Rupasree Y, Divyya S, Deepti S, Digumarti RR, Gottumukkala SR, Prayaga A and Kutala VK (2012) Association of aberrations in one-carbon metabolism with molecular phenotype and grade of breast cancer. *Mol Carcinog* 5:E32-41.
- Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA *et al.* (2000) Molecular portraits of human breast tumours. *Nature* 406:747-52.

- Sangrajrang S, Sato Y, Sakamoto H, Ohnami S, Khuhaprema T and Yoshida T (2010) Genetic polymorphisms in folate and alcohol metabolism and breast cancer risk: a case-control study in Thai women. *Breast Cancer Res Treat* 123:885-93.
- Scaglione F and Panzavolta G (2014) Folate, folic acid and 5-methyltetrahydrofolate are not the same thing. *Xenobiotica* 44:480-8.
- Sharp L, Little J, Schofield AC, Pavlidou E, Cotton SC, Miedzybrodzka Z, Baird Jo, Haites NE, Heys SD and Grubb DA (2002) Folate and breast cancer: the role of polymorphisms in methylenetetrahydrofolate reductase (MTHFR). *Cancer Lett* 181:65-71.
- Siegel RL, Miller KD, Fuchs HE and Jemal A (2021) *Cancer Statistics, 2021*. *CA Cancer J Clin* 71:7-33.
- Sobin LH, Gospodarowicz MK and Wittekind C (2010) *TNM Classification of Malignant Tumors*. 7th edition. Blackwell Publishing, Chichester, 310 pp.
- Song MA, Brasky TM, Marian C, Weng DY, Taslim C, Llanos AA, Dumitrescu RG, Liu Z, Mason JB, Spear SL *et al.* (2016) Genetic variation in one-carbon metabolism in relation to genome-wide DNA methylation in breast tissue from healthy women. *Carcinogenesis* 37:471-80.
- Suzuki T, Matsuo K, Hirose K, Hiraki A, Kawase T, Watanabe M, Yamashita T, Iwata H and Tajima K (2008) One-carbon metabolism-related gene polymorphisms and risk of breast cancer. *Carcinogenesis* 29:356-62.
- Varela-Rey M, Woodhoo A, Martinez-Chantar ML, Mato JM and Lu SC (2013) Alcohol, DNA methylation, and cancer. *Alcohol Res* 35:25-35.
- Weiner AS, Boyarskikh UA, Voronina EN, Selezneva IA, Sinkina TV, Lazarev AF, Petrova VD and Filipenko ML (2012) Polymorphisms in the folate-metabolizing genes MTR, MTRR, and CBS and breast cancer risk. *Cancer Epidemiol* 36:e95-e100.
- Weisberg I, Tran P, Christensen B, Sibani S and Rozen R (1998). A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol Genet Metab* 64:169-72.
- Wittke-Thompson JK, Pluzhnikov A and Cox NJ (2005) Rational inferences about departures from Hardy-Weinberg equilibrium. *Am J Hum Genet* 76:967-86.
- Zara-Lopes T, Gimenez-Martins AP, Nascimento-Filho CH, Castanhole-Nunes MM, Galbiatti-Dias AL, Padovani-Júnior JA, Maniglia JV, Francisco JL, Pavarino EC and Goloni-Bertollo EM (2016) Role of MTHFR C677T and MTR A2756G polymorphisms in thyroid and breast cancer development. *Genet Mol Res* 15.

Associate Editor: Jorge Lopez-Camelo

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License (type CC-BY), which permits unrestricted use, distribution and reproduction in any medium, provided the original article is properly cited.