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Associations of polymorphisms of folate cycle enzymes and risk of breast cancer in a Brazilian population are age dependent

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Abstract Polymorphisms in genes involved in folate metabolism have been shown to be implicated in breast cancer risk but with contradictory results. In this case–control study, we investigated the association between *MTHFR* C677T and A1298C, TYMS 5′-UTR, *MTR* A2756G and c*SHMT* C1420T and also the folate carrier (*RFC1* G80A) and breast cancer risk in a northeastern Brazilian population. The study included 183 women diagnosed with breast cancer and 183 controls volunteers without any history of cancer. Also a significant number of healthy individuals were included for allelic frequency in the population studied. Risk of breast cancer was estimated by conditional logistic regression. An association with risk was found for women carrying the *MTR* A2756G polymorphic allele (AG, $P = 0.0036$; AG/GG, $P = 0.0040$), and a protective effect in carriers of the *RFC1* G80A polymorphic allele (GA, $P = 0.0015$; AA, $P = 0.0042$). Stratifying the data by age (cutoff point of 50 years old), different distributions were observed for breast cancer risk. For women ≤50 years, the risk observed in the presence of the polymorphic allele *MTR* 2756 (AG/GG) in the general analysis was, restricted to this age group ($P = 0.0118$). Conversely, for women over 50, the risk of breast cancer development was statistically associated with the *MTHFR* 677CT genotype, but especially significant was risk associated with the presence of the polymorphic allele of *cSHMT* C1420T ($P = 0.0120$) and the protective effect associated with the *RFC1* G80A polymorphism allele ($P = 0.0021$), was restrict to this age group. These data indicate that the cutoff age used (50 years old) was appropriate, since it was able to discriminate risk in each age group in the population studied and also to point to the importance of age in the analyses of cancer-associated polymorphisms.

Keywords Breast cancer · Folate metabolism · Single nucleotide polymorphism · Age

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Introduction

Breast cancer is the leading cause of cancer mortality in women worldwide [1, 2]. The etiology of the disease is poorly understood, some risk factors include familial history of the disease, age of menarche and of menopause, diet, reproductive history, high estrogen exposure as well as genetic factors [3, 4]. Studies suggest that the effect determined by low-penetrance genes, may provide a plausible explanation for breast cancer susceptibility. Sequence variants or polymorphisms are associated with a risk of or protection against the disease, especially the polymorphisms in genes encoding enzymes involved in folic acid metabolism [5, 6].

Folate metabolism imbalances have been implicated in predisposition to various kind of cancer [7] because it may influence DNA stability as a one-carbon donor through two different pathways: DNA methylation and nucleotide synthesis. Those crucial roles in the DNA metabolism involve several enzymes in the folate biological network, such as Methylenetetrahydrofolate reductase (*MTHFR*),

which catalyzes the critical reduction of 5,10 methylene-tetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-methylTHF). Nevertheless, 5,10-MTHF is also used in the conversion of dUMP to dTMP by *TYMS*, and the product, 5-methylTHF, is the methyl donor for remethylation of homocysteine to methionine, which is mediated by methionine synthase (*MTR*) [8]. Methionine is the precursor of *S*-adenosylmethionine (SAM), the universal methyl donor for several biological methylation reactions including DNA methylation [9, 10]. The concentration of 5,10-MTHF also depends on the enzyme serine hydroxymethyltransferase (*SHMT*), specifically cytosolic *SHMT* (*cSHMT*), which is responsible for the reversible conversion of serine and tetrahydrofolate to glycine and 5,10-MTHF, thus playing an important role in providing carbon units for the synthesis of purine, thymidylate and methionine [11]. In addition to the above enzymes, the protein reduced folate carrier 1 (*RFC1*) plays an essential role in folate metabolism, being responsible for the entrance of reduced folate into the cell [12].

Although several studies have been published related to breast cancer and polymorphisms of the folate cycle enzymes, the majority are restricted to *MTHFR* polymorphisms, but with controversial results [13–16]. Some studies found the variant *MTHFR* C677T (rs1801133) genotype associated with an increase in breast cancer risk [15, 17, 18], while no significant association was found in others [16, 19]. On the other hand, the *MTHFR* 1298C allele of *MTHFR* A1298C (rs1801131) was associated with increased risk in the studies of Ergul et al. [17] and Stevens et al. [20], while reduced risk of breast cancer for the polymorphic allele (AC+CC) was found by Chou et al. [21]. The same conflicting results are found in the literature for the *MTR* A2756G (rs1805087) polymorphism; risk was found in some studies [22, 23], but others did not show any association [15, 24, 25]. In relation to *SHMT*, only the cytosolic form is related to cancer risk, but few studies have associated polymorphisms with predisposition to breast cancer. Cheng et al. [26], showed that the C1420T polymorphism *cSHMT* (rs 1979277) have an independent role in association with reduced risk of breast cancer. However, Bentley et al. [27] do not support any association between the genotype *cSHMT* C1420T and breast cancer risk. Regarding *TYMS*, the most studied polymorphism in this enzyme is the 28-bp tandem repeat sequence, identified in the 5′promoter region (5′-UTR). However, no significant association with breast cancer risk has been reported. Besides its importance, only two studies were conducted considering the *RFC1* (SLC19A1) G80A (rs1051266) polymorphism and breast cancer risk [25, 28], also with contradictory results.

Conflicting results are also observed related to the analysis of some of these polymorphism and menopause status. For example, considering the *MTHFR* C677T, the *MTHFR* 677 T allele was found to be associated with premenopausal risk by Campbell et al. [29], Ergul et al. [17] and Semenza et al. [30]. In contrast, Suzuki et al. [31], Ericson et al. [32] and Maruti et al. [33] found risk for postmenopausal women, while Justenhoven et al. [16] and Platek et al. [19] did not find any significant associations. The importance of the menopause state is related to the reduction in hormone levels; however, the hormonal changes happen even before this event. Therefore, menopause itself is not an accurate marker. On the other hand, age is a well-accepted risk factor for cancer including breast cancer.

From this standpoint, we were prompted to investigate breast cancer risk and six common polymorphisms related to the four major enzymes of folate metabolism (*MTHFR*, *TYMS*, *MTR* and *cSHMT*) and also the folate carrier protein (*RFC1*) in a population in Ceará state (northeastern Brazil). Also, we investigated our data stratifying it by age using as a cut-off the average age in which occurs the menopause (50 years old). To our knowledge, this is the first study to evaluate this set of polymorphisms with regard to the risk of breast cancer in the same population.

Materials and methods

Subjects

This case–control study consisted of 183 women with a diagnosis of breast cancer, aged 31–79 years, who were recruited from the Integrated Regional Oncology Center Clinic (CRIO), taking as criteria for inclusion age older than 18 years with a pathological diagnosis of breast carcinoma stage I, II, III or IV, had no previous breast cancer or metastasized cancer originating from other organs and who were not previously exposed to chemo- and/or radiotherapy.

The controls were composed by 183 healthy volunteers recruited from blood bank donors and volunteers belonging to the staff of the university, without any history of cancer. All subjects were genetically unrelated, from the same geographical region (Ceará State, Northeast Brazil) and they were matched by age (±2 years) for the conditional logistic regression analysis. Additionally, a higher number of healthy individual than the one used on the case control study analysis was recruited to enable us to verify the polymorphism frequencies of all genes in the studied population.

All participants signed an informed consent and the study was approved by the Ethics Committee of the Hospital Complex of the Federal University of Ceará under the protocol No. 702/04, according to the Resolution 306/04 of the National Council of Health, Ministry of Health/Brazil.

At the time, informed consent was obtained from all participants, and information about demographic characteristics and family history of breast cancer was provided by a trained interviewer through a questionnaire. 4 ml of blood were harvested for analysis.

Genotype analysis

Genomic DNA was extracted, soon after blood sampling, using a salting out method [34]. DNA quality was determined by 1% agarose gel electrophoresis and the amount was determined using the NanoDropTM® 3300 fluorospectrometer (Wilmington, DE, USA). The *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, c*SHMT* C1420T and *RFC*1 G80A polymorphisms were determined by PCR–RFLP and the *TYMS* 5′-UTR (28 bp) was detected by simple PCR. The reaction conditions were carried out according to the references cited in Table 1.

All PCR products, except those of *TYMS* analysis, were verified by 1% agarose gel electrophoresis with ethidium bromide staining. The digested fragments and the *TYMS* PCR products were visualized in 6% polyacrylamide gels with silver stained, in order to better determine the fragment size. Quality control samples were included in all laboratory analyses. Random samples (10% of cases and control samples) were reanalyzed for control of the laboratory procedures with unknown identification by the laboratory staff. The concordance of the analysis was 99.5% for all polymorphisms. To the discordant samples, the genotype assay was repeated by two independent researchers to achieve 100% of concordance.

Table 1 PCR and restriction protocols

Polymorphism	Product (bp)	Restriction enzyme	Patterns	Protocol references
MTHFR C677T	198	*Hin*fI	C allele (198 bp)	[35]
			T allele (175 and 23 bp)	
MTHFR A1298C	237	*Mbo*II	A allele (210 and 27 bp)	[36]
			C allele (182, 28 and 27 bp)	
TYMS 5′-UTR	–	None	2R (220 bp)	[37, 38]
			3R (248 bp)	
MTR A2756G	211	*Hae*III	A allele (211 bp)	[39]
			G allele (131 and 80 bp)	
SHMT C1420T	217	*Ear*I	C allele (131 and 86 bp)	[40]
			T allele (217 bp)	
*RFC*1 G80A	231	*Hha*I	A allele (162 and 68 bp)	[41]
			G allele (125, 37 and 68 bp)	

Statistical analysis

Conditional logistic regression analysis was done for all the cases and the age-matched control population, being calculated the odds ratio (OR) in a 95% confidence interval (CI), in order to investigate the association between the studied polymorphisms and breast cancer risk. A '*p*' value less than 0.05 was taken to be significant. All statistical tests were performed with software Epi Info, v. 3.5.1. To detect signs of genotyping error or confounding factor due to population admixtures, the deviation from Hardy–Weinberg equilibrium, among the controls, was tested with Pearson's test.

Results

The mean age of the patients included in this study was 51.7 (median of 51 years, ranging from 31 to 79) and 41.9 for the population study group (median of 42 years, ranging from 16 to 80 years). Most of the patients were postmenopausal (54.9%, median age of 58 years) and the majority of the cases were diagnosed with invasive ductal carcinoma in stage III (Table 2).

Table 3 shows the genotype distribution of *MTR, MTHFR,* c*SHMT, RFC*1 and *TYMS* polymorphisms for the population studied, patients and matched controls, also the distribution considering their odds ratios (OR) for breast cancer risk. The population genotype analysis from same enzymes was different due to difficulties in amplifying some samples. No statically difference was found between both healthy populations groups. Genotype distributions in control groups for all polymorphisms were in accordance with Hardy–Weinberg equilibrium. A very low frequency of the homozygous polymorphic genotype for methionine synthase (*MTR* 2756GG) was found for both patient and control groups, although the polymorphic allele was present in heterozygosis at a substantial frequency. On the other hand, the genotype frequencies of *MTHFR* 1298CC, c*SHMT* 1420TT and *MTR* 2756AG were higher in the patient group rather than in the control subjects.

Breast cancer risk (Table 3) was found only in women carrying the polymorphic allele *MTR* 2756G (AG or AG+GG). The lack of association with the homozygous polymorphic genotype (GG) may be explained by the presence of only one case carrying this genotype. A strong protective effect was found associated with the presence of

Table 2 Histopathological classification and clinical staging of patients with breast cancer

Histopathological classification	No. of patients (%)	Stage I	Stage II	Stage III	Stage IV
Invasive ductal carcinoma	164 (89.6)	8 (4.9)	58 (35.4)	84 (51.2)	14 (8.5)
Invasive lobular carcinoma	8 (4.4)	0	3 (37.5)	5 (62.5)	0
Mucinous carcinoma	2 (1.1)	0	1 (50)	1 (50)	0
Apocrine carcinoma	3 (1.6)	0	0	3 (100)	0
Papillary carcinoma	1 (0.6)	0	1 (100)	0	0
Medullary carcinoma	5 (2.7)	0	3 (60)	1 (20)	1 (20)
Total (%)	183 (100)	8 (4.4)	66 (36.1)	94 (51.3)	15 (8.2)

Table 3 Genotype frequencies of MTR A2756G, MTHFR A1298C, MTHFR C677T, cSHMT C1420T, RFC1 G80A, TYMS 5′-UTR and risk of breast cancer in all individuals

Genotype	Total population (%)	No. of patients (%)	No. of controls (%)	OR (95% CI)	*P*-value
TYMS 5′-UTR(28 bp), $N = 510$					
2R/2R[a]	92 [18]	30 [17]	29 (16.5)	1.00	–
2R/3R	272 (53.4)	97 (55.1)	96 (54.5)	0.98 (0.56–1.81)	0.9374
3R/3R	146 (28.6)	49 (27.8)	51 (29.0)	0.94 (0.51–1.72)	0.8345
2R/3R+3R/3R	418 (82)	146 (82.9)	147 (83.5)	0.96 (0.56–1.65)	0.8908
MTHFR C677T, $N = 514$					
CC[a]	251 (48.8)	76 (43.2)	87 (49.4)	1.00	–
CT	224 (43.6)	83 (42.7)	70 (39.8)	1.39 (0.87–2.20)	0.1652
TT	39 (7.6)	17 (9.7)	19 (10.8)	1.06 (0.51–2.17)	0.8806
CT+TT	263 (51.2)	100 (52.4)	89 (50.6)	1.31 (0.85–2.04)	0.2231
MTHFR A1298C, $N = 490$					
AA[a]	224 (45.7)	68 (41.2)	72 (43.6)	1.00	–
AC	241 (49.2)	80 (48.5)	84 (50.9)	1.01 (0.67–1.52)	0.9491
CC	25 (5.1)	17 (10.3)	09 (5.5)	1.90 (0.82–4.42)	0.1338
AC+CC	266 (54.3)	97 (58.8)	93 (56.4)	1.08 (0.73–1.61)	0.6863
MTR A2756G, $N = 491$					
AA[a]	323 (65.8)	82 (47.1)	109 (62.6)	1.00	–
AG	166 (33.8)	91 (52.3)	64 (36.8)	1.96 (1.25–3.10)	0.0036*
GG	2 (0.4)	01 (0.6)	01 (0.6)	1.00 (0.06– 15.99)	1.0000
AG+GG	168 (34.2)	92 (52.9)	65 (37.4)	1.93 (1.23–3.02)	0.0040*
cSHMT C1420T, $N = 256$					
CC[a]	86 (33.6)	32 (26.7%)	48 (40.0%)	1.00	–
CT	136 (53.1)	62 (51.7%)	56 (46.7%)	1.39 (0.87–2.20)	0.1652
TT	34 (13.3)	26 (21.7%)	16 (13.3%)	1.06 (0.51–2.17)	0.8806
CT+TT	170 (66.4)	88 (73.4%)	72 (60%)	1.31 (0.85–2.04)	0.2231
RFC1 G80A, $N = 426$					
GG[a]	112 (26.3)	58 (37.2%)	30 (19.2%)	1.00	–
GA	225 (52.8)	71 (45.5%)	89 (57.1%)	0.39 (0.22–0.70)	0.0015*
AA	89 (20.9)	27 (17.3%)	37 (23.7%)	0.35 (0.17–0.72)	0.0042*
GA+AA	314 (73.7)	98 (62.8%)	126 (80.8%)	0.38 (0.22–0.66)	0.0006*

* $P < 0.05$

[a] Reference genotype

*RFC*1 G80A polymorphism. No statistical significance was found for the *MTHFR* C677T, *MTHFR* A1298C, c*SHMT* C1420T and *TYMS* 5′-UTR polymorphisms and breast cancer risk.

Stratifying the analysis by age (50 years old as cutoff point), different distributions were observed, and the susceptibility for breast cancer showed a different pattern of risk according to age.

Table 4 Genotype frequencies of *MTR* A2756G, *MTHFR* A1298C, *MTHFR* C677T, *cSHMT* C1420T, *RFC1* G80A, *TYMS* 5′-UTR and risk of breast cancer in patients aged ≤50 years old

Genotype	No. of patients (%)	No. of controls (%)	OR (95% CI)	*P*-value
TYMS 5′-UTR(28 bp)				
2R/2R[a]	17 (19.8%)	17 (19.3%)	1.00	–
2R/3R	51 (59.3%)	46 (52.3%)	1.05 (0.50–2.20)	0.8998
3R/3R	18 (20.9%)	25 (28.4%)	0.74 (0.32–1.73)	0.4864
2R/3R+3R/3R	69 (80.2%)	71 (80.7%)	0.94 (0.46–1.90)	0.8575
MTHFR C677T				
CC[a]	40 (46.5%)	38 (43.2%)	1.00	–
CT	34 (39.5%)	42 (47.7%)	0.67 (0.33–1.37)	0.2740
TT	12 (14.0%)	08 (9.1%)	1.26 (0.39–3.26)	0.6324
CT+TT	46 (53.5%)	50 (56.8%)	0.81 (0.43–1.53)	0.5172
MTHFR A1298C				
AA[a]	37 (47.4%)	30 (37.5%)	1.00	–
AC	30 (38.5%)	47 (58.8%)	0.53 (0.28–1.02)	0.0577
CC	11 (14.1%)	03 (3.8%)	4.05 (0.87–18.93)	0.0754
AC+CC	41 (52.6%)	50 (62.6%)	0.69 (0.38–1.26)	0.2304
MTR A2756G				
AA[a]	41 (48.2%)	59 (67.8%)	1.00	–
AG	44 (51.8%)	28 (32.2%)	2.31 (1.20–4.42)	0.0118*
GG	00 (0.0%)	00 (0.0%)	n.d.	n.d.
AG+GG	44 (51.8%)	28 (32.2%)	2.31 (1.20–4.42)	0.0118*
cSHMT C1420T				
CC[a]	16 (29.1%)	17 (29.8%)	1.00	–
CT	26 (47.3%)	30 (52.6%)	0.93 (0.36–2.40)	0.8786
TT	13 (23.6%)	10 (17.5%)	1.30 (0.40–4.21)	0.6651
CT+TT	39 (70.9%)	40 (70.1%)	1.00 (0.40–2.52)	1.0000
RFC1 G80A				
GG[a]	27 (36.5%)	18 (23.7%)	1.00	–
GA	32 (43.2%)	42 (55.3%)	0.45 (0.19–1.03)	0.0602
AA	15 (20.3%)	16 (21.1%)	0.54 (0.20–1.47)	0.2293
GA+AA	47 (63.5%)	58 (76.4%)	0.47 (0.21–1.05)	0.0648

* $P < 0.05$

[a] Reference genotype

For women aged 50 years or under (Table 4), a risk was associated with the presence of the polymorphic allele *MTR* 2756G ($P = 0.0118$) and a tendency for the protective effect of the heterozygous genotype of *MTHFR* A1298C was observed ($P = 0.0577$). Conversely, for woman aged above 50 (Table 5), the risk of breast cancer development was statistically associated with the *MTHFR* C677T heterozygous genotype, being also associated when considering the allele T carries (CT+TT). Also, a risk associated with the presence of the c*SHMT* polymorphic 1420T allele was especially significant. On the other hand, a protective effect associated with *RFC*1 G80A polymorphism was found.

Considering others epidemiological data related to breast cancer we found among the patients that, women who had menarche at age ≥14 were correlated to the genotype 2R/3R+3R/3R of TYMS and GA+AA of *RFC*. The presence of these *RFC* genotypes (GA+AA) was also associated with patients who were smokers. On the other hand, the *RFC* genotype GG were correlated with patients who had children at age ≥30 and had family history. Additionally, was analyzed the correlation considering the tumors stage regarding to Invasive Ductal Carcinoma due to small number of the others histological classification, but no significant results were found.

Discussion

Although the polymorphisms in *MTHFR* and *MTR* genes are the most studied, the results are still conflicting. One important parameter considered in some studies is the menopause status, however the hormonal changes, and consequently the metabolism that are dependent of them, already happen in the perimenopause, a period of changing ovarian function, and precedes the final menses by between 2 and 8 years [42]. This fact can be corroborated by

Table 5 Genotype frequencies of *MTR* A2756G, *MTHFR* A1298C, *MTHFR* C677T, *cSHMT* C1420T, *RFC1* G80A, TYMS 5′-UTR and risk of breast cancer in patients aged >50 years old

Genotype	No. of patients (%)	No. of controls (%)	OR (95% CI)	*P*-value
TYMS 5′-UTR (28 bp)				
2R/2R[a]	13 (14.4%)	12 (13.6%)	1.00	–
2R/3R	46 (51.1%)	50 (56.8%)	0.86 (0.35–2.08)	0.7332
3R/3R	31 (34.4%)	26 (29.5%)	1.00 (0.39–2.54)	1.0000
2R/3R+3R/3R	77 (85.5%)	76 (86.3%)	0.91 (0.39–2.14)	0.8273
MTHFR C677T				
CC[a]	36 (40.0%)	49 (55.7%)	1.00	–
CT	49 (54.4%)	28 (31.8%)	2.36 (1.20–4.64)	0.0127*
TT	05 (5.6%)	11 (12.5%)	0.67 (0.19–2.32)	0.5287
CT+TT	54 (60%)	39 (44.3%)	2.00 (1.05–3.80)	0.0342*
MTHFR A1298C				
AA[a]	31 (35.6%)	50 (57.5%)	1.00	–
AC	50 (57.5%)	36 (43.5%)	1.55 (0.88–2.72)	0.1261
CC	06 (6.9%)	01 (7.1%)	1.29 (0.40–4.42)	0.6717
AC+CC	56 (64.4%)	37 (50.6%)	1.52 (0.88–2.64)	0.1337
MTR A2756G				
AA[a]	41 (46.1%)	50 (57.5%)	1.00	–
AG	47 (52.8%)	36 (41.4%)	1.67 (0.88–3.16)	0.1178
GG	01 (1.1%)	01 (1.1%)	1.00(0.06–15.99)	1.0000
AG+GG	48 (53.9%)	37 (42.5%)	1.62 (0.87–3.03)	0.1265
cSHMT C1420T				
CC[a]	16 (24.6%)	31 (49.2%)	1.00	–
CT	36 (55.4%)	26 (41.3%)	2.26 (1.10–4.63)	0.0266*
TT	13 (20.0%)	06 (9.5%)	4.12 (1.16–14.66)	0.0286*
CT+TT	49 (75.4%)	32 (50.8%)	2.45 (1.22–4.95)	0.0120*
RFC1 G80A				
GG[a]	31 (37.8%)	12 (15.0%)	1.00	–
GA	39 (47.6%)	47 (58.8%)	0.30 (0.13–0.71)	0.0062*
AA	12 (14.6%)	21 (26.2%)	0.20 (0.06–0.59)	0.0040*
GA+AA	51 (62.2%)	68 (85%)	0.27 (0.12–0.62)	0.0021*

* $P < 0.05$

[a] Reference genotype

contradictory findings between some studies that show risk of breast cancer being related to menopausal status. From this standpoint, and also considering that age is a well-accepted risk factor for cancer, including breast cancer, the data in this study were also stratified by age using the average age cohort that occurred at menopause (≤50 years and >50 years) as done by some other authors [25, 43].

First, considering the analysis without a cutoff, no statistically significant association was found related to the *MTHFR* C677T, *MTHFR* A1298C and *TYMS* 5′-UTR polymorphisms. Table 6 lists the studies of breast cancer and polymorphisms of folate metabolism genes, indicating the concordant and discordance to our data. Studies in which dietary folate intake was considered was not compared.

Unlike the *MTHFR* C677T and *MTHFR* A1298C and c*SHMT* C1420T polymorphism, the lack of association of *TYMS* 5′-UTR polymorphism is in accordance with all studies, independently on the studied population.

The most interesting finding in this analysis was the associated risk found in the presence of the polymorphic allele *MTR* 2756G, contrasting with the protection observed for the *RFC*1 polymorphic allele 80A. In our study, a very low frequency of the *MTR* A2756G homozygous polymorphic genotype (GG) was observed which can explain the lack of association between homozygous polymorphism and risk. The frequency of *MTR* 2756GG in the literature varies from 2 to 5.4%; furthermore, two studies from southeastern Brazil [23, 51] found a frequency of 3.2 and 3.7% for this polymorphism in the control population. The difference observed between the frequency in our study (0.3%) for a Northeastern population and the two other Southern Brazilian studies frequencies reflects the differences in the ethnic background, lifestyle and

Table 6 Association of previous studies with our present, six of polymorphisms of genes involved in folate transport or metabolism and the risk of breast cancer

Polymorphism	Results	Analyzed population	References
MTHFR C677T	No association[b]	Brazil, China, Korea, Australia	[23, 43, 44, 45[a], 46]
	Protection (TT genotype)	Taiwan	[21]
	Risk (TT genotype)	Turkey, USA	[15, 18, 47[a]]
MTHFR A1298C	No association[b]	Canada	[25]
	Protection (CC genotype)	Taiwan	[21]
TYMS 5′-UTR	No association[b]	China, Australia, Spain	[43, 46, 48]
c*SHMT* C1420T	No association[b]	USA, Taiwan	[22, 27]
	Protection (TT genotype)	Taiwan, Indian	[26, 49]
RFC1 G80A	No association	Canada	[25]
	Risk (AA genotype)[b]	Indian	[49]
MTR A2756G	No association	Taiwan	[22, 50[a]]

[a] Meta-analysis

[b] Similar results to this study

environmental factors of these regions. Brazilian population is heterogeneous, formatted by European, African, Asian, as well Indian groups, moreover, with differences in its composition among regions of the country. Ceará is a Northeast state and has an evident Indian component in its population formation, and different from the Southern state, the African black was almost absent and the Asian was not present. Europeans, besides Portuguese, were the Dutch settlers, while in the south, predominantly Germans and Italians [52]. In a recent meta-analysis by Qi et al. [53] an increased risk was found in an East Asian population but not in a Caucasian population, pointing to the importance of ethnic background for some polymorphisms.

Discordances' data was also found in the literature regarding the *MTR A*2756G however, a meta-analysis by Lu et al. [50] found some evidences of an association between the *MTR* A2756G polymorphism and breast cancer risk among Europeans, but not among Asians. No consensus was found in the few paper was found related to the *RFC*1 *G*80A polymorphism and breast cancer.

When the data was stratified by age (cutoff of 50 years old), most polymorphisms showed differences in the risk association pattern. In women aged ≤50 years, the breast cancer risk associated to the presence of the *MTR* 2756G allele in the overall analysis was, in fact, restricted to that group of age, and only a trend of a protective effect was observed, regarding to *MTHFR* 1298AC genotype. In this age group, conversely, Kotsopoulos et al. [25], in this age group, found no association of breast cancer risk related to *MTHFR* A1298C or *MTR* A2756G polymorphisms. Moreover, in women aged >50 years old, a risk association was found with the presence of the polymorphic alleles of *MTHFR C*677T and c*SHMT* C1420T. The protection association observed with the *RFC*1 80A allele in the general sample was found only in this group of age.

The association of breast cancer risk and the *MTHFR* 677T allele and c*SHMT* C1420T in women aged >50 years old, is in contrast with Kotsopoulos et al. [25]. As long as most studies did not stratify the data by age, we assumed that the cutoff of 50 years old should be comparable to a distinguishable point between pre- and postmenopausal status. So that, the association of the *MTHFR* 677CT genotype to breast cancer risk is in accordance to some previous studies, such as Suzuki et al. [31], Ericson et al. [32] and Maruti et al. [33] who found an increased risk of 34% in breast cancer among the postmenopausal Swedish when carrying the CT and TT genotypes.

The importance of each polymorphism for the redirection and compensation of the metabolic pathways involved in the folate cycle is not explained. There is only speculation considering the peculiarity of each one. Both *MTHFR*C677T and *cSHMT* C1420T polymorphism have been associated with decreased folate levels in plasma and in red blood cells [40, 44]. Therefore, it is speculated that a decrease in the enzyme activity could mimic a folate deficiency by lowering one-carbon availability for remethylation of homocysteine to methionine and DNA synthesis (repair) and methylation.

Considering the *RFC*1 G80A polymorphism, it has been described as having no impact on plasma homocysteine level, but it appears to increase the folate level of the extracellular environment [54]. Polymorphism in the *RFC1* gene seems to have an impact on the absorption and cellular translocation of dietary folate [55]. The protection associated with *RFC*1 G80A polymorphism may not be entirely unexpected, as long as it has been suggested that folate is able to prevent tumor development before the establishment of pre-neoplastic lesions; however, once the lesions are established, this nutrient might promotes tumorigenesis [28, 56, 57]. Therefore, pre-lesions in patients who have cell folate intake deficiency would have less chances of disease progression. Most of the patients included in this study were in advanced stages (III, IV) which could contribute to this result. Also, the protection was associated to the women aged >50 years, that who already have metabolic changes compared with younger women. The complexity of the role of *RFC* in folate metabolism can be observed by the statistical associations found with other factors linked to breast cancer, in which, the presence of the polymorphic allele of this enzyme was

significantly correlated with patients who had menarche age ≥14, and also patients smokers. Inversely, the wild type genotype was correlated with patients who had children age ≥30 and had family history. Together, our data points to the fine regulation existing in the folate balance at the intracellular level and points to differences in this metabolism according to age.

In conclusion, although we found protective effect and risk association for breast cancer involved with folate metabolism in the general analysis, these effects were better differentiated when we stratified our data by age. In women ≤50 years old, of risk association was found with the *MTR* 2756G allele and a tendency *MTHFR* 1298AC genotype, while in women >50 years old, breast cancer risk was associated with the presence of the polymorphic alleles of *MTHFR* C677T and c*SHMT* C1420T. Protection effect was associated with the *RFC*1 80A allele and it was restricted to women >50 years old. The cutoff age (50 years old) used was shown to be appropriate as long as it was able to discriminate breast cancer risk or protection in each group of age in the studied population with a high statistical significance. This was probably due to the fact that the age used as cutoff joined two parameters: the genetic susceptibility considering cancer arising in early age and the methylation pattern which changes with age, being, indeed, relevant to cancer.

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References

1. Parkin DM (2001) Global cancer statistics in the year 2000. Lancet Oncol 2:533–543
2. Ferlay J, Bray F, Pisani P, Parkin DM (2004) Cancer incidence, mortality and prevalence worldwide IARC Cancer Base No 5, version 2.0
3. Hankinson SE, Colditz GA, Willett WC (2004) Towards an integrated mod breast cancer etiology. The lifelong interplay of genes, lifestyle, and hormones. Breast Cancer Res 6:213–218
4. Dumitrescu RG, Cotarla I (2005) Understanding breast cancer risk—where do we stand in 2005. J Cell Mol Med 9:208–221
5. Weber BL, Nathanson KL (2000) Low penetrance genes associated with increased risk for breast cancer. Eur J Cancer 36:1193–1199
6. Ponder BA (2001) Cancer genetics. Nature 411:336–341
7. Forsti A, Angelini S, Festa F et al (2004) Single nucleotide polymorphisms in breast cancer. Oncol Rep 11:917–922
8. Födinger M, Hörl WH, Sunder-Plassmann G (2000) Molecular biology of 5,10-methylenetetrahydrofolate reductase. Nephrol 13:20–33
9. Choi SW, Mason JB (2000) Folate and carcinogenesis: an integrated scheme. J Nutr 130:129–132
10. Stern LL, Bagley PJ, Rosenberg IH, Selhub J (2000) Conversion of 5-formyltetrahydrofolic acid to 5-methyltetrahydrofolic acid is unimpaired in folate-adequate persons homozygous for the C677T mutation in the methylenetetrahydrofolate reductase gene. J Nutr 130:2238–2242
11. Skibola CF, Smith MT, Hubbard A, Shane B, Roberts AC, Law GR, Rollinson S, Roman E, Cartwright RA, Morgan GJ (2002) Polymorphisms in the thymidylate synthase and serine hydroxymethyltransferase genes and risk of adult acute lymphocytic leukemia. Blood 99:3786–3791
12. Dervieux T, Furst D, Lein DO et al (2004) Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum 50:2766–2774
13. Luo L, Callaway EM, Svoboda K (2008) Genetic dissection of neural circuits. Neuron 57:634–660
14. Süzen HS, Yüce N, Güvenç G, Duydu Y, Erke T (2005) TYMS and DPYD polymorphisms in a Turkish population. Eur J Clin Pharmacol 61:881–885
15. Deligezer U, Akisik EE, Dalay N (2005) Homozygosity at the C677T of the MTHFR gene is associated with increased breast cancer risk in the Turkish population. In Vivo 19:889–893
16. Justenhoven C, Hamann U, Pierl CB, Rabstein S, Pesch B, Harth V, Baisch C, Vollmert C, Illig T, Brüning T, Ko Y, Brauch H (2005) One-carbon metabolism and breast cancer risk: no association of MTHFR, MTR, and TYMS polymorphisms in the GENICA study from Germany. Cancer Epidemiol Biomark Prev 14:3015–3018
17. Ergul E, Sazci A, Utkan Z, Canturk NZ (2003) Polymorphisms in the MTHFR gene are associated with breast cancer. Tumour Biol 24:286–290
18. Chen J, Gammon MD, Chan W, Palomeque C, Wetmur JG, Kabat GC, Teitelbaum SL, Britton JA, Terry MB, Neugut AI, Santella RM (2005) One-carbon metabolism, MTHFR polymorphisms, and risk of breast cancer. Cancer Res 65:1606–1614
19. Platek ME, Shields PG, Marian C, McCann SE, Bonner MR, Nie J, Ambrosone CB, Millen AE, Ochs-Balcom HM, Quick SK et al (2009) Alcohol consumption and genetic variation in Methylenetetrahydrofolate reductase and 5-methyltetrahydrofolate homocysteine methyltransferase in relation to breast cancer risk. Cancer Epidemiol Biomark Prev 18:2453–2459
20. Stevens VL, McCullough ML, Pavluck AL, Talbot JT, Feigelson HS, Thun MJ, Calle EE (2007) Association of polymorphisms in one-carbon metabolism genes and postmenopausal breast cancer incidence. Cancer Epidemiol Biomark Prev 16:1140–1147
21. Chou YC, Wu MH, Yu JC, Lee MS, Yang T, Shih HL, Wu TY, Sun CA (2006) Genetic polymorphisms of the methylenetetrahydrofolate reductase gene, plasma folate levels and breast cancer susceptibility: a case–control study in Taiwan. Carcinogenesis 27:2295–2300
22. Yu CP, Wu MH, Chou YC, Yang T, You SL, Chen CJ, Sun CA (2007) Breast cancer risk associated with multigenotypic polymorphisms in folate-metabolizing genes: a nested case–control study in Taiwan. Anticancer Res 27:1727–1732
23. Ma E, Iwasaki M, Junko I, Hamada GS, Nishimoto IN, Carvalho SM, Motola J Jr, Laginha FM, 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
24. Shrubsole MJ, Gao YT, Cai QY, Shu XO, Dai Q, Jin F, Zheng W (2006) MTR and MTRR polymorphisms, dietary intake, and breast cancer risk. Cancer Epidemiol Biomark Prev 15:586–588
25. Kotsopoulos J, Zhang WW, Zhang S, McCready D, Trudeau M, Zhang P, Sun P, Narod SA (2008) Polymorphisms in folate metabolizing enzymes and transport proteins and the risk of breast cancer. Breast Cancer Res Treat 112:585–593
26. Cheng CW, Yu JC, Huang CS, Shieh JC, Fu YP, Wang HW, Wu PE, Shen CY (2008) Polymorphism of cytosolic serine

hydroxymethyltransferase, estrogen and breast cancer risk among Chinese women in Taiwan. Breast Cancer Res Treat 111:145–155
27. Bentley AR, Raiszadeh F, Stover PJ, Hunter DJ, Hankinson SE, Cassano PA (2010) No association between cSHMT genotypes and the risk of breast cancer in the Nurses' Health Study. Short communication. Eur J Clin Nutr 64:108–110
28. Xu X, Gammon MD, Wetmur JG, Bradshaw PT, Teitelbaum SL, Neugut AI, Santella RM, Chen J (2008) B-vitamin intake, one-carbon metabolism, and survival in a population-based study of women with breast cancer. Cancer Epidemiol Biomark Prev 17:2109–2116
29. Campbell IG, Baxter SW, Eccles DM, Choong DYH (2002) Methylenetetrahydrofolate reductase polymorphism and susceptibility to breast cancer. Breast Cancer Res 4:1–4
30. Semenza JC, Delfino RJ, Ziogas A, Anton-Culver H (2003) Breast cancer risk and methylenetetrahydrofolate reductase polymorphism. Breast Cancer Res Treat 77:217–223
31. Suzuki T, Matsuo K, Hirose K, Hiraki A, Kawase T, Watanabe M, Yamashita T, Iwata H, Tajima K (2008) One-carbon metabolism-related gene polymorphisms and risk of breast cancer. Carcinogenesis 29:356–362
32. Ericson U, Sonestedt E, Ivarsson MI, Gullberg B, Carlson J, Olsson H, Wirfalt E (2009) Folate intake, methylenetetrahydrofolate reductase polymorphisms, and breast cancer risk in women from the Malmo diet and cancer cohort. Cancer Epidemiol Biomark Prev 18:1101–1110
33. Maruti SS, Ulrich CM, Jupe ER et al (2009) MTHFR C677T and postmenopausal breast cancer risk by intakes of one-carbon metabolism nutrients: a nested case–control study. Breast Cancer Res 11:R91
34. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215
35. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH, den Heijer M, Kluijtmans LAJ, van den Heuve LP, Rozen R (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10:111–113
36. Cicek M, Nock N, Li L, Conti D, Casey G, Witte J (2004) Relationship between methylenetetrahydrofolate reductase C677T and A1298C genotypes and haplotypes and prostate cancer risk and aggressiveness. Cancer Epidemiol Biomark Prevent 13:1331–1336
37. Etienne MC, Chazal M, Laurent-Puig P et al (2002) Prognostic value of tumoral thymidylate synthase and p53 in metastatic colorectal cancer patients receiving fluorouracil-based chemotherapy: phenotypic and genotypic analyses. J Clin Oncol 20:2832–2843
38. Etienne MC, Ilc K, Formento JL, Laurent-Puig P, Formento P, Cheradame S, Fischel JL, Milano G (2004) Thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphisms: relationships with 5-fluorouracil sensitivity. Br J Cancer 90: 526–534
39. Matsuo K, Suzuki R, Hamajima N, Ogura M, Kagami Y, Taji H, Kondoh E, Maeda S, Asakura S, Kaba S, Nakamura S, Seto M, Morishima Y, Tajima K (2001) Association between polymorphisms of folate and methionine metabolizing enzymes and susceptibility to malignant lymphoma. Blood 15:3205–3209
40. Heil SG et al (2001) Is mutated serine hydroxymethyltransferase (SHMT) involved in the etiology of neural tube defects? Mol Genet Metab 73:164–172
41. Winkelmayer WC, Eberle C, Sunder-Plassmann G, Fodinger M (2003) Effects of the glutamate carboxypeptidase II (GCP2 1561CNT) and reduced folate carrier (RFC1 80GA) allelic variants on folate and total homocysteine levels in kidney transplant patients. Kidney Int 63:2280–2285
42. Greendale GA, Lee NP, Arriola ER (1999) The menopause. Lancet 353:571–580
43. Zhai X et al (2006) Polymorphisms in thymidylate synthase gene and susceptibility to breast cancer in a Chinese population: a case–control analysis. BMC Cancer 6:138
44. Shrubsole MJ, Gao YT, Cai Q, Shu XO, Dai Q, Hébert JR, Jin F, Zheng W (2004) *MTHFR* polymorphisms, dietary folate intake, and breast cancer risk: results from the Shanghai Breast Cancer Study. Cancer Epidemiol Biomark Prev 13:190–196
45. Lewis SJ, Harbord RM, Harris R, Smith GD (2006) Meta-analyses of observational and genetic association studies of folate intakes or levels and breast cancer risk. J Natl Cancer Inst 98: 1607–1622
46. Grieu F, Powell B, Beilby J, Iacopetta B (2004) Methylenetetrahydrofolate reductase and thymidylate synthase polymorphisms are not associated with breast cancer risk or phenotype. Anticancer Res 24:3215–3220
47. Macis D, Maisonneuve P, Johansson H, Bonanni B, Botteri E, Iodice S, Santillo B, Penco S, Gucciardo G, D'Aiuto G, Rosselli Del Turco M, Amadori M, Costa A, Decensi A (2007) Methylenetetrahydrofolate reductase (MTHFR) and breast cancer risk: a nested-casecontrol study and a pooled meta-analysis. Breast Cancer Res Treat 106:263–271
48. Henriquez-Hernández LA, Murias-Rosales A, Hernandez Gonzalez A, Cabrera De Leon A, Diaz-Chico BN, Mori De Santiago M, Fernandez Perez L (2009) Gene polymorphisms in TYMS, MTHFR, p53 and MDR1 as risk factors for breast cancer: a case-control study. Oncol Rep 22:1425–1433
49. Mohammad NS, Yedluri R, Addepalli P, Gottumukkala SR, Digumarti RR, Kutala VK (2011) Aberrations in one-carbon metabolism induce oxidative DNA damage in sporadic breast cancer. Mol Cell Biochem 349:159–167
50. Lu M, Wang F, Qiu J (2010) Methionine synthase A2756G polymorphism and breast cancer risk: a meta-analysis involving 18,953 subjects. Breast Cancer Res Treat 123:213–217
51. Lima CS, Ortega MM, Ozelo MC, Araujo RC, De Souza CA, Lorand-Metze I, Annichino-Bizzacchi JM, Costa FF (2008) Polymorphisms of methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR), and thymidylate synthase (TYMS) in multiple myeloma risk. Leuk Res 32:401–405
52. Arruda VR, Grignolli CE, Goncalves MS, Soares MC, Menezes R, Saad STO, Costa FF (1998) Prevalence of homozygosity for the deleted alleles of glutathione S-transferase mu (GSTMl) and theta (GSTTl) among distinct ethnic groups from Brazil: relevance to environmental carcinogenesis? Clin Genet 54:210–214
53. Qi X, Ma X, Yang X, Fan L, Zhang Y, Zhang F, Chen L, Zhou Y, Jiang J (2010) Methylenetetrahydrofolate reductase polymorphisms and breast cancer risk: a meta-analysis from 41 studies with 16,480 cases and 22,388 controls. Breast Cancer Res Treat 123:499–506
54. Yates Z, Lucock M (2005) G80A reduced folate carrier SNP modulates cellular uptake of folate and affords protection against thrombosis via a non homocysteine related mechanism. Life Sci 77:2735–2742
55. Chango A, Emery-Fillon N, de Courcy GP, Lambert D, Pfister M, Rosenblatt DS, Nicolas JP (2000) A polymorphism (80G-A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia. Mol Genet Metab 70:310–315
56. Kim YI (2006) Does a high folate intake increase the risk of breast cancer? Nutr Rev 64:468–475
57. Lin J, Lee IM, Cook NR, Selhub J, Manson JE, Buring JE, Zhang SM (2008) Plasma folate, vitamin B-6, vitamin B-12, and risk of breast cancer in women. Am J Clin Nutr 87:734–743