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ANA PAULA D'ALARME GIMENEZ MARTINS

MARCADORES MOLECULARES ENVOLVIDOS NO METABOLISMO DO FOLATO EM PACIENTES COM CÂNCER DE MAMA

São José do Rio Preto

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ANA PAULA D'ALARME GIMENEZ MARTINS

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Dissertação apresentada à Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Mestre no curso de Pós-graduação do Programa de Enfermagem

Orientadora: Profª Drª Eny Maria Goloni Bertollo

Co-Orientadora: Profaª Drª Márcia Maria Urbanin Castanhole-Nunes

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ANA PAULA D'ALARME GIMENEZ MARTINS

MARCADORES MOLECULARES ENVOLVIDOS NO METABOLISMO DO FOLATO EM PACIENTES COM CÂNCER DE MAMA

BANCA EXAMINADORA

DISSERTAÇÃO PARA OBTENÇÃO DE TÍTULO DE MESTRE

Presidente e Orientadora

Profª Drª Eny Maria Goloni Bertollo

Examinadores

Profª Drª Ana Lívia Silva Galbiatti

Profª Drª Edenir Inêz Palmero

Suplentes

Profª Drª Lídia Maria Rebolho Batista Arantes

Profº Dr° Rodrigo Castro

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SUMÁRIO

DEDICATÓRIA

Aos meus pais Mauro e Selma

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"Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível".

(Charles Chaplin)

LISTA DE FIGURAS

INTRODUÇÃO

LISTA DE TABELAS

INTRODUÇÃO

ARTIGO CIENTÍFICO I

ARTIGO CIENTÍFICO II

LISTA DE ABREVIATURAS E SÍMBOLOS

5,10- MTHF – 5,10- metilenotetrahidrofolato *(5,10- metylenotetrahydrofolate)*

5- MTHF- 5- metiltetrahidrofolato (5- *metyletrahydrofolato)*

bp- pares de base

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CH_3- Grupo Metil

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico

CONEP- Conselho Nacional de Saúde

DNA – Ácido Desoxirribonucleico *(deoxyribonucleic acid)*

DHFR- Dihidrofolato redutase *(Dihydrofolate reductase)*

DHF – Dihidrofolato (*Dihydrofolate)*

EDTA - *Ethylenediamine tetraacetic acid*

ER- *estrogen receptor*

FAMERP – Faculdade de Medicina de São José do Rio Preto

FAPESP – Fundação de Pesquisa do Estado de São Paulo

FUNFARME – Fundação da Faculdade de Medicina de São José do Rio Preto

HER2- Receptor tipo 2 do fator de crescimento Epidérmico Humano *(Human Epidermal growth factor Receptor-type 2)*

HWE- *Hardy-Weinberg Equilibrium*

Hcy - Homocisteína *(Homocysteine)*

IC – Intervalo de Confiança (*Confidence interval)*

IMC- Índice de Massa Corpórea *(Body-mass index)*

M- metástase

mL- mililitro

***MTHFR* -** metilenotetrahidrofolato redutase *(methylenotetrahydrofolate reductase)*

***MTR* -** metionina sintase *(methionine syntase)*

***MTRR* -** metionina sintase redutase *(methionine syntase reductase)*

N- envolvimento de linfonodo

OR – Odds ratio

PCR-RFLP*- Polymerase Chain Reaction- Restriction Fragment Length Polymorphism*

PR- Progesterone Receptor

RP – Receptor de Progesterona

RE- Receptor de Estrogênio

SNPs – Polimorfismo de Nucleotídeo Simples *(single nucleotide polymorphism)*

SHMT - Serina Hidroximetiltransferase *(serine-hydroxymetyltransferase)*

SAM - S-Adenosilmetionina *(S-adenosylmethionine)*

SAH - S-Adenosilhomocisteína *(S-Adenosylhomocysteine)*

T- Tamanho do tumor

THF - Tetrahidrofolato *(Tetrahydrofolate)*

UICC- União Internacional Contra o Câncer *(Union for International Cancer Control)*

UPGEM- Unidade de Pesquisa em Genética e Biologia Molecular

RESUMO

Introdução: O câncer de mama é o segundo tipo de câncer mais frequente no mundo, sendo o mais comum entre as mulheres. Esta doença é multifatorial envolvendo o estilo de vida, fatores hormonais, ambientais e genéticos. O metabolismo do folato pode estar associada ao desenvolvimento do câncer de mama, já que o folato desempenha papel crucial na síntese, regulação e metilação do DNA. Polimorfismos nos genes participantes desse metabolismo, como *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G e *MTRR* A66G modificam a eficiência das enzimas, causando alterações anormais na expressão gênica, inativação dos genes supressores de tumor e ativação da oncogênese. **Objetivos:** Investigar a frequência dos polimorfismos nos genes *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G (rs1805087) e *MTRR* A66G (rs1801394) em pacientes com câncer de mama, comparando-a com aquela observada em indivíduos sem história de neoplasia; avaliar a associação entre os polimorfismos e os fatores de risco (idade, tabagismo, consumo de álcool, número de gestações, IMC e terapia hormonal) e as características clinico-patológicas (classificação TNM e fenotípica) no desenvolvimento do câncer de mama. **Métodos:** O presente estudo caso-controle envolveu 606 mulheres, sendo 128 no grupo caso e 478 no grupo controle. Para a análise molecular, o DNA genômico foi extraído a partir de sangue periférico. A técnica de Reação em Cadeia da Polimerase e digestão enzimática (PCR-RFLP) foi utilizada na genotipagem dos polimorfismos nos genes *MTHFR* e *MTR* e a técnica de PCR em tempo real para o polimorfismo no gene *MTRR*. Os dados clínico-histopatológicos foram obtidos por meio de prontuário médico. Para a análise estatística foram utilizados os programas MINITAB 14.0 (Regressão Logística Múltipla) e SNPstats (modelos de herança e Equilíbrio de Hardy-Weinberg). **Resultados:** Mulheres com 50 anos ou mais (OR: 2,65; 95% IC: 1,65-4,26; p<0,001) e que ingerem bebida alcoólica (OR: 1,76; 95% IC: 1,09-2,85; p=0,021) possuem risco aumentado para desenvolver câncer de mama. O hábito tabagista (OR: 1,07; 95% IC: 0,65-1,79; p=0,782), número de gestações (OR: 0,86 95% IC: 0,54-1,38; p=0,536), índice de massa corpórea (OR: 1,24 95% IC: 0,75-2,06; p=0,405) e terapia hormonal (OR: 1,41 95% IC: 0,86-2,33; p=0,174) não foram associados com o risco de câncer de mama. Quanto ao polimorfismo *MTHFR* A1298C (rs1801131), foi observado uma redução no risco de desenvolver a doença no modelo codominante (genótipo CC - OR: 0,22; 95% IC: 0,06-0,74; p=0,01), modelo recessivo (OR: 0,22; 95% IC: 0,07-0,76; p=0,004) e modelo log-aditivo (OR: 0,70; 95% IC: 0,49-0,98; p=0,03), enquanto que os polimorfismos *MTHFR* C677T (rs1801133), *MTR* A2756G (rs1805087) e *MTRR* A66G (rs1801394) não foram associados ao risco de câncer de mama. Com relação aos parâmetros clínicos-patológicos, não foi encontrado associação entre os polimorfismos estudados e o os tumores de mama. **Conclusões:** Mulheres com 50 anos e que ingerem bebida alcoólica possuem risco aumentado para desenvolver câncer de mama. O polimorfismo *MTHFR* A1298C está associado com a diminuição do risco no câncer de mama. Este é o primeiro estudo de associação entre perfil genotípico

desses polimorfismos e clínico-patológico das mulheres brasileiras do noroeste do estado de São Paulo, Brasil.

Palavras chave: Neoplasia de mama; polimorfismo genético; ácido fólico; fatores de risco.

ABSTRACT

Introduction: Breast cancer is the second most common cancer in the world, being the most common among women. This disease is multifactorial involving lifestyle, hormonal, environmental and genetic factors. The folate metabolism may be associated with the development of breast cancer, since folate plays a crucial role in the synthesis, regulation and DNA methylation. Polymorphisms in genes involved in metabolism, such as *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G modify the efficiency of the enzymes, causing abnormal changes in gene expression, inactivation of tumor suppressor genes and activation of oncogenesis. **Objectives:** To investigate the frequency of polymorphisms in *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G (rs1805087) and *MTRR* A66G (rs1801394) genes in patients with breast cancer comparing to individuals with no history neoplasia; to evaluate the association between polymorphisms and risk factors (age, smoking habits, alcohol consumption, number gestations, body mass index and hormone therapy) and the clinical histopathological parameters (tumor size, node involvement, metastasis and cancer subtypes) of breast cancer. **Materials and methods:** The present case-control study involved 606 Brazilian women, 128 case group and 478 control group. For molecular analysis, genomic DNA was extracted from peripheral blood leukocytes. Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) was used in the genotyping of polymorphisms in the genes *MTHFR* and *MTR* and PCR real time for polymorphsm in gene *MTRR*. The clinical and pathological data were obtained from medical records. For statistical analysis, program used were MINITAB 14.0 (multiple logistic regression), and SNPstats (inheritance models and Hardy-Weinberg Equilibrium) program. **Results:** Women aged 50 and over (OR: 2.65; 95% IC: 1.65-4.26; p<0.001) and alcohol consumption (OR: 1.76; 95% IC: 1.09-2.85; p=0.021) are associated increased risk for breast cancer. Smoking habits (OR:1.07; 95%CI:0.65-1.79; p=0.782), number of pregnancies (OR:0.86; 95%CI:0.54-1.38; p=0.536), BMI ≥25 Kg/m^2 (OR:1.24; 95%CI:0.75-2.06; p=0.405), and hormone therapy (OR: 1.41; 95%CI:0.86-2.33; p=0.174) are not associated with the risk of breast cancer. For *MTHFR* A1298C (rs1801131), we observed reduced risk of developing disease in 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), however no significant associations was found between *MTHFR* C677T (rs1801133), *MTR* A2756G (rs1805087), and *MTRR* A66G (rs1801394) polymorphisms and breast cancer risk. In relation to clinical histopathological parameters, we not found significant association between polymorphisms studies and breast tumors. **Conclusions:** Women aged 50 and over and who drink alcohol have a higher risk of developing breast cancer. The MTHFR A1298C polymorphism was associated with decreased risk in breast cancer. This is the first study the association between the genotypic these polymorphisms, and clinical histopathological parameters involving women population from the Northwest region of Sao Paulo State, Brazil.

Keywords: Breast Neoplasms; Polymorphism, Genetic; Folic Acid; Risk Factors.

RESUMEN

Introducción: El cáncer de mama es el segundo tipo de cáncer más frecuente en el mundo, siendo el más común entre las mujeres. Esta enfermedad es multifactorial incluyendo el estilo de vida, factores hormonales, ambientales y genéticos. El metabolismo del folato puede estar asociada al desarrollo del cáncer de mama, ya que el folato desarrolla papel crucial en la síntesis, regulación y metilación del DNA. Polimorfismos en los genes participantes de ese metabolismo, como *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G y *MTRR* A66G modifican la eficiencia de las enzimas, causando alteraciones anormales en la expresión génica, inactivación de los genes supresores de tumor y activación de la oncogénesis. **Objetivos:** Investigar la frecuencia de los polimorfismos en los genes *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G (rs1805087) y *MTRR* A66G (rs1801394) en pacientes con cáncer de mama, comparándola con la observada en individuos sin historia de neoplasia; evaluar la asociación entre los polimorfismos y los factores de riesgo (edad, tabaquismo, consumo de alcohol, número de gestaciones, IMC y terapia hormonal) y las características clínico-patológicas (clasificación TNM y fenotípica) en el desarrollo del cáncer de mama. **Materiales y Métodos:** El presente estudio caso-control ha incluido a 606 mujeres, siendo 128 en el grupo caso y 478 en el grupo control. Para el análisis molecular, el DNA genómico ha sido extraído a partir de sangre periférica. La técnica de Reacción en Cadena de la Polimerasa y digestión enzimática (PCR-RFLP) ha sido utilizada en la genotipificación de los polimorfismos en los genes *MTHFR* y *MTR* y la técnica de PCR en tiempo real para el polimorfismo en el gen *MTRR*. Los datos clínico-histopatológicos han sido obtenidos por medio de prontuario médico. Para el análisis estadístico han sido utilizados los programas MINITAB 14.0 (Regresión Logística Múltiple) y SNPstats (modelos de herencia y Equilibrio de Hardy-Weinberg). **Resultados:** Mujeres con 50 años o más (OR:2.65; 95%CI:1.65-4.26; p<0.001) y que ingieren bebida alcohólica (OR:1.76; 95%CI:1.09-2.85; p=0.021) poseen riesgo aumentado para desarrollar cáncer de mama. El consumo de tabaco (OR:1.07; 95%CI:0.65-1.79; p=0.782), número de embarazos (OR:0.86; 95%CI:0.54-1.38; p=0.536), IMC ≥25 Kg/m^2 (OR:1.24; 95%CI:0.75-2.06; p=0.405), y la terapia hormonal (OR: 1.41; 95%CI:0.86-2.33; p=0.174) no han sido asociados al riesgo de cáncer de mama. En cuanto al polimorfismo *MTHFR* A1298C (rs1801131), se ha observado una reducción en el riesgo de desarrollar la enfermedad en modelo codominante (genotype CC – OR:0.22; 95%CI:0.06-0.74; p=0.014), modelo recesivo (OR:0.22; 95%CI:0.07-0.76; p=0.004) y modelo log-additivo (OR:0.70; 95%CI:0.49-0.98; p=0.035), mientras que para los polimorfismos *MTHFR* C677T (rs1801133), *MTR* A2756G (rs1805087) y *MTRR* A66G (rs1801394) no han sido asociados al riesgo de cáncer de mama. Respecto a los parámetros clínicos-patológicos, no se ha encontrado asociación entre los polimorfismos estudiados y los tumores de mama. **Conclusiones:** Mujeres con 50 años y que ingieren bebida alcohólica poseen mayor riesgo de desarrollar cáncer de mama. El polimorfismo *MTHFR* A1298C ha sido asociado con la disminución del riesgo en el cáncer de mama. Este es

el primer estudio de asociación entre el perfil genotípico estos polimorfismos y clínico-patológico de las mujeres brasileñas del noroeste del Estado de São Paulo, Brasil.

Palabras-clave: Neoplasias de la Mama; Polimorfismo Genético; Ácido Fólico; Factores de Riesgo.

1. INTRODUÇÃO

1. INTRODUÇÃO

O câncer de mama é o segundo tipo de câncer mais frequente no mundo, sendo o mais comum entre as mulheres, [1,2] representando 25% do total de casos de câncer no mundo em 2012. [1] No Brasil, excluindo os tumores de pele não melanoma, o câncer de mama é o mais incidente em mulheres de todas as regiões, com exceção da região Norte, onde predomina o câncer de colo de útero. Para 2014, foi estimado 57.120 casos novos, representando uma taxa de incidência de 56,1 casos por 100 mil mulheres. [1]

O diagnóstico, em países em desenvolvimento, ainda é tardio, contribuindo com o aumento da taxa de mortalidade. Em países desenvolvidos, a sobrevida em 5 anos é de 85%, já para os países em desenvolvimento é de 50% a 60%. [1,3]

O câncer de mama também é uma doença multifatorial que envolve o estilo de vida, fatores hormonais, ambientais e genéticos. Os principais fatores de risco da doença estão associados ao gênero feminino, idade avançada, aspectos endócrinos, baixa paridade, idade tardia da primeira gestação (>30 anos), curto período de lactação, idade precoce da menarca (<12 anos), idade tardia da menopausa (>55 anos), alto Índice de Massa Corpórea (IMC), consumo de álcool e ingestão inadequada de vitaminas do complexo B. [4-9]

O câncer de mama é uma doença complexa, de alta heterogeneidade clínica, morfológica e biológica. [10,11] Tumores mamários inicia-se no tecido mamário que é composto por lóbulos (glândulas de leite) e ductos (que conectam os lóbulos ao mamilo), o restante da mama é formado por tecido adiposo, conectivo e linfático. [1,3,11,12] O câncer de mama é classificado

utilizando-se de fatores prognósticos tradicionais como tipo e grau histológico, tamanho do tumor (T), presença de linfonodos regionais (N), metástase a distância (M) e classificação fenotípica. [1,10,13]

Os carcinomas mamários são classificados histologicamente em: carcinoma *in situ* (tumor localizado nos ductos ou nos lóbulos, sem invasão de tecidos vizinhos) e carcinoma invasivo ou infiltrante (inicia-se nos ductos ou nos lóbulos, invadindo o tecido mamário vizinho). [3,12] Já a classificação TNM leva em consideração a extensão anatômica da doença que são: tamanho do tumor, presença/ausência de linfonodos regionais e metástase à distância. Essa classificação é realizada de acordo com a União Internacional Contra o Câncer (UICC). A tabela 1 mostra como essa classificação é aplicada aos tumores de mama [13].

Tumores mamários com histologia e clínica semelhantes podem apresentar diferentes prognósticos e respostas terapêuticas. [1,10,14] A classificação prognóstica atual considera, além das classificações histológicas e TNM, os subtipos moleculares do carcinoma de mama que podem exigir terapêutica específica. [10,14,15] Basicamente, essa classificação leva em conta a presença/ausência de receptores hormonais (estrogênio e progesterona), amplificação e/ou superexpressão do receptor tipo 2 do fator de crescimento epidérmico humano (HER2) e índice de proliferação celular (Ki-67). Essa classificação fenotípica consiste em: [10,14,16]

- Luminal A: tumores positivos para receptor de estrogênio (RE) e/ou receptor de progesterona (RP), negativos para amplificação e/ou superexpressão de HER2 e Ki-67 <14%. [10,14,16]

- Luminal B: tumores positivos para RE e/ou RP, positivo para HER2 ou quando HER2 negativo ter Ki-67 >14%. [10,14,16]
- Superexpressão de HER2: tumores negativos para RE e RP e positivo para HER2. [10,14,16]
- Triplo Negativo: tumores negativos para RE, RP e HER2. [10,14,16]

1.1. Câncer de mama e o metabolismo do folato

O metabolismo do folato desempenha papel crucial na síntese, regulação e metilação do DNA. [7,17-20] A metilação do DNA é a transferência de grupos metil (CH_3) por meio de reações catalisadas por proteínas denominadas DNA metiltransferases. Os níveis de folato podem ser alterados, não só pela ingestão desse nutriente, mas também por meio de polimorfismos genéticos que codificam enzimas responsáveis pela metabolização desse nutriente intracelular. [20-22]

O metabolismo do folato se inicia com a conversão de ácido fólico sintético, presente nos alimentos e suplementos vitamínico, em dihidrofolato (DHF) através da enzima dihidrofolato redutase (DHFR). Essa enzima também catalisa a conversão de DHF em tetrahidrofolato (THF), que é a forma metabolicamente ativa do folato. [21,25] A enzima serina-hidroximetiltransferase (SHMT) catalisa a reação reversível de THF em 5,10-metilenotetrahidrofolato (5,10-MTHF), que participa das reações de síntese de DNA. A enzima Metilenotetrahidrofolato redutase (MTHFR) catalisa a conversão do 5,10-MTHF para 5-metiltetrahidrofolato (5-MTHF), a principal forma circulante de folato, que atua como doador de grupos metil (CH3) para a remetilação da homocisteína

(Hcy) para metionina. [2,8,18,26,27] Esta reação de remetilação é catalisada pela enzima Metionina sintase (MTR), que requer a vitamina B_{12} como cofator, e resulta na formação de SAM (S-adenosilmetionina), que participa das reações de metilação do DNA. A enzima metionina sintase redutase (MTRR) é responsável pela manutenção do estado ativo da enzima MTR. Por uma reação de desmetilação, forma-se a S-adenosil-homocisteína (SAH) a partir da SAM, com posterior hidrólise para liberar adenosina e Hcy completando, assim, o ciclo do folato. [2,19,21,26,27] A Figura 1 mostra o ciclo do metabolismo do folato.

Há três mecanismos pelos quais as alterações no metabolismo do folato podem favorecer a carcinogênese: (i) hipometilação de DNA, subsequente ativação dos proto-oncogenes e indução da transformação maligna; (ii) erro de incorporação da uracila durante a síntese de DNA que leva a um descontrole no ciclo de reparo, causando quebras frequentes na molécula de DNA e danos cromossômicos, aumentando o risco de adquirir câncer; (iii) aumento na desaminação de citosina nos sítios de metilação do DNA. [21,23,24]

Polimorfismos nos genes participantes do metabolismo do folato podem levar a uma diminuição da eficiência das enzimas, causando alterações anormais na expressão gênica, principalmente, a inativação dos genes supressores de tumor e a ativação da oncogênese, assim como erros na divisão cromossômica durante a divisão celular. [5,23,28] A importância do gene *metilenotetrahidrofolato redutase* (*MTHFR)* na suscetibilidade ao câncer deve-se à presença dos polimorfismos C677T e A1298C do gene (o primeiro ocorre uma toca de uma citosina por uma timina na posição 677 do nucleotídeo; e o segundo ocorre uma troca de adenina por citosina no nucleotídeo 1298), que

pode estar associado à diminuição da atividade enzimática em câncer de mama. [24,29,30,31]

Estudos mostram que indivíduos com combinação heterozigota *MTHFR* C677T [8,9,18,30,32] possuem risco aumentado para desenvolver câncer de mama, assim como o polimorfismo *MTHFR* A1298C, [31,33,34] uma vez que se instala uma instabilidade genômica que pode ser crítica para transformação oncogênica de células humanas, favorecendo uma possível ligação entre a enzima MTHFR e a carcinogênese,[24,31] associada à baixa ingestão de folato.[7,17]

Os genes *MTR e MTRR* desenvolvem um papel importante na regulação da reação de metilação da Hcy. A enzima MTRR é responsável pelo estado ativo da enzima MTR, [26,35] caso ocorra a inativação da enzima MTR, a remetilação da Hcy em metionina será prejudicada, tendo como consequência a diminuição da síntese de SAM prejudicando diretamente na metilação do DNA. [23,35] Um polimorfismo comum no gene *MTR* é o A2756G, onde ocorre a substituição de adenina por guanina no nucleotídeo 2756. [24,28] Estudos relatam que essa variante leva à diminuição da formação se SAM devido ao aumento dos níveis plasmáticos de Hcy, consequentemente ocorre a hipometilação do DNA, [17,23,24] favorecendo a carcinogênese. Em relação ao câncer de mama os estudos são controversos, uma vez que o polimorfismo *MTR* A2756G foi associado ao risco aumentado de desenvolver câncer de mama nos estudos de Ma et al.,[17] Weiwei et al.[18] e Naushad et al. [15], enquanto que nos estudos de Weiner et al., [23] Kakkoura et al. [35] e He et al.[7] essa associação não foi encontrada.

O polimorfismo A66G no gene *MTRR* implica na substituição de adenina para guanina no nucleotídeo 66 tem sido investigado como fator de para o desenvolvimento de câncer de mama. [20,23,25] Esse gene codifica a enzima MTRR, então, o polimorfismo *MTRR* A66G gera baixa afinidade pela enzima MTR que diminui sua atividade. [24,36] Estudos mostram que a variante *MTRR* A66G está associada ao aumento do risco de desenvolver câncer de mama.[25,37] Porém, outros estudos não encontraram essa associação. [6,23]

A identificação de associação dos polimorfismos *MTHFR* C677T, *MTHFR* A1298C, *MTRR* A66G e *MTR* A2756G associados ao metabolismo do folato e fatores clínicos-patológicos em mulheres com câncer de mama, auxilia no entendimento dos mecanismos envolvidos no processo neoplásico, além de colaborar na etiologia, prevenção, prognóstico e tratamento da doença.

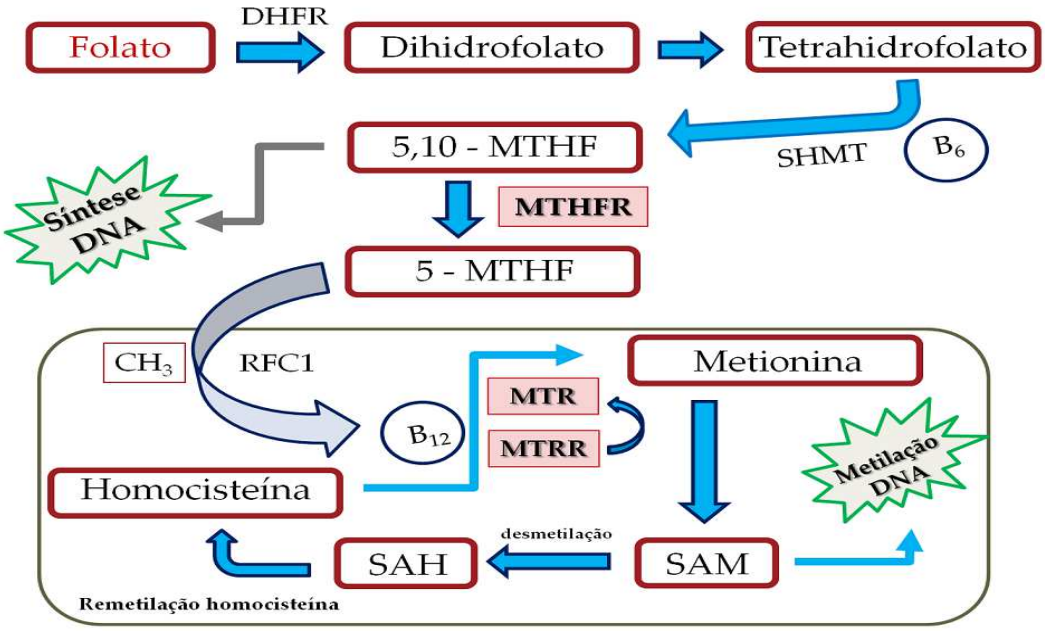

Figura 1. Esquema representado o metabolismo do folato. DHFR: Dihidrofolato redutase; 5,10–MTHFR: 5,10-metilenotetrahidrofolato; MTHFR: metilenotetrahidrofolato redutase; 5-MTHF: -metiltetrahidrofolato; CH_3: grupo metil; RFC1: Carreador de folato reduzido; MTR: metionina sintase; MTRR: metionina sintase redutase; SAM: S-adenosilmetionina; SAH: S-adenosilhomocisteína.

TABELA 1 – Classificação TNM dos tumores de mama de acordo com a UICC.[13]

Classificação	Característica
Tx	Tumor não pode ser avaliado
T0	Não há evidência de tumor primário
T*is*	Carcinoma *in situ*
T1	≤ 2 cm na maior dimensão
T1mic	Microinvasão ≤ 0,1 cm na maior dimensão
T1a	> 0,1 cm até 0,5 cm em sua maior dimensão
T1b	> 0,5 cm até 1 cm em sua maior dimensão
T1c	>1 cm até 2 cm em sua maior dimensão
T2	> 2 cm até 5 cm em sua maior dimensão
T3	> 5 cm em sua maior dimensão
T4	Qualquer tamanho com extensão direta à parede torácica ou à pele
T4a	Extensão à parede torácica
T4b	Edema ou ulceração da pele da mama, ou nódulos cutâneos satélites confinados à mesma mama
T4c	Ambos T4a e T4b
T4d	Carcinoma inflamatório
Nx	Linfonodos regionais não podem ser avaliados
N0	Ausência de metástase em linfonodos regionais
N1	Metástase em linfonodo(s) axilar(es), homolateral(ais), móvel(eis)
N2	Metástase em linfonodo(s) axilar(es) homolateral(is) fixo(s) ou metástase clinicamente aparente
N2a	Metástase em linfonodo(s) axilar(es) fixos
N2b	Metástase clinicamente aparente
N3	Metástase em linfonodo(s) infraclavicular(es) homolateral(ais) ou clinicamente aparente em linfonodo(s) mamário(s) interno(s) homolateral(is) e axilares
N3a	Metástase em linfonodo(s) infraclavicular(es)
N3b	Metástase em linfonodo(s) mamário(s) interno(s) e axilares
N3c	Metástase em linfonodo(s) supraclavicular(es)
Mx	Metástase à distância não pode ser avaliada
M0	Ausência de metástase à distância
M1	Metástase à distância

T – tamanho do tumor; N – linfonodos regionais; M – metástase

1.2. Objetivos

1- Investigar a frequência dos polimorfismos nos genes *MTHFR* (C677T e A1298C), *MTR* A2756G e *MTRR* A66G em pacientes com câncer de mama, comparando-a com aquela observada em indivíduos sem história de neoplasia.

2- Avaliar a associação entre os polimorfismos e os fatores de risco (idade, tabagismo, consumo de álcool, número de gestações, IMC e terapia hormonal) no desenvolvimento do câncer de mama.

3- Verificar a associação entre os polimorfismos e as características clinico-patológicas (classificação TNM e fenotípica) no desenvolvimento da doença.

2. ARTIGOS CIENTÍFICOS

2. ARTIGOS CIENTÍFICOS

Os resultados dessa dissertação estão apresentados em forma de artigos científicos. No total são 2 artigos que estão submetidos a revistas internacionais.

Artigo 1

Título: Role of *MTHFR* C677T and *MTR* A2756G polymorphisms in thyroid and breast cancer development

Autores: Tairine Zara-Lopes*, Ana Paula D'Alarme Gimenez-Martins*, Carlos Henrique Viesi Nascimento-Filho; Márcia Maria Urbanin Castanhole-Nunes, Ana Lívia Silva Galbiatti, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo.

*Os dois autores tiveram a mesma colaboração na produção do artigo.

Periódico: Cancer Science; Online ISSN: 1349-7006.

Artigo 2

Título: Breast cancer: polymorphisms in folate metabolism and clinical histopathological features in case-control study

Autores: Ana Paula D'Alarme Gimenez-Martins, Márcia Maria Urbanin Castanhole-Nunes, Carlos Henrique Viesi Nascimento-Filho, Stéphanie Piacenti dos Santos, Ana Lívia Silva Galbiatti, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo.

Periódico: Molecular Carcinogenesis; Online ISSN: 1098-2744

2.1. ARTIGO CIENTÍFICO I

COMPROVANTE DE SUBMISSÃO

Cancer Science

Submission Confirmation

Thank you for submitting your manuscript to *Cancer Science*.

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Authors:	Zara-Lopes, Tairine Gimenez-Martins, Ana Paula Nascimento-Filho, Carlos Henrique Castanhole-Nunes, Márcia Maria Galbiatti, Ana Lívia Pavarino, Érika Goloni-Bertollo, Eny
Date Submitted:	07-Jul-2015

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Role of *MTHFR* C677T and *MTR* A2756G polymorphisms in thyroid and breast cancer development

Tairine Zara-Lopes*, **Ana Paula D'Alarme Gimenez-Martins***, Carlos Henrique Viesi Nascimento-Filho; Márcia Maria Urbanin Castanhole-Nunes, Ana Lívia Silva Galbiatti, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo**.

São José do Rio Preto Medical School – FAMERP/FUNFARME, Molecular Biology, Genetics and Research Unit (UPGEM), Brazil

Corresponding author: Dr. Eny Maria Goloni-Bertollo, Av. Brigadeiro Faria Lima, - 5416 - Vila São Pedro CEP: 15090-000 - São José do Rio Preto – SP, Phone number: +55 17 3201 5906, Fax number: +55 17 3229 1777, Email address: eny.goloni@famerp.br

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* The two authors had the same collaboration in producing the article

Summary: Folate metabolism is essential for DNA synthesis and repair. Alterations in genes that participating metabolism folate can be associated with several types of malignant neoplasms, including thyroid and breast cancer. In the present case-control study, we examined the association of methylenetetrahydrofolate reductase (*MTHFR* C677T - rs1801133) and methionine synthase (*MTR* A2756G - rs1805087) polymorphisms in thyroid and breast cancer and risk factors in 100 women with thyroid cancer, 100 women with breast cancer compared with 144 women controls. The Polymerase Chain Reaction-Restriction Fragment Length (RFLP) technique was used for genotyping of the polymorphisms. The statistical analysis was performed by multiple logistic regression test. We found a association of MTHFR C677T polymorphsm with increased risk for thyroid cancer (OR: 2.50; 95% CI: 1.15-5.46; *p*=0.02) and breast cancer (OR: 2.53; 95% CI: 1.08-5.93; *p*=0.03). Tobacco consumption and Body-mass index were also associated with thyroid cancer. In addition, age ≥50 years and alcohol consumption are associated with breast cancer. Our results indicated that *MTHFR* C677T is significantly associated with thyroid and breast cancer risk in the present casuistic. Thus, it may be a possible prognostic marker for these cancers.

Keywords: Breast cancer; folate; genes; genetic polymorphism; thyroid cancer

ABBREVIATIONS

BMI - Body-mass index

bp- base pair

CAPES - Coordination for the Improvement of Higher Level

CI – Confidence interval

CNPq - National Counsel of Technological and Scientific Development

DNA - deoxyribonucleic acid

EDTA - Ethylenediamine tetraacetic acid

FAMERP - São José do Rio Preto Medical School

FAPESP - São Paulo Research Foundation

FUNFARME - São José do Rio Preto Medical School Foundation

mL- mililiter

***MTHFR* -** methylenetetrahydrofolate reductase

***MTR* -** methionine synthase

OR – Odds ratio

PCR-RFLP- Polymerase Chain Reaction- Restriction Fragment Length Polymorphism

REC- Research Ethics Committee

SNPs - single nucleotide polymorphism

INTRODUCTION

Thyroid and Breast Cancers affect mainly women. Thyroid Cancer is the most common malignancy of the endocrine system. It may be noted a continuous increased of disorder. In recent years, the malignant thyroid tumor has been increased the number of diagnosis, and it is the fifth most common type of cancer in women. The estimate of Worldwide is approximately 300,000 new cases. Of these, 230,000 are females. The estimate for Breast Cancer was approximately 57,120 new cases, 56.09 cases per 100,000 women, representing 25% of total types of cancer diagnosed in women. [1, 2] Nowadays, it is observed a significant increase in the number of cases. Breast Cancer ranks second as a cause of death by cancer in women. [1, 3]

Multiple risk factors contribute to the development of thyroid and breast cancer such as hormonal factors, family history of cancer, tobacco and alcohol consumption, obesity, poor diet in folic acid and genetic variations. [1, 3, 4] Studies with single nucleotide polymorphisms (SNPs) involved in folate metabolism have been performed in several cancer types. Available data in literature are inconsistent and contradictory strengthening further studies are required in this area. [5-8] In thyroid cancer, researches addressing the folate pathways are poorly studied. [9, 10]

Low folate levels cause genomic instability through DNA synthesis, methylation and repair alterations. Consequently, low folate levels can induce carcinogenesis. [11-13] Several enzymes, including methylenetetrahydrofolate reductase (*MTHFR*) and methionine synthase (*MTR*) regulate this metabolism. [13, 14]

The MTHFR enzyme, encoded by *MTHFR* gene is responsible for catalyzes the irreversible reaction of 5,10- methylenetetrahydrofolate to 5-methyltetrahydrofolate what is involved in DNA methylation process, important factor for regulation of gene expression. Alterations in DNA methylation due to polymorphisms in *MTHFR* gene may be associated to cancer development. [11, 14, 15] The *MTR* enzyme encoded by *MTR* gene, is responsible for catalyzes the homocysteine remethylation to methionine such as have cofactor vitamin B_{12}. Therefore, polymorphisms in this gene increase homocysteine in the plasma changing folate pathway inducing carcinogenesis process. [15, 16] According to authors, *MTHFR* C677T and *MTR* A2756G polymorphisms are able to change folate metabolism important for DNA synthesis and methylation, responsible for genomic stability. [11, 14-16]

The aims of the present study were to investigate associations between *MTHFR* C677T and *MTR* A2756G polymorphisms involved in folate metabolism and thyroid and breast cancers compared with subjects without neoplasia and the interaction between these polymorphisms and risk factors (age, alcohol consumption, tobacco and Body Mass Index – BMI) in the disease.

MATERIALS AND METHODS

1. Subjects

A total of 344 women were evaluated in this case-control study, 200 patients (100 women with thyroid cancer and 100 with breast cancer), and 144 women controls without historic of cancer from January 2013 to January 2015.

Patients admitted to Hospital de Base with thyroid and breast cancers regardless of the age. The hospital is located in the city of São José do Rio Preto, São Paulo State, Brazil. The physicians responsible made the definitive diagnosis by examining the results of imaging studies, histopathological analysis, and biopsies. Patients with other neoplasms were excluded from the case group. The control group included healthy blood donors from the Hemoterapy Center of the city of São José do Rio Preto. Women were excluded if they presented with family history of cancer, other neoplasms, and chronic diseases described in Resolution RDC 34 [17] of the National Health Surveillance Agency/Brazil. All individuals involved in this study signed the Written Informed Consent Form. This study was approved by the Faculdade de Medicina de São José do Rio Preto (FAMERP) Research Ethics Committee (Thyroid cancer REC approval: 389.380; Breast Cancer REC approval: 84397).

2. Genotyping

Peripheral blood samples were collected from all the subjects using EDTA (Ethylenediamine tetraacetic acid). Genomic DNA was extracted by the method described by MILLER *et al*. [18] with modifications. The *MTHFR* C677T (rs1801133) and *MTR* A2756G (rs1805087) polymorphisms were determined by Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (*PCR-RFLP)*, using primers: *MTHFR* C677T - sense 5'- TGA AGG AGA AGG TGT CTG CGG GA 3'; a-sense 5'- AGG ACG GTG CGG TGA GAG TG 3'; *MTR* A2756G - sense 5'- CCA GGG TGC CAG GTA TAC AG 3'; a-sense 5'- GCC TTT TAC ACT CCT CAA AAC 3'. The genotyping *MTHFR* C677T

polymorphism was accomplished by restriction enzyme *Hinf I.* This analysis showed the following fragments: 198 bp (C allele) and 175, 23 bp (T allele). The *MTR* A2756G polymorphism was genotyped using restriction enzyme *Hae III* resulting in the fragments of *413*, 85 bp (A allele) and 290, 123 and 85 bp (G allele). [19-21] The genotyping confirmation was accomplished in 10% random samples of each group, and we observed 100% of the concordance.

3. Statistical Analysis

The Hardy-Weinberg equilibrium was evaluated by chi-square test using BioEstat 5.4 computer program. Multiple regression logistic test was performed by Minitab/Version 14.0 computer program, adjusting for age (thyroid cancer - reference: <49 years and breast cancer - reference <50 years), alcohol consumption (reference: not consume alcohol), tobacco (reference: nonsmoking), BMI (reference: <24.9), *MTHFR* C677T (reference: genotype CC-CT) and *MTR* A2756G (reference: genotype AA-AG). In this study, we considered smokers, those who smoked >100 cigarettes in their lifetime and a female drinker who has at least four drinks per week. One drink is equivalent to 30 mL of liquor; 102 mL of wine, and 340 mL of beer. [22-25] The subjects with BMI ≥ 25.0 were considered overweight. [26-28]

SNPstat online computer program (available: (<http://bioinfo.iconcologia.net/SNPstats>) was used to analyze the polymorphisms' effect in models (1) codominant (heterozygous *versus* homozygous wild type and polymorphic homozygous *versus* homozygous wild type), (2) dominant (heterozygous more polymorphic homozygous *versus*

homozygous wild type), (3) recessive (polymorphic homozygous *versus* homozygous wild type more heterozygous), (4) overdominant (wild homozygous *versus* heterozygous more polymorphic homozygote) and (5) additive (weight polymorphic homozygote *versus* heterozygote 2 more homozygous wild-type).

SNPstat online computer program was used to investigate the interaction between *MTHFR* C677T and *MTR* A2756G polymorphisms, as well as alcohol and tobacco consumption and BMI on the risk of thyroid and breast cancer. The results of both analyses were presented in odds ratio (OR), confidence interval 95% (CI – 95%) and value of p <0.05 was considered significant.

RESULTS

Table 1 and Table 2 show association of *MTHFR* C677T and *MTR* A2756G polymorphisms to thyroid and breast cancer according to heritage models. The 677TT genotype was associated with increased risk for development thyroid cancer (OR: 2.50; 95% CI: 1.15-5.46; p=0.02) and breast cancer (OR: 2.53; 95% CI: 1.08-5.93; p=0.03). We observed no association with risk to development of both types of cancers in other models. No statistical significance was observed for the *MTR* A2756G polymorphism in the risk of thyroid and breast cancers.

Hardy-Weinberg equilibrium for thyroid cancer and controls individuals showed that genotype frequencies were in equilibrium within the case group (χ^2=2.02, p=0.15) and control group (χ^2=0.28, p=0.59) for *MTHFR* C677T polymorphism. For *MTR* A2756G polymorphism, the equilibrium was only the

control group (χ^2=0.11, p=0.73); the case group presented disequilibrium (χ^2=4.38, p=0.03) (Table 1). In Breast Cancer and controls individuals both polymorphisms were in equilibrium (*MTHFR* C677T case group: χ^2=0.006, p=0.93 and control group: χ^2=0.28, p=0.59; *MTR* A2756G case group: χ^2=1.56, p=0.21 and control group: χ^2=0.11, p=0.73) (Table 2).

Multiple logistic regression showed that tobacco consumption (OR: 1.82; 95% CI= 1.02-3.25; *p*= 0.04) and BMI (OR: 1.81; 95% CI= 1.00-3.25; *p*= 0.04) were risk predictors for thyroid cancer. On the other hand, patients 49 years and over, as well as alcohol drinking was found no statistically significant. Patients 50 years and over (OR: 3.14; 95% CI= 1.79-5.51; *p*<0.001) and alcohol drinking (OR: 1.87; 95% CI= 1.05-3.34; *p*= 0.03) was more frequently in case group than in the control group. However, there was not an association between tobacco use and BMI for thyroid cancer (Table 3).

Table 4 and Table 5 show interaction analysis between *MTHFR* C677T and *MTR* A2756G polymorphisms and variables studied (alcohol consumption, tobacco consumption and BMI) on the risk thyroid and breast cancers. There was no interaction between the variables with both cancers (*p* interaction ≥ 0.05).

DISCUSSION

In the present study, we evaluated the association of *MTHFR* C677T and *MTR* A2756G polymorphism involved in folate metabolism and thyroid and breast cancers. We also investigated the interaction of the polymorphisms and possible risk factors for referred disorders. We found an association of the

MTHFR C677T polymorphism variant genotype (TT) and increased risk to both cancers. Tobacco consumption and BMI were associated with thyroid cancer development. The age ≥ 50 years and alcohol consumption were observed as a positive association to breast cancer.

Furthermore, in our study we have not observed the Hardy Weinberg equilibrium in the thyroid cancer group. This is due to random selection samples, model, and complexity disease that involved biological effects and genetic features. [21, 29]

Some polymorphism in the folate pathway altered the enzyme activity. It interfered in DNA methylation, in the synthesis of purines and pyrimidine, as well as in the genomic instability by inducing higher susceptibility of the carcinogenesis process. [16, 30] *MTHFR* gene reduces the enzymatic activity by limiting the conversion of 5,10 methylenetetrahydrofolate into 5-*MTHFR,* which is the only form of folate required to make the DNA methylation reaction. This reduction is important because it leads to cancer susceptibility. DNA hypomethylation associate with several cancers occurred as a result of a decrease in the concentration of 5-*MTHFR* [11, 14, 31]

The association of the recessive model (genotype 677TT) *MTHFR* gene with the increased risk for thyroid and breast cancers was observed in the present study (OR: 2.50; 95% CI: 1.15–5.46; p = 0.02) and (OR: 2.53; 95% CI: 1.08–5.93; p = 0.03), respectively. We suggested the relation this metabolic pathway for the disease development. Regarding thyroid cancer, an study by Ozdemir *et al*. [10] involving 60 cases and 50 controls found an increased risk of 2.33-fold for homozygous recessive genotype (677TT). A similar risk (2.08-fold)

for the same genotype was achieved by Fard-Esfahani et *al*. [9] in a study involving 154 cases and 198 controls. Both studies included men and women. In a breast cancer study, an increased risk for 677TT genotype was found in three case-control studies involving Chinese women. [15, 32,33] These results are according our present findings.

The genotype 677CT+TT and 677CT in breast cancer showed an increase risk of 1.2-fold and 1.3-fold in Kazakhstan's population, respectively. [31] Another study in Moroccan population conducted by Diakite *et al*. [34] involving 96 women found an association of at least one polymorphic allele, and breast cancer increased risk, contrary to our findings. In our study, we found no statistically significant difference between 677CT+TT and 677CT genotypes in both types of cancers studied. Our results were similar to other studies addressing thyroid and breast cancers. [35-38]

For the *MTR* A2756G polymorphism, ours results have shown no association between this SNP and thyroid and breast cancers, which met the results of four case-controls studies in breast cancer. [15, 27, 32, 33] A meta-analysis by Zhong *et al.* [6] including 16 case-controls studies and Weiner *et al.* [16] that evaluated 15 studies found no association *MTR* A2756G polymorphism in breast cancer as well. However, a Brazilian case-control study performed in Northeast region [30] and a study by Hosseini [39], which evaluated the Iran population, found an association of at least one polymorphic allele (2756G) in breast cancer, discordantly to our study. This polymorphism is associated with decrease *MTR* enzyme causing elevation of homocysteine level and DNA hypomethylation. [39,40]

Studies with polymorphisms and cancer risk presented controversial results due to several factors such as a measurement sample, ethnicity and population study, features hormones, and environmental factors such as folate intake. [34, 41]

Many studies have shown importance the smoking habit for risk cancer. [19-21] The association between tobacco consumption and thyroid cancer was found during this study (OR: 1.82; 95% CI: 1.02-3.25, *p*= 0.04) in agreement with a meta-analysis, which included 25 case-controls studies and six cohort studies concluding that the tobacco consumption is a predictor factor to several thyroid malignancies. [42] Another factor described in literature as a predictor for the development of several types of cancers is obesity, which was statistically significant in our study for thyroid cancer (OR: 1.81; 95% CI: 1.00-3.25, *p*= 0.04). According to present study, some studies confirmed an association between obesity and increased risk for thyroid cancer. This might influence the tumor size, extrathyroidal invasion, increase aggressiveness and even metastasis. [43-45] Guinard e*t al.* [46] in a study case-control involving men and women found no evidence between alcohol consumption and thyroid cancer risk in New Caledonia (Oceania) population, as reported in our study.

In this study, we suggested that women, 50 ≥ years constituted a risk group for developing breast cancer. It has been strongly related to the postmenopausal period in accordance with the literature. [32, 36] The alcohol consumption is a predictor factor for breast cancer development in women (OR: 1.87; 95% CI: 1.05–3.34; *p* = 0.03). This association was found in two case-control studies carried out in China (*p*=0.002) [15] and Malmo (South of Sweden)

(*p*=0,001) [47]; both of them involving women. The intake of alcoholic beverages causes poor absorption of B-complex vitamins, modifying folate metabolism, causing oxidative injury, and damaging the DNA strand. Studies in Breast Cancer and our results confirmed this fact. [48] The relation between tobacco and BMI was not statistically significant for breast cancer as well as in other studies. [36, 41, 47]

The limitation of our study was the sample size, once the implementation period was relatively short. Nevertheless, our study combined to others studies should provide a comprehensive understanding between the folate pathway and both types of cancer. It is noteworthy emphasizing that studies regarding thyroid cancer, and its association to folate pathway is still scarce in the literature.

Our case-control study shows that women presenting the *MTHFR* 677TT genotype have an increased risk for thyroid and breast cancers. Additionally, tobacco consumption and obesity are related to thyroid cancer. Alcohol consumption indicates an association to breast cancer development in women 50 ≥ years old. Thus, further investigation of gene-gene interactions between folate metabolism and studies of different populations can contribute towards the understanding regarding the polymorphisms' effect on the risk of breast and thyroid cancers.

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DISCLOSURE STATEMENT

All authors have no conflict of interest.

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Table 1. Association between *MTHFR* C677T and *MTR* A2756G polymorphisms and thyroid cancer.

SNP	Model	Genotype	Cases n (%)	Controls n (%)	OR (95% CI)	*p value*†
MTHFR C677T	*Codominant*	C/C	40 (40)	66 (45.83)	1.00 (ref)	
		C/T	41 (41)	65 (45.13)	1.10 (0.62-1.96)	0.06
		T/T	19 (19)	13 (9.04)	2.63 (1.14-6.04)	
		Allele C	121 (60.5)	197 (68.4)		
		Allele T	79 (39.5)	91 (31.6)		
		HWE test	*p = 0,15*	*p = 0,59*		
	Dominant	C/C	40 (40)	66 (45.83)	1.0 (ref)	
		C/T-T/T	60 (60)	78 (54.17)	1.36 (0.79-2.33)	0.26
	Recessive	C/C-C/T	81 (81)	131 (90.96)	1.0 (ref)	
		T/T	19 (19)	13 (9.04)	2.50 (1.15-5.46)	**0.02***
	Overdominant	C/C-T/T	59 (59)	79 (54.9)	1.0 (ref)	
		C/T	41 (41)	65 (45.1)	0.87 (0.51-1.49)	0.62
	Aditive	---	---	---	1.47 (1.00-2.16)	0.05
MTR A2756G	*Codominant*	A/A	63 (63)	88 (61.11)	1.00 (ref)	
		A/G	28 (28)	50 (34.72)	0.82 (0.46-1.47)	0.39
		G/G	9 (9)	6 (4.17)	1.82 (0.60-5.50)	
		Allele A	154 (77)	226 (78.4)		
		Allele G	46 (23)	62 (21.6)		
		HWE test	*p = 0,03*	*p = 0,73*		
	Dominant	A/A	63 (63)	88 (61.11)	1.00 (ref)	
		A/G-G/G	37 (37)	56 (38.89)	0.94 (0.55-1.62)	0.83
	Recessive	A/A-A/G	91 (91)	138 (95.83)	1.0 (ref)	
		G/G	9 (9)	6 (4.17)	1.93 (0.65-5.76)	0.23
	Overdominant	A/A-G/G	72 (72)	95 (66)	1.0 (ref)	0.40
		A/G	28 (28)	49 (34)	0.78 (0.44-1.38)	0.76
	Aditive	---	---	---	1.07 (0.70-1.64)	

OR, odds Ratio; Adjusted for age, alcohol and smoking consumption, BMI (Body-mass index) and polymorphisms; *HWE*, Hardy - Weinberg equilibrium; **p* values significant.

Table 2. Association between *MTHFR* C677T and *MTR* A2756G polymorphisms and breast cancer.

SNP	Model	Genotype	Cases n (%)	Controls n (%)	OR (95% CI)	*p value*
MTHFR C677T	*Codominant*	C/C	35 (35)	66 (45.83)	1.00 (ref)	
		C/T	48 (48)	65 (45.13)	1.09 (0.59-2.03)	0.09
		T/T	17 (17)	13 (9.04)	2.65 (1.07-6.58)	
		Allele C	118 (59)	197 (68.4)		
		Allele T	82 (41)	91 (31.6)		
		HWE test	*p = 0,93*	*p = 0,59*		
	Dominant	C/C	35 (35)	66 (45.83)	1.00 (ref)	
		C/T-T/T	65 (65)	78 (54.17)	1.33 (0.75-2.37)	0.33
	Recessive	C/C-C/T	83 (83)	131	1.00 (ref)	
		T/T	17 (17)	(90.96)	2.53 (1.08-5.93)	**0.03***
				13 (9.04)		
	Overdominant	C/C-T/T	52 (52)	79 (54.9)	1.0 (ref)	
		C/T	48 (48)	65 (45.1)	0.86 (0.49-1.53)	0.61
	Aditive	---	---	---	1.46 (0.96-2.23)	0.07
MTR A2756G	*Codominant*	AA	68 (68)	88 (61.11)	1.00 (ref)	
		AG	31 (31)	50 (34.72)	1.01 (0.55-1.85)	0.35
		GG	1 (1)	6 (4.17)	0.24 (0.03-2.17)	
		Allele A	167 (83.5)	226 (78.4)		
		Allele G	33 (16.5)	62 (21.6)		
		HWE test	*p = 0,21*	*p = 0,73*		
	Dominant	A/A	68 (68)	88 (61.11)	1.00 (ref)	
		A/G-G/G	32 (32)	56 (38.89)	0.91 (0.51-1.65)	0.77
	Recessive	A/A-A/G	99 (99)	138	1.0 (ref)	
		G/G	01 (01)	(95.83)	0.24 (0.03-2.15)	0.15
				6 (4.17)		
	Overdominant	A/A-G/G	69 (69)	94 (65.3)	1.0 (ref)	
		A/G	31 (31)	50 (34.7)	1.06 (0.58-1.94)	0.84
	Aditive	---	---	---	0.83 (0.50-1.40)	0.49

OR, odds Ratio; Adjusted for age, alcohol and smoking consumption, BMI (Body-mass index) and polymorphisms; *HWE*, Hardy - Weinberg equilibrium; **p* values significant.

Table 3. Risk factors and odds ratio (OR) for thyroid and breast cancer.

Cancer	Variable	Patients (n=100) n (%)	Controls (*n*=144) *n* (%)	OR (95% CI)	*p value*†
Thyroid Cancer	**Age (years)**				
	< 49	44 (44)	77 (53.48)	1.00 (ref)	
	≥ 49	56 (56)	67 (46.52)	1.43 (0.84-2.45)	0.19
	Alcohol consumption				
	No	81 (81)	103 (71.58)	1.00 (ref)	
	Yes	19 (19)	41 (28.42)	0.53 (0.28-1.02)	0.06
	Tobacco consumption				
	No	62 (62)	106 (73.62)	1.00 (ref)	
	Yes	38 (38)	38 (26.38)	1.82 (1.02-3.25)	**0.04***
	BMI				
	<25.0	26 (26)	54 (37.5)	1.00 (ref)	
	≥ 25.0	74 (74)	90 (62.5)	1.81 (1.00-3.25)	**0.04***
Breast Cancer	**Age (years)**				
	< 50	32 (32)	84 (58.34)	1.00 (ref)	
	≥ 50	68 (68)	60 (41.66)	3.14 (1.79-5.51)	**<0.001***
	Alcohol consumption				
	No	54 (54)	103 (71.58)	1.00 (ref)	
	Yes	46 (46)	41 (28.42)	1.87 (1.05-3.34)	**0.03***
	Tobacco consumption				
	No	64 (64)	106 (73.62)	1.00 (ref)	
	Yes	36 (36)	38 (26.38)	1.28 (0.70-2.35)	0.42
	BMI				
	<25.0	31 (31)	54 (37.5)	1.00 (ref)	
	≥ 25.0	69 (69)	90 (62.5)	1.31 (0.73-2.33)	0.36

OR, Odds Ratio; Adjusted for age, alcohol and smoking consumption, BMI (Body-mass index) and polymorphisms in the recessive model; **p* values

Table 4: Interaction between *MTHFR* C677T and *MTR* A2756G polymorphisms and alcohol and tobacco consumption and BMI on the risk of Thyroid Cancer.

	***MTHFR* C677T**		***MTR* A2756G**	
	CC/CT	**TT**	**AA/AG**	**GG**
Alcohol consumption	N (%)	N (%)	N (%)	N (%)
No				
Case	65 (65)	15 (15)	73 (73)	07 (07)
Control	93 (64.6)	10 (6.9)	98 (68)	05 (3.5)
OR (95% CI)	1.00	**2.50 (1.03-6.04)**	1.00	1.64 (0.49-5.51)
Yes				
Case	16 (16)	04 (04)	18 (18)	02 (02)
Control	38 (26.4)	03 (2.1)	40 (27.8)	01 (0.7)
OR (95% CI)	0.56 (0.28-1.12)	1.71 (0.36-8.17)	0.56 (0.29-1.07)	2.32 (0.20-26.98)
p interaction	0.84		0.50	
Tobacco consumption				
No				
Case	47 (47)	15 (15)	56 (56)	06 (06)
Control	95 (65.9)	11 (7.6)	104 (72.2)	02 (1.4)
OR (95% CI)	1.00	**2.64 (1.11-6.26)**	1.00	**5.52 (1.06-28.73)**
Yes				
Case	34 (34)	04 (04)	35 (35)	03 (03)
Control	36 (25)	02 (1.4)	34 (23.6)	04 (2.8)
OR (95% CI)	**1.94 (1.06-3.53)**	4.83 (0.83-28.20)	**2.02 (1.11-3.65)**	1.30 (0.28-6.08)
p interaction	0.96		0.06	
Body-mass index				
< 25 Kg/m^2				
Case	21 (21)	05 (05)	24 (24)	02 (02)
Control	49 (34)	05 (3.5)	52 (36.1)	02 (1.4)
OR (95% CI)	1.00	2.48 (0.64-9.66)	1.00	1.60 (0.21-12.45)
≥ 25 Kg/m^2				
Case	60 (60)	14 (14)	67 (67)	07 (07)
Control	82 (56.9)	08 (5.6)	86 (59.7)	04 (2.8)
OR (95% CI)	1.67 (0.90-3.11)	**4.46 (1.60-12.42)**	1.65 (0.92-2.97)	3.56 (0.93-13.71)
p interaction	0.93		0.81	

OR, Odds Ratio; Adjusted for age, alcohol consumption, tobacco consumption and Body-mass index. *p values significant.

Table 5: Interaction between *MTHFR* C677T and *MTR* A2756G polymorphisms and alcohol and tobacco consumption and BMI on the risk of Breast Cancer.

	***MTHFR* C677T**		***MTR* A2756G**	
	CC/CT	**TT**	**AA/AG**	**GG**
Alcohol consumption				
No				
Case	48 (48)	06 (06)	54 (54)	00 (00)
Control	93 (64.6)	10 (6.9)	98 (68)	05 (3.5)
OR (95% CI)	1.00	1.28 (0.39-4.16)	1.00	0.00
Yes				
Case	35 (35)	11 (11)	45 (45)	01 (01)
Control	38 (26.4)	03 (2.1)	40 (27.8)	01 (0.7)
OR (95% CI)	1.74 (0.91-3.31)	**11.06 (2.73-44.75)**	**2.09 (1.15-3.80)**	1.97 (0.12-32.76)
p interaction	0.08		0.15	
Tobacco consumption				
No				
Case	55 (55)	09 (09)	63 (63)	01 (01)
Control	95 (65.9)	11 (7.6)	104 (72.2)	02 (1.4)
OR (95% CI)	1.00	1.56 (0.54-4.45)	1.00	1.24 (0.09-16.46)
Yes				
Case	28 (28)	08 (08)	36 (36)	00 (00)
Control	36 (25)	02 (1.4)	34 (23.6)	04 (2.8)
OR (95% CI)	1.13 (0.58-2.21)	**8.57 (1.62-45.39)**	1.57 (0.85-2.93)	0.00
p interaction	0.11		0.09	
Body-mass index				
< 25 Kg/m^2				
Case	24 (24)	07 (07)	31 (31)	00 (00)
Control	49 (34)	05 (3.5)	52 (36.1)	02 (1.4)
OR (95% CI)	1.00	3.44 (0.89-13.26)	1.00	0.00
≥ 25 Kg/m^2				
Case	59 (59)	10 (10)	68 (68)	01 (01)
Control	82 (56.9)	08 (5.6)	86 (59.7)	04 (2.8)
OR (95% CI)	1.41 (0.74-2.66)	2.64 (0.86-8.17)	1.23 (0.68-2.21)	0.44 (0.04-4.45)
p interaction	0.49		0.39	

OR, Odds Ratio; Adjusted for age, alcohol consumption, tobacco consumption and Body-mass index. *p values significant.

2.2. ARTIGO CIENTÍFICO II

COMPROVANTE DE SUBMISSÃO

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Molecular Carcinogenesis

Submission Confirmation

Thank you for submitting your manuscript to *Molecular Carcinogenesis*.

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Authors:	Gimenez-Martins, Ana Paula Castanhole-Nunes, Márcia Nascimento-Filho, Carlos dos Santos, Stéphanie Galbiatti, Ana Lívia Pavarino, Érika Goloni-Bertollo, Eny Maria
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Breast cancer: polymorphisms in folate metabolism and clinical histopathological features in case-control study

Ana Paula D'Alarme Gimenez-Martins, Márcia Maria Urbanin Castanhole-Nunes, Carlos Henrique Viesi Nascimento-Filho, Sthéphanie Piacenti dos Santos, Ana Lívia Silva Galbiatti, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo*.

Faculdade de Medicina de São José do Rio Preto – FAMERP/FUNFARME; Molecular Biology Department; Unidade de Pesquisa em Genética e Biologia Molecular (UPGEM), Brazil.

***Corresponding author**: Dr. Eny Maria Goloni-Bertollo.

Av. Brigadeiro Faria Lima, - 5416 - Vila São Pedro CEP: 15090-000 - São José do Rio Preto – SP, Phone number: +55 17 3201 5906, Fax number: +55 17 3229 1777.

Email address: eny.goloni@famerp.br

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ABBREVIATIONS

BMI - Body-mass index
CAPES - Coordination for the Improvement of Higher Level
CAAE*- Certificate of Presentation for Ethical Consideration*
CI – Confidence interval
CNPq - National Counsel of Technological and Scientific Development
CONEP- National Health Council
DNA - deoxyribonucleic acid
ER- estrogen receptor
FAMERP - São José do Rio Preto Medical School
FAPESP - São Paulo Research Foundation
FUNFARME - São José do Rio Preto Medical School Foundation
HER2- Human Epidermal growth factor Receptor-type 2
HWE- Hardy-Weinberg Equilibrium
M- metastase
mL- milliliter
***MTHFR* -** methylenetetrahydrofolate reductase
***MTR* -** methionine synthase
***MTRR* -** methionine synthase reductase
N- node involvement
OR – Odds ratio
PCR-RFLP- Polymerase Chain Reaction- Restriction Fragment Length Polymorphism
PR- progesterone receptors
SNPs - single nucleotide polymorphism
T- tumor size
UICC- Union International Control Cancer

KEY WORDS: Methylenetetrahydrofolate Reductase (MTHFR); Methionine Synthetase (MTR); Methionine Synthase Reductase (MTRR); Genetic Polymorphism; Breast Carcinoma.

ABSTRACT

Methylenetetrahydrofolate Reductase (MTHFR), Methionine Synthetase (MTR) and Methionine Synthase Reductase (MTRR) genes are responsible by encode enzymes involved in the folate pathway. Polymorphisms these genes can modify enzyme activity to modulate risk of breast cancer. The present case-control study involved 606 Brazilian women, 128 cases and 478 controls to investigated associations between *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G polymorphisms and breast cancer risk. Further, we showed the association these polymorphisms with both risk factors and clinical histopathological parameters of breast cancer. Polymorphisms in genes *MTHFR* and *MTR* were genotyping using Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (*PCR-RFLP)* and *MTRR* A66G polymorphism by Real Time PCR technique. For statistical analysis, program used were MINITAB 14.0 (multiple logistic regression test) and SNPstats (inheritance models and Hardy-Weinberg Equilibrium) program. Women with ≥50 years old (OR: 2.65; 95% CI: 1.65-4.26; p<0.001) and alcohol consumption (OR: 1.76; 95% CI: 1.09-2.85; p=0.021) are associated with increased risk for breast cancer. For *MTHFR* A1298C, we observed reduced risk of developing breast cancer in 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 associations were found among *MTHFR* C677T, *MTR* A2756G, and *MTRR* A66G polymorphisms and models. In relation to clinical histopathological parameters, we did not observe significant association between polymorphisms

with clinical features and polymorphisms with cancer subtypes. Although we found no association with increased breast cancer risk, this is the first study involving women population from the Northwest region of Sao Paulo State, Brazil, which showed the relation between polymorphisms in folate metabolism and clinical histopathological parameters.

INTRODUCTION

Breast cancer is the most common cancer among women and second leading cause of death in women in the worldwide [1-4]. For Brazil, 25% of total types of cancer diagnosed in women is breast cancer. The estimate was approximately 57,120 new cases (56.09 cases per 100,000 women) [2].

The risk factors for breast cancer are multiples involving genetic and environmental factors [5-6]. Among the risk factors are: obesity, hormone therapy, menopausal status, age and lifestyle [3,7-8]. In relation of lifestyle, some researchers have studies the association between folate ingestion and breast cancer risk [5,9-10]. Regarding of genetic factors, single nucleotide polymorphisms (SNPs) in genes involved in the folate pathway may be associated with breast cancer development [6,11-14] and can lead to a decrease in the effect of enzymes, modifications in the methylation, and the synthesis of DNA. These can cause abnormal gene expression and errors in chromosome segregation during cell division favoring carcinogenesis [12,14-16].

The 5, 10-methylenetetrahydrofolate reductase (MTHFR) is responsible by converting 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (circulating form of folate in plasma) [17,11,18]. The enzyme methionine synthase (MTR) catalyze the reaction in the remethylation of homocysteine into methionine, the essential amino acid. The methionine synthase reductase (MTRR) is responsible for maintaining active the enzyme MTR [9,19-20].

The aims of the present study were to investigate associations between *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G

(rs1805087) and *MTRR* A66G (rs1801394) polymorphisms involved in folate metabolism and breast cancer risk. Furthermore, we showed the association these polymorphisms with risk factors (age, smoking habits, alcohol consumption, number pregnancies, body mass index, and hormone therapy) and with clinical histopathological parameters (tumor size, node involvement, metastasis, and cancer subtypes) of breast cancer.

MATERIALS AND METHODS

1. Subjects

The case-control study was approved of by National Ethics Committee (CAAE – 04069612.1.0000.5415), Faculdade de Medicina de São José do Rio Preto (FAMERP), São Paulo, Brazil. We evaluated 606 women, 128 with breast cancer (case group) and 478 volunteers (control group). All individuals agreed with Informed Consent Form, according to the Resolution 466/12 the National Health Council – CONEP.

We included in the case group female patients, regardless of the age with definitive diagnosis for breast cancer, through results of imaging exams, histopathological analysis, and biopsy at the Hospital de Base, Faculdade de Medicina de São José do Rio Preto, São Paulo, Brazil. The exclusion criterion was patients with other neoplasms.

All women the case group answered a questionnaire to obtain the following data: age, smoking habits, alcohol consumption, number pregnancies, body mass index (BMI), and hormone use. Additional information about the

diagnosis, histological type, tumor location, tumor size (T), node involvement (N), metastasis (M), progesterone receptors (PR) estrogen receptor (ER), and Human Epidermal growth factor Receptor-type 2 (HER2) were collected from the medical records and pathology reports. The physicians performed the TNM classification in accordance with the parameters of the Union International Control Cancer (UICC) and the breast cancer subtypes to according to a study by Sikandar et al. [21]. The breast cancer subtypes are: Luminal A (ER+ and/or PR+, HER2- and Ki-67 <14%), Luminal B (ER+ and/or PR+, HER2- and Ki-67 ≥14%), Overexpression HER2 (ER-, PR- and HER2+), and triple negative (ER-, PR- and HER2-) [21,22].

The control group involved healthy women blood donors at the Blood Therapy Service, in the city of São José do Rio Preto, São Paulo, Brazil. The inclusion criterion was women aged 40 and over. The exclusion criteria were personal and cancer family history and chronic diseases describe in Resolution – RDC nº34 of the National Health Surveillance Agency/Brazil [23]. Of our total study sample (478 women in the control group), we found data of 219 women, such as age, smoking habits, alcohol consumption, number pregnancies, BMI, and hormone therapy. Regarding the remaining 259 women, we could collect data such as age, smoking habits, and alcohol consumption.

We considered smokers women who consumed at least 100 cigarettes during their lifetime and alcohol consumers those who drink more than 4 drinks weekly, corresponding 30 mL liquor; 102 mL wine, and 340 mL beer [24-27].

2. Genotyping

DNA genomic was extracted the peripheral blood leukocytes of cases and controls according to Miller et al [28], with modifications. DNA samples were quantification by spectrophotometer Picodrop Pico200TM *(Thermo Scientific)*.

The *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131) and *MTR* A2756G (rs1805087) polymorphisms were performed using Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (*PCR-RFLP)*. Table 1 show primers sequence, restriction enzyme and fragments size, according to Barbosa et al. [29], and Galbiatti et al. [17].

The genotyping of *MTRR* A66G (rs1801394) polymorphisms were performed by Real Time PCR – SNP Genotyping Assay *(Applied Biosystems – Life Technologies)* – Assay ID: C___3068176_10 (20X), according manufacturer instructions. The reactions were performed in equipment Step One Plus TM Real-Time PCR System [17]. For each polymorphism was performed the genotyping in 10% of the samples for confirmation. We found a 100% concordance.

3. Statistical Analysis

Multiple regression logistic test was used to determine the effects of variables on breast cancer risk and clinical histopathological parameters, using the Minitab/Windows program - Version 14.0. The model include variables: age (reference: <50 years old; median), smoking habits (reference: no smokers), alcohol consumption (reference: non-drinkers), number pregnancies (reference:

≥ 3 gestations), BMI (reference: <25 Kg/m²), hormone use (reference: no), *MTHFR* C677T (reference: genotype CC), *MTHFR* A1298C (reference: genotype AA), *MTR* A2756G (reference: genotype AA), and *MTRR* A66G (reference: genotype AA). The clinical histopathological parameters were T classification (reference: T1 and T2), N Classification (reference: negative), and M classification (reference: negative).

The Hardy-Weinberg equilibrium (HWE) was assessed using the chi-square test using the program SNPStats online computer program (available at: <http://bioinfo.iconcologia.net/SNPstats_web>). The polymorphisms were examined with codominant, dominant, recessive, overdominate and additive models through the program SNPStats. The relation between polymorphisms and clinical histophatological features were assessed by SNPStats online computer program. The results are presented as odds ratios (OR) and confidence interval of 95% (95% CI). P-value less than 0.05 were considered statistically significant.

RESULTS

The relationships between risk factors and breast cancer development are in Table 2. Multiple logistic regression (128 cases and 219 controls) shows than women with ≥50 years old (OR:2.65; 95%CI:1.65-4.26; p<0.001) and alcohol consumption (OR:1.76; 95%CI:1.09-2.85; p=0.021) are associated with increased risk for breast cancer in this population. Smoking habits (OR:1.07; 95%CI:0.65-1.79; p=0.782), number of pregnancies (OR:0.86; 95%CI:0.54-1.38; p=0.536), BMI ≥25 Kg/m^2 (OR:1.24; 95%CI:0.75-2.06; p=0.405), and

hormone use (OR: 1.41; 95%CI:0.86-2.33; p=0.174) are not associated with the risk of breast cancer. The Multiple logistic regression (adjusted for age, smoking habits and alcohol consumption) did not show different results (data not shown) for both cases (n=128) and controls (n=478).

Hardy-Weinberg Equilibrium analysis (Table 3) shows that the case and control group of *MTHFR* C677T (rs1801133) (case p=1.00; control p=0.76) and *MTR* A2756G (rs1805087) (case p=0.07; control p=0.7) polymorphisms are in equilibrium. In the control group, the *MTHFR* A1298C (rs1801131) and *MTRR* A66G (rs1801394) polymorphisms showed disequilibrium *p*=0.01 and *p*=0.03, respectively. Frequencies of variant alleles for SNPs (Table 3) were: allele C - 60% case and 66% control the *MTHFR* C677T (rs1801133) polymorphism, allele A - 80% case and 72% control the *MTHFR* A1298C (rs1801131) polymorphism, allele A - 82% case and 79% control the *MTR* A2756G (rs1805087) and allele A - 60% case and 57% control the *MTRR* A66G (rs1801394).

The genotype distributions of *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G (rs1805087) and *MTRR* A66G (rs1801394) polymorphisms and breast cancer risk are present in Table 3 (128 cases and 478 controls). No correlation is observed between *MTHFR* C677T (rs1801133) polymorphism and breast cancer risk in codominant (p=0.25), dominant (p=0.21), recessive (p=0.15), overdominat (p=0.82) and log-additive (p=0.1) models. We observed that *MTHFR* A1298C (rs1801131) polymorphism reduced the risk of developing breast cancer in 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 *MTR* A2756G (rs1805087), and *MTRR* A66G (rs1801394) polymorphisms analyzed and models (p>0.05).

In relation to clinical histopathological parameters, we observed that 94.6% breast cancer cases were ductal involvement. Lobular and mixed (ductal and lobular) involvements account for 4.6% and 0.8% breast cancers, respectively. Regarding TNM classification, we found 62.5% of the cases with low extension tumors (T1-T2), 42.2% of the cases are positive for lymph node involvement, and 34.4% of the cases are positive for metastasis. The high frequency for breast cancer subtype is 48.4% for luminal B. Luminal A, overexpression HER2, and triple negative are 26.6%, 10.1%, and 12.5%, respectively.

The analyses of *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G (rs1805087), and *MTRR* A66G (rs1801394) polymorphisms and the clinical tumor size (T1-T2 vs T3-T4), lymph nodes involvement, and metastasis are shown in Table 4. We did not find significant association between polymorphisms with clinical features (TNM) parameters. In relation to SNPs and breast cancer subtypes, we did not observe an association among luminal A, luminal B, overexpression HER2, and triple negative subtypes and polymorphisms (Table 5).

DISCUSSION

This study states that women aged 50 and over (OR: 2.65; 95%CI:1.65-4.26; p<0.001) and alcohol consumption (OR:1.76; 95%CI:1.09-2.85; p=0.021)

are predictor factors for breast cancer. Women aged 50 and over was associated with breast cancer increased risk in other studies [3,6,12,19,30]. Sangrajrang et al. [19] found that Thailand women with mean age of 46 (±10.6) years were associated with significant increase of breast cancer, $p < 0.001$. This p-value was also found by He et al. [12], Akilzhanova [6], Jiang-Hua et al. [30] and Gong et al. [3] with a different mean age. The age can be associated with menopause period, BMI, and hormone therapy due to hormone influence during women's life. Hvidtfeldt et al. [7] found in their pooled cohort study that overweight women (median age of 56 ± 5.0) under hormone therapy present increase the absolute risk of breast cancer (p interaction=0.003). Regarding postmenopausal women, Key et al. [31] found that the risk of a breast cancer increase with levels of endogenous estradiol. Ericson et al. [32] in their case-control study (313 cases and 626 controls) confirmed the association between menopausal hormone therapy and breast cancer risk ($p<0.001$) in Malmo women. The present study did not evaluate the menopausal hormone therapy. We evaluated the hormone use during women's life. We did not find a statically significant association between hormone use and breast cancer risk, as well as between BMI ≥ 25 Kg/m^2, smoking habits, and the number of pregnancies and breast cancer risk/development. In the present study, alcohol consumption is associated with breast cancer risk. This association was found in studies by Ma et al. [11] and Ericson et al. [32], both with $p<0.001$. Alcohol could be an important factor for induced carcinogenesis because it may induce epigenetic alterations and aberrant DNA methylation, moreover, alcohol reduced folate absorption [9].

Regarding polymorphisms, we did not find a significant association between *MTHFR* C677T, *MTR* A2756G and *MTRR* A66G polymorphisms, and breast cancer risk in this study. We observed reduction in the risk of breast cancer in the presence of *MTHFR* A1298C polymorphism. MTHFR enzyme is responsible by the production of the main circulating folate, which is the donor of methyl groups for the reaction of homocysteine remethylation. In this reaction, MTR and MTRR enzymes are included. Studies with C677T and A1298C polymorphisms in *MTHFR* gene observed that these SNPs decreased the enzyme activity, changing folate levels, which leads to carcinogenesis [18,29-30,32-34]. Sohn et al. [35] in their in vitro research, found that cells expressing the mutant 677T *MTHFR* reduced the enzyme activity by 35% in colon and breast cancer ($p<0.001$). Additionally, we found in the literature studies that showed increase breast cancer risk in women with 677 CT and 677 TT genotypes [1,6,12,14,18] in the *MTHFR* gene. In the study with Brazilian population, they found that women aged 50 and over and heterozygotes for polymorphisms in the *MTHFR* gene (677CT+1298AC) have increased of risk breast cancer [29]. The meta-analysis by Li et al. [1], involving 57 cases-control studies, suggested that 677 CT polymorphism in the *MTHFR* gene may contribute for breast cancer development, while the 1298 AC polymorphism was not associated with risk of the disease. In the present study, we did not observe an association between *MTHFR* C677T polymorphism and breast cancer risk, as well as in the study by Ma et al. [11], Sangrajrang et al. [19] and Gong et al. [3], reinforcing our findings.

For *MTHFR* A1298C SNP, SHARP et al. [36] reported in their study case-control (with 62 women case and 64 controls) reduced breast cancer risk in women with 1298CC genotype compared to AA, in Scotland population. Moreover, a study with Kazakhstan's women found a 1.2-fold decrease in breast cancer risk in a dominant model [6]. These results are in accordance with the present study, in which we observed that *MTHFR* A1298C polymorphism reduced the breast cancer risk in a codominant model, recessive model, and log-additive model.

The MTR enzyme is responsible by catalyzes the homocysteine remethylation to methionine and MTRR enzyme product maintains the activity of MTR [18,30,37]. S-adenosylmethionine (SAM) is the precursor of methionine, and it is important for methylation reactions (including DNA methylation) [20]. However, polymorphisms these genes (*MTR* and *MTRR)* may not maintain adequate intracellular concentrations of methionine and SAM levels [16,20]. The SNPs *MTR* A2756G and *MTRR* A66G may influence cancer susceptibility [19] due to alterations in the plasma levels of homocysteine [29,33]. Studies have shown the association between *MTR* A2756G and *MTRR* A66G polymorphisms and breast cancer risk [15,19,34].

In this study, we did not observe an association between *MTR* A2756G and *MTRR* A66G polymorphisms, and breast cancer development. The meta-analysis by Weiner et al. [20] (involving 12 case-control studies for *MTR* A2756G SNP and 7 studies for *MTRR* A66G polymorphisms) and two studies involving Chinese women also showed no association of *MTR* A2756G polymorphism with breast cancer [12,30]. Other studies involving Japanese

women [19,38] and the study by Naushad et al. [8] (involving 244 case-control pairs of Indian women) also found no significance between *MTRR* A66G polymorphism and breast cancer risk. All these studies agree with our results.

On the other hand, Weiwei et al. [14] with 296 cases and 306 controls observed that women with the 2756 AG genotype have increased the risk for breast cancer and SuzukI et al. [34] showed that *MTRR* A66G polymorphism was associated with breast cancer risk in Japanese premenopausal women (p trend = 0.04).

Additionally, some researchers have studied the association between polymorphisms on folate metabolism and clinical histopathological parameters [8,18,39]. In our study, *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G polymorphisms were not associated with TNM classification. However, Babyshkina et al. [18], in their study with 300 women with breast cancer, found a significant association between 677CT and 677CT+TT and tumor size, but did not found association with *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G polymorphisms and tumor size. Data show also association of lymph node involvement ($p<0.05$) and *MTHFR* C677T in Brazilian population [39]. On the other hand, Martin et al. [40] did not observe an association between TNM classification and *MTHFR* C677T, and *MTHFR* A1298C polymorphisms, according to the present study. The genetic variants of the genes coding key enzymes (MTHFR, MTR and MTRR) in the folate metabolism influence enzyme function [29-30,32,34]. This causes the inhibition of DNA synthesis and inadequate DNA methylation, which favors the tumor progression [8,18].

In relation to breast cancer subtype, our study observed no association between polymorphisms studied and luminal A, luminal B, overexpression HER2, and triple negative subtypes. Naushad et al. [10] showed that the *MTHFR* C677T polymorphism was association with risk for luminal B subtype, and the *MTR* A2756G polymorphism was associated with increased risk for luminal A subtype. For *MTRR* A66G polymorphism, Naushad et al. [10] also did not observe an association between any breast cancer subtypes, as well as in this study. Batschauer et al. [39] found no association between *MTHFR* C677T polymorphism and HER2 receptor or ER and PR. Babyshkina et al. [18] found that patients with at least one polymorphic allele for *MTHFR* C677T polymorphism were significantly associated with a positive ER. For *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G polymorphisms Babyshkina et al. [18] did no observe an association with breast cancer subtype. Tumor with negative hormone receptor and positive HER2 has a worse prognosis in breast cancer [39,41], but they may not be associated with *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G polymorphisms on the folate metabolism.

Our study is first one that shows women's profile of the Northwest population of São Paulo State, Brazil, since few studies have performed the analysis between polymorphisms involved in folate metabolism and clinical histopathological features in breast cancer. In conclusion, this study states that women aged 50 and over and alcohol consumption have increased risk for breast cancer. We did not find a significant association between *MTHFR* C677T, *MTR* A2756G and *MTRR* A66G polymorphisms, and breast cancer development. However, women with 1298CC genotype in the *MTHFR* gene

have decreased risk for breast cancer development. The polymorphisms studied are not associated with clinical histopathological parameters in the present study.

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TABLE 1 - Primers sequence, restriction enzyme and fragments size

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

MTHFR - Methylenetetraydrofolate reductase; MTR - methionine synthase, bp- base-pair

TABLE 2- Relationship between risk factors and breast cancer development.

Variables	Cases (N= 128) N (%)	Controls (N= 219) N (%)	OR (95% CI)	*P* - value
Age				
< 50 years	41 (32.03)	126 (57.53)	1.00	
≥ 50 years	87 (67.97)	93 (42.47)	**2.65 (1.65-4.26)**	**<0.001***
Smoking Habits				
No	84 (65.62)	159 (72.60)	1.00	
Yes	44 (34.38)	60 (27.40)	1.07 (0.65-1.79)	0.782
Alcohol Consumption				
No	66 (51.56)	146 (66.67)	1.00	
Yes	62 (48.44)	73 (33.33)	**1.76 (1.09-2.85)**	**0.021***
Gestations				
< 3 gestations	64 (50)	131 (59.82)	1.00	
≥ 3 gestations	64 (50)	88 (40.18)	0.86 (0.54-1.38)	0.536
BMI				
< 25 Kg/m^2	36 (28.13)	71 (32.42)	1.00	
≥ 25 Kg/m^2	92 (71.87)	148 (67.58)	1.24 (0.75-2.06)	0.405
hormone use				
No	85 (66.40)	159 (72.60)	1.00	
Yes	43 (33.60)	60 (27.40)	1.41 (0.86-2.33)	0.174

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

TABLE 3- Analyze 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
		***MTHFR* C677T**					***MTHFR* A1298C**			
Codominant	CC	46 (35.9)	207 (43.3)	1.00		AA	79 (61.7)	261 (54.6)	1.00	
	CT	61 (47.7)	218 (45.6)	1.21 (0.77-1.89)	0.25	AC	46 (35.9)	170 (35.6)	0.90 (0.59-1.39)	**0.01***
	TT	21 (16.4)	53 (11.1)	1.71 (0.91-3.22)		CC	3 (2.3)	47 (9.8)	**0.22 (0.06-0.74)**	
	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		AA	79 (61.7)	261 (54.6)	1.00	
	CT+TT	82 (64.1)	271 (56.7)	1.31 (0.86-2.00)	0.21	AC+CC	49 (38.3)	217 (45.4)	0.76 (0.50-1.16)	0.20
Recessive	CC+CT	107 (83.6)	425 (88.9)	1.00		AA+AC	125 (97.7)	431 (90.2)	1.00	
	TT	21 (16.4)	53 (11.1)	1.55 (0.87-2.75)	0.15	CC	3 (2.3)	47 (9.8)	**0.22 (0.07-0.76)**	**0.004***
Overdominant	CC+TT	67 (52.3)	260 (54.4)	1.00		AA+CC	82 (64.1)	308 (64.4)	1.00	
	CT	61 (47.7)	218 (45.6)	1.05 (0.70-1.58)	0.82	AC	46 (35.9)	170 (35.6)	1.02 (0.67-1.57)	0.92
Log-additive	---	---	---	1.28 (0.95-1.74)	0.1	---	---	---	**0.70 (0.49-0.98)**	**0.03***
		***MTR* A2756G**					***MTRR* A66G**			
Codominant	AA	83 (64.8)	300 (62.8)	1.00		AA	48 (37.5)	168 (35.1)	1.00	
	AG	44 (34.4)	156 (32.6)	1.22 (0.79-1.89)	0.12	AG	57 (44.5)	211 (44.1)	0.99 (0.63-1.56)	0.59
	GG	1 (0.8)	22 (4.6)	0.23 (0.03-1.77)		GG	23 (18.0)	99 (20.7)	0.76 (0.42-1.35)	
	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		AA	48 (37.5)	168 (35.1)	1.00	
	AG+GG	45 (35.2)	178 (37.2)	1.11 (0.72-1.71)	0.63	AG+GG	80 (62.5)	310 (64.8)	0.91 (0.60-1.39)	0.66
Recessive	AA+AG	127 (99.2)	456 (95.4)	1.00		AA+AG	105 (82)	379 (79.3)	1.00	
	GG	1 (0.8)	22 (4.6)	0.21 (0.03-1.64)	0.06	GG	23 (18)	99 (20.7)	0.76 (0.45-1.29)	0.30
Overdominant	AA+GG	84 (65.6)	322 (67.4)	1.00		AA+GG	71 (55.5)	267 (55.9)	1.00	
	AG	44 (34.4)	156 (32.6)	1.28 (0.83-1.98)	0.27	AG	57 (44.5)	211 (44.1)	1.09 (0.72-1.65)	0.68
Log-additive	---	---	---	0.98 (0.67-1.43)	0.91	---	---	---	0.89 (0.67-1.17)	0.40

TABLE 4- 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 N (%)	**CT+TT N (%)**	**AA N (%)**	**AC+CC N (%)**	**AA N (%)**	**AG+GG N (%)**	**AA N (%)**	**AG+GG 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)
p 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)
p 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)
p 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 use and polymorphisms.

TABLE 5- Association between *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G polymorphisms and breast cancer subtype

	***MTHFR* C677T**		***MTHFR* A1298C**		***MTR* A2756G**		***MTRR* A66G**	
Breast Cancer Subtype	**CC N (%)**	**CT+TT N (%)**	**AA N (%)**	**AC+CC N (%)**	**AA N (%)**	**AG+GG N (%)**	**AA N (%)**	**AG+GG 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)
p 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)
p 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)
p 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)
p 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 use and polymorphisms.

3. CONCLUSÕES

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As frequências dos polimorfismos no grupo caso são: 64,1% para o polimorfismo *MTHFR* C677T; 38,3% para *MTHFR* A1298C; 35,2% para *MTR* A2756G e 62,5% para a variante *MTRR* A66G. Para o grupo controle as frequências são: 56,7% para *MTHFR* C677T; 45,4% para o polimorfismo *MTHFR* A1298C; 37,2% para *MTR* A2756G e 64,8% para a variante *MTRR* A66G.

Os polimorfismos *MTHFR* C677T, *MTR* A2756G e *MTRR* A66G não estão associados ao risco de desenvolver câncer de mama. A variável *MTHFR* A1298C está associado à redução no risco da doença.

O genótipo TT do polimorfismo *MTHFR* C677T está associado ao risco de desenvolver câncer de mama na casuística avaliada no artigo científico I.

Mulheres com idade ≥ 50 anos e que consomem bebida alcoólica possuem risco aumentado para desenvolver câncer de mama.

Os polimorfismos *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G e *MTRR* A66G não estão associados aos parâmetros clínicos-patológicos (TNM e classificação fenotípica).

4. REFERÊNCIAS

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5. ANEXOS

ANEXO 1

METODOLOGIA

1. Indivíduos

No total, foram estudadas 606 mulheres, sendo 128 pacientes com diagnóstico confirmado de câncer de mama (grupo caso), procedentes do Serviço de Ginecologia e Obstetrícia do Hospital de Base/ Faculdade de Medicina de São José do Rio Preto – SP e 478 mulheres voluntárias, saudáveis e sem história de neoplasia do Hemocentro da Faculdade de Medicina/ Hospital de Base de São José do Rio Preto – SP. Os indivíduos foram incluídos no estudo após a obtenção do Termo de Consentimento Livre e Esclarecido (Anexo 2) e todas as informações foram obtidas por meio de questionário padronizado (Anexo 3) e mantidas em sigilo. As amostras do material genético foram codificadas e armazenadas em arquivos na Unidade de Pesquisa em Genética e Biologia Molecular (UPGEM) da Faculdade de Medicina de São José do Rio Preto - FAMERP, conforme aprovado pelo CEP-FAMERP (CAAE: 04069612.1.0000.5415 – Anexo 4).

2. Extração de DNA genômico

O DNA genômico foi extraído de leucócitos a partir do sangue periférico, segundo a técnica proposta por Miller *et al*. (1988), com modificações, e armazenado em freezer -20ºC para a genotipagem. Foram coletados aproximadamente 7,0 mL de sangue periférico e transferidos para um tubo de 15 mL estéreis já contendo 7 mL de Ficoll (proporção 1:1). Posteriormente, o

tubo foi centrifugado a 1500 rpm por 30 minutos. Após a formação de três fases (plasma, leucócitos e hemácias), os leucócitos foram transferidos para um novo tubo com a utilização de uma pipeta Pasteur estéril descartável. Em seguida, foi adicionado tampão PBS até completar o volume de 15 ml. O tubo foi centrifugado novamente a 1200 rpm por 15 minutos. O sobrenadante foi descartado e foi adicionado novamente PBS até completar o volume de 15 mL. Após centrifugação, o sobrenadante foi descartado e adicionado 3 mL de tampão de lise, 200 uL de SDS 10% e 50 uL de proteinase K (20 mg/mL). A solução foi incubada *overnight* a 37ºC. Após a digestão proteica, foi adicionado 1 mL de NaCl 6M, com posterior agitação e em seguida foi colocado no gelo por 15 minutos. Após este período, a solução foi homogeneizada e centrifugada a 2000 rpm por 15 minutos. O sobrenadante foi transferido para um tubo novo de 15 mL, descartando-se o *pellet*. Após a adição de etanol 100% gelado, o tubo foi fechado e homogeneizado por inversão. O DNA precipitado foi removido para um tubo *eppendorf* contendo 500 uL etanol 70%. Este foi centrifugado a 12000 rpm por 5 minutos e o etanol 70% descartado. Para secagem do *pellet* de DNA, os microtubos ficaram em temperatura ambiente e em seguida *o* DNA foi ressuspendido em 200 uL de tampão TE para quantificação e posteriormente armazenado em freezer -20ºC.

3. Análise Molecular - Genotipagem

3.1. Polimorfismos C677T no gene *MTHFR*

O polimorfismo C677T no gene *MTHFR* foi genotipado por meio da técnica de PCR-RFLP (Polimerase Chain Reaction-Restriction Fragment

Lenght Polimorphism). Os reagentes utilizados na reação de PCR foram água Ultra-pura, glicerol a 50%, tampão 1X, 0,8 mM de dNTP, 0,2 pmol/µl de cada primer, sense (5' TGA AGG AGA AGG TGT CTG CGG GA 3') e anti-sense (5' AGG ACG GTG CGG TGA GAG TG 3'), 2,0 mM de $MgCl_2$, 1U da enzima Taq DNA polimerase e 200 ng de DNA. A ciclagem utilizada para amplificação consistiu de um ciclo inicial a 94°C por 4 minutos, seguido de 30 ciclos a 94°C por 1 minuto, 59°C por 50 segundos e 72°C por 50 segundos, e uma etapa final a 72°C por 10 minutos.

Os produtos de PCR foram submetidos à digestão com a enzima *Hinf I*. Para essa reação foram utilizados água Ultra-pura, tampão 1X e 5U de *Hinf I*. O produto de amplificação de 198 pares de base (pb) derivado do alelo selvagem 677C não é digerido pela *Hinf I*. O produto do mesmo tamanho derivado do alelo polimórfico 677T é digerido pela enzima em fragmentos de 175 pb e 23 pb. Os indivíduos heterozigotos apresentam os três fragmentos (198 pb, 175 pb e 23 pb). A eletroforese ocorreu em gel de agarose 2,5%, a 110 Volts por 90 minutos.

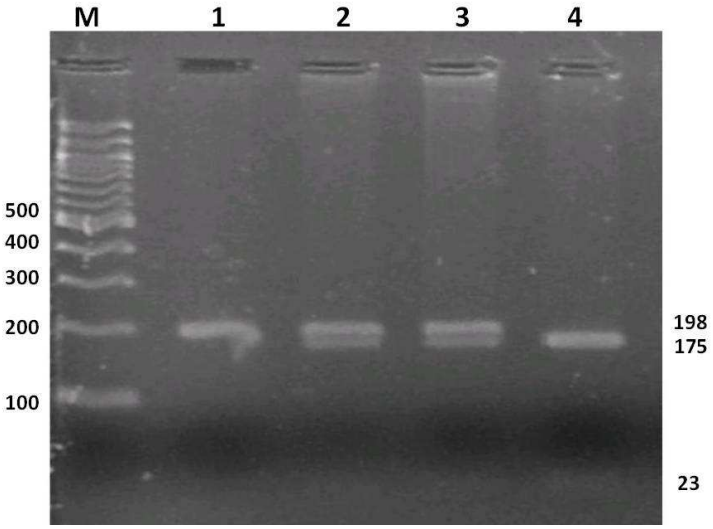


Figura 1. Gel de agarose 2,5% apresenta os genótipos do polimorfismo C677T do gene *MTHFR.* **Coluna M:** marcador de peso molecular 100 pb. **Coluna 1:** genótipo homozigoto selvagem CC. **Colunas 2 e 3:** genótipo heterozigoto CT. **Coluna 4:** genótipo homozigoto polimórfico TT.

3.2. Polimorfismo A1298C no gene *MTHFR*

O DNA genômico foi amplificado pela técnica de PCR-RFLP. Para a PCR, os reagentes utilizados foram água Ultra-pura, tampão 1X, 0,8 mM de dNTP, 0,2 pmol/µl de cada primer, sense (5' CAA GGA GGA GCT GCT GAA GA 3') e anti-sense (5' CAA CTC CAG CAT CAC TCA CT 3'), 2,0 mM de $MgCl_2$, 1U da enzima Taq DNA polimerase e 200 ng de DNA. A ciclagem utilizada para amplificação consistiu de um ciclo inicial a 94°C por 4 minutos, seguido de 30 ciclos a 94°C por 1 minuto, 60°C por 1 minuto e 72°C por 1 minuto, e uma etapa final a 72°C por 10 minutos.

Os produtos de PCR foram submetidos à digestão com a enzima Mbo *II*. Para essa reação foi utilizado água Ultra-pura, tampão 1X e 5U de *Mbo II*. O tamanho do amplificado foi de 128 pb e após a digestão enzimática foram gerados fragmentos de 72 pb e 2 fragmentos de 28 pb na presença do alelo

selvagem A, e fragmentos de 100 e 28 pb na presença do alelo polimórfico C. A eletroforese ocorreu em gel de agarose 2,5%, a 110 Volts por 90 minutos.

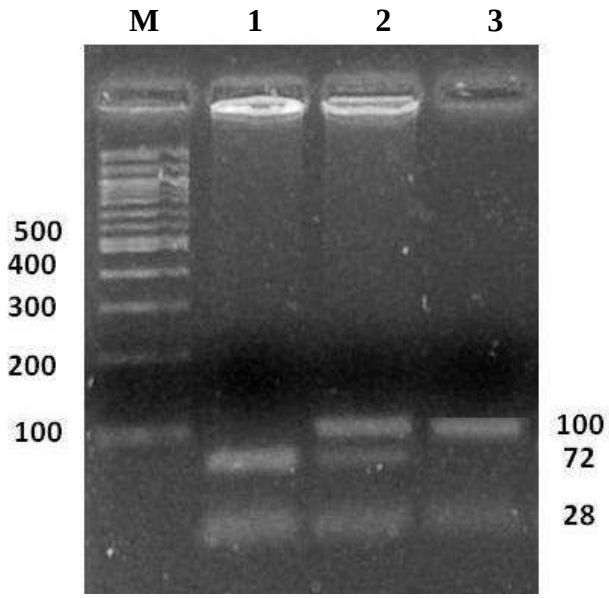


Figura 2. Gel de agarose 2,5% apresenta os genótipos do polimorfismo A1298C do gene *MTHFR.* **Coluna M:** marcador de peso molecular 100 pb. **Coluna 1:** genótipo homozigoto selvagem AA. **Coluna 2:** genótipo heterozigoto AC. **Coluna 3:** genótipo homozigoto polimórfico CC.

3.3. Polimorfismo A2756G no gene *MTR*

A investigação da variante *MTR* A2756G foi realizada através da técnica de PCR-RFLP. Os reagentes utilizados na reação de PCR foram água Ultra-pura, tampão 1X, 0,8 mM de dNTP, 0,2 pmol/µl de cada primer, sense (5' CCA GGG TGC CAG GTA TAC AG 3') e anti-sense (5' GCC TTT TAC ACT CCT CAA AAC 3'), 1,5 mM de $MgCl_2$, 1U da enzima Taq DNA polimerase e 100 ng de DNA. A ciclagem utilizada para amplificação consistiu de um ciclo inicial a 94°C por 4 minutos, seguido de 30 ciclos a 94°C por 1 minuto, 56°C por 1 minuto e 72°C por 1 minuto, e uma etapa final a 72°C por 10 minutos.

Os produtos de PCR foram submetidos à digestão com a enzima *Hae III*. Para essa reação foi utilizado água Ultra-pura, tampão 1X e 5U de *Hae III*. O tamanho do amplificado foi de 498 pb e após a digestão enzimática foram gerados fragmentos de 290, 123 e 85 pb na presença do alelo polimórfico G, e fragmentos de 413 e 85 pb na presença do alelo selvagem A. A eletroforese ocorreu em gel de agarose 2,0%, a 110 Volts por 90 minutos.

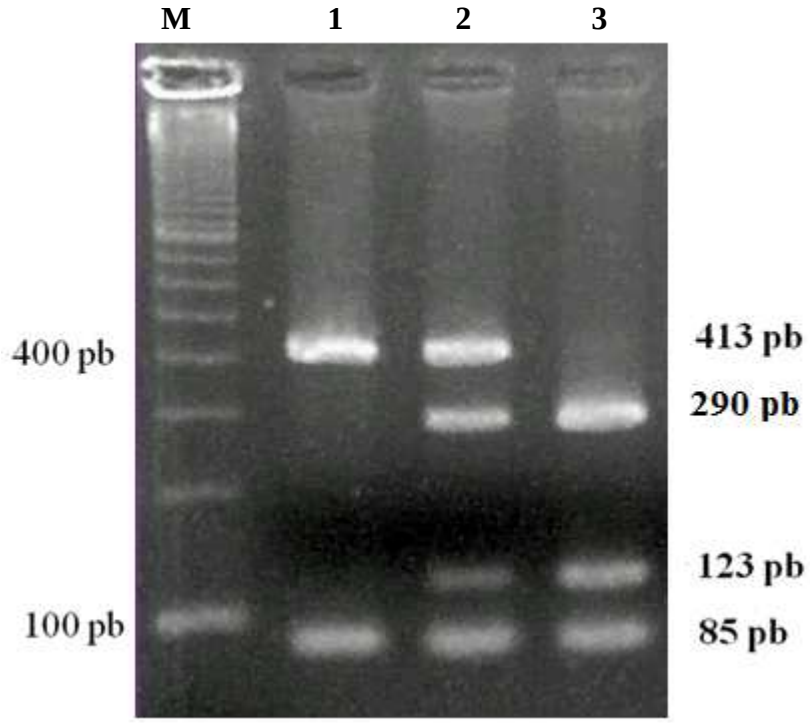


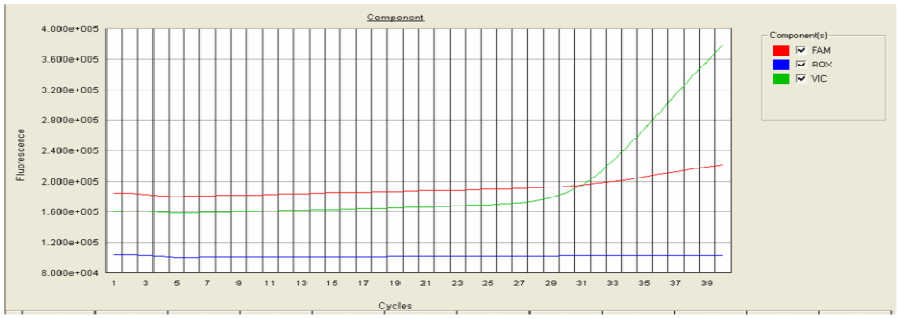
Figura 3. Gel de agarose 2,0% apresenta os genótipos do polimorfismo A2756G do gene *MTR.* **Coluna M:** marcador de peso molecular 100 pb. **Coluna 1:** genótipo homozigoto selvagem AA. **Coluna 2:** genótipo heterozigoto AG. **Coluna 3:** genótipo homozigoto polimórfico GG.

3.4. *MTRR* A66G

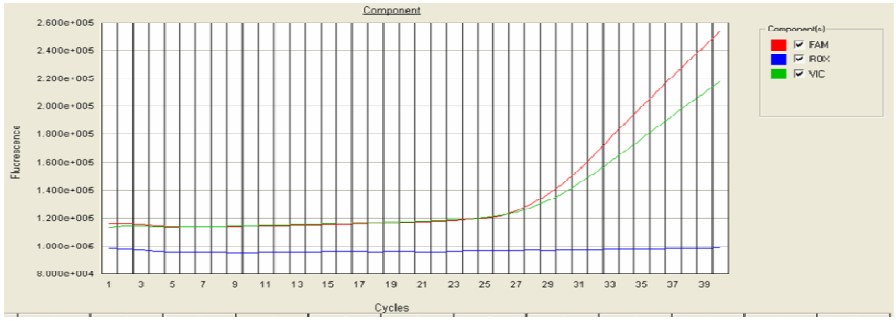
A genotipagem do polimorfismo *MTRR* A66G foi realizada pela técnica de Discriminação Alélica por PCR em Tempo Real utilizando-se o *SNP Genotyping Assay* (*Applied Biosystems*), com *primers* e sondas específicas para cada alelo (*MTRR* A66G: C__3068176_10 – 20X). A reação foi realizada com água tratada com dietilpirocarbonato (DEPC), *Master Mix Genotyping* 0,8X, *Assay* 0,8X. As reações foram submetidas ao equipamento *Step One Plus TM Real-Time PCR System*, com a ciclagem de 95°C por 10 minutos, seguido de 50 ciclos a 92°C por 15 segundos e 60°C por 1 minuto.

O resultado da reação de PCR em tempo real dá-se através da emissão de fluorescência emitida pelas sondas e captada pelo equipamento. Uma vez que cada sonda é marcada com um fluoróforo diferente, foi possível determinar o genótipo da amostra por meio da leitura da fluorescência emitida, visualizada na tela do equipamento com cores distintas. Utilizou-se o software SDS versão 2.0 para analisar a fluorescência emitida em tempo real ao final da reação de PCR (leitura *endpoint*).

I



II



III

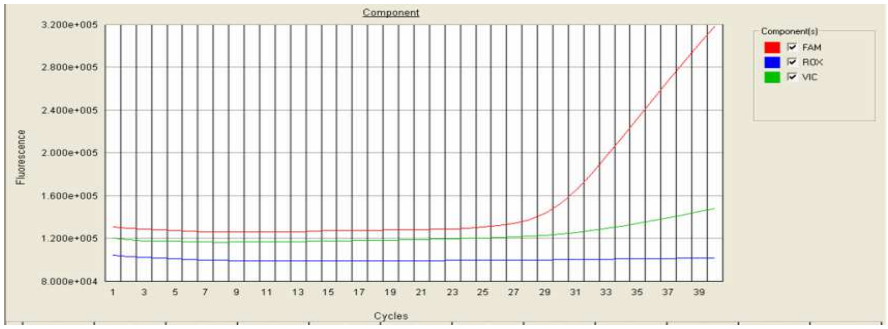


Figura 4. Curvas da discriminação alélica produzidas pela análise no software *Step One*. O eixo X representa o número de ciclos de amplificação e o eixo y, o valor da fluorescência. **I:** genótipo homozigoto selvagem (AA); **II:** genótipo heterozigoto (AG); **III:** genótipo homozigoto polimórfico (GG). O fluoróforo VIC (verde) representa o alelo A e o fluoróforo FAM (vermelho) representa o alelo G.

4. Classificação clínico-patológica

Os tumores foram classificados de acordo com os parâmetros da Union International Control Cancer (UICC) em três critérios: tamanho do tumor (T), presença de linfonodos regionais comprometidos (N) e presença de metástase à distância (M), conforme descrito na Tabela 1 da Introdução (página 8). Além da classificação TNM, os tumores de mama também foram classificados nos subtipos: Luminal A (RE+ e/ou RP+. HER2- e Ki-67 <14%), Luminal B (RE+ e/ou RP+, HER2 negativo ou positivo e Ki-67 ≥14%), Superexpressão HER2 (RE-, RP- e HER2+) e Triplo Negativo (RE-, RP- e HER2+), de acordo com Perou et al. (2000), Cirqueira et al. (2011) e Sikandar et al. (2015).

O diagnóstico, a classificação do TNM e a classificação fenotípica foram obtidos por meio do prontuário médico do paciente. Os dados obtidos foram depositados em banco de dados informatizado construído para o projeto na Unidade de Pesquisa em Genética e Biologia Molecular (UPGEM).

5. Estatística

O teste de Regressão Logística Múltipla foi utilizado para determinar o efeito das variáveis no risco de Câncer de mama e os parâmetros clínicos-patológicos, utilizando o programa MINITAB/Windows – versão 14.0. As variáveis são: idade (referência, mediana, <50 anos), hábito tabagista (referência: não fumante), consumo de bebida alcoólica (referência: não consumidores de álcool), número de gestações (referência: ≥ 3 gestações), IMC (referência: <25 Kg/m^2), terapia hormonal (referência: não uso), *MTHFR* C677T (referência: genótipo CC), *MTHFR* A1298C (referência: genótipo AA),

MTR A2756G (referência: genótipo AA), *MTRR* A66G (referência: genótipo AA), Tamanho do tumor (referência: T1 e T2), Linfonodos (referência: negativo) e metástase (referência: ausência). Foram considerados tabagistas indivíduos que consumiram cerca de 100 cigarros durante toda a vida (Ahrendt et al., 2000) e etilistas aqueles que consomem mais de quatro drinks por semana (cada drink equivale aproximadamente a 30 ml de licor, 102 ml de vinho e 340 ml de cerveja). (Carpenter et al., 1998; Kjaerhein et al., 1998; Ahrendt ET al., 2000; Russo et al., 2013)

O Equilíbrio de Hady-Weinberg (HWE) foi avaliado por meio do teste Qui-quadrado, utilizando o programa online SNPstats (http:// http://bioinfo.iconcologia.net/SNPstats). Os polimorfismos foram avaliados nos modelos codominante, domintante, recessivo, *overdominat* e aditivo, utilizando o programa SNPstatis. A relação entre os polimorfismos e os fatores clínicos-patológicos também foram avaliados pelo programa SNPstatis. Os resultados foram apresentados em odds ratio (OR) e intervalo de confiança (IC) de 95%. O valor de p menor que 0,05 foi considerado estatisticamente significante para todos os resultados.

ANEXO 2

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

(Conselho Nacional de Saúde, resolução 466/12)

Título da Pesquisa: Avaliação de marcadores moleculares e clínicos em câncer de mama

Pesquisadora Responsável: Profa. Dra. Eny Maria Goloni-Bertollo - UPGEM: Unidade de Pesquisa em Genética e Biologia Molecular da FAMERP;

A) Este estudo tem como objetivos: 1) Coletar informações da história e obter dados clínicos dos prontuários médicos dos pacientes com câncer de mama atendidos no Serviço de Atendimento Ambulatorial vinculado ao Departamento de Ginecologia e Obstetricia/Mastologia do Hospital de Base de São José do Rio Preto. 2) Analisar alterações em genes (material hereditário) com a finalidade de esclarecer o papel de fatores genéticos no desenvolvimento do tumor;

B) Para este estudo serão utilizados dois grupos de pessoas: 1) pacientes com câncer de mama; 2) indivíduos do grupo controle, não portadores do tumor;

C) O estudo será feito utilizando-se sangue, que será colhido com seringa descartável por enfermeira e o risco da colheita pode incluir inchaço e vermelhidão no local , sem qualquer outro risco para minha saúde;

D) O material (sangue) será identificado no laboratório por código formado por números e letras e, portanto, minha privacidade e identidade serão preservadas;

E) O material genético (DNA), ou seja hereditário, extraído do sangue será armazenado no laboratório, compondo um banco de amostras biológicas, podendo ser utilizado em futuros estudos;

F) Todas as informações por mim fornecidas por meio do questionário e os resultados serão mantidos em sigilo e que, estes últimos só serão utilizados para divulgação em reuniões e revistas científicas;

G) Se eu concordar em participar desta pesquisa e se eu concordar com a retirada e uso do meu sangue, do modo descrito acima, não terei quaisquer benefícios ou direitos financeiros sobre os eventuais resultados decorrentes da pesquisa. Se eu não concordar, em doar o sangue para a pesquisa ou decidir retirar meu consentimento em qualquer momento, minha decisão não influenciará, de nenhum modo, o meu tratamento;

H) Esse estudo é importante porque pode colaborar para conhecimento científico dos mecanismos envolvidos no desenvolvimento do tumor.

Declaro que, após ter convenientemente esclarecido pelo pesquisador, consinto em participar livre e espontaneamente deste estudo sem que tenha sido submetido a qualquer tipo de pressão.

Assim, consinto em participar do projeto de pesquisa em questão.

Nome do(a) participante:

Representante legal:

RG do prontuário médico:

Data:...../...../...../ Assinatura:.....

Declaração de responsabilidade: Expliquei a natureza, objetivos, riscos e benefícios deste estudo. Coloquei-me a disposição para perguntas e respondi a todas. Obtive o consentimento de maneira livre e me coloquei à disposição para esclarecimento de qualquer dúvida sobre o estudo pelos endereços abaixo indicados.

Nome do(a) pesquisador:

Data...../...../...../ Assinatura:.....

Inscrição no Conselho Regional: .....

Profa. Dra. Eny Maria Goloni-Bertollo – Departamento de Biologia Molecular

Av. Brigadeiro Faria Lima, no. 5416

FAMERP - Faculdade de Medicina de S.J. Rio Preto

São José do Rio Preto, SP - CEP 15090-000 Fone: (17) 3201-5720 **e-mail:** **eny.goloni@famerp.br**

Anexo 3

QUESTIONÁRIO

Dados Gerais:

Nome:_____

Prontuário:_____Telefone:_____

Data de Nascimento e local:_____

Endereço:_____

Nº____Bairro:_____Cidade:_____

CEP:_____

Etnia:_____Profissão:_____Escolaridade:_____

Data da coleta:_____

Histórico Médico

Fatores de risco:

Exposição ao Álcool: ( ) Sim ( ) Não Duração:_____

Exposição ao Tabaco: ( ) Sim ( ) Não ( ) Ex-fumante Duração:_____

Casos na família e Local:_____

Peso:_____ Altura:_____

Terapia Hormonal Anticoncepcional: ( ) Sim ( ) Não Outros:_____

Número de Gestações:____ Filhos Vivos:_____ Abortos:_____ Natimorto:____

Diabetes: ( ) Sim ( ) Não Hipertensão: ( ) Sim ( ) Não

Outras:_____

Dados do Tumor:

Diagnostico: ( ) Image ( ) Biopsia

Tipo:_____

TRATAMENTO RECEBIDO:

Quimioterapia: ( ) Sim ( ) Não Radioterapia: ( ) Sim ( ) Não

Cirurgia: ( ) Sim ( ) Não Qual:_____

Linfonodo: ( ) Sim ( ) Não

Metástase: ( ) Sim ( ) Não Local:_____

Responsável pela entrevista:_____

Anexo 4

Aprovação do Comitê de Ética em Pesquisa

FACULDADE DE MEDICINA DE SÃO JOSE DO RIO PRETO- FAMERP - SP

Plataforma Brasil

PROJETO DE PESQUISA

Título: Avaliação de marcadores moleculares e clínicos em câncer de mama

Área Temática:

Versão: 4

CAAE: 04069612.1.0000.5415

Pesquisador: Eny Maria Goloni-Bertollo

Instituição: Faculdade de Medicina de São Jose do Rio Preto- FAMERP - SP

PARECER CONSUBSTANCIADO DO CEP

Número do Parecer: 84397

Data da Relatoria: 14/08/2012

Apresentação do Projeto:

O câncer de mama é uma preocupação mundial, sendo esta a neoplasia mais comum entre as mulheres e a quinta maior causa de morte relacionada ao câncer. Algumas alterações genéticas podem desencadear esse tipo de tumor, como a metilação do DNA associadas à deficiência de folato. Polimorfismos em genes que participam da via do folato e metabolizadores de xenobióticos tem sido investigados como fatores de risco para susceptibilidade ao câncer, entre eles, polimorfismos nos genes MTHFR, MTR, MTRR, GSTT1, GSTM1, CYP1A1*2A e CYP1A1*2C. Os objetivos deste projeto são avaliar a freqüência dos polimorfismos nestes genes em pacientes com câncer de mama comparando-a com aquela observada em indivíduos sem história de neoplasia (controles) e avaliar a associação destes polimorfismos com os parâmetros clínicos/epidemiológicos. A identificação de associação desses polimorfismos com o desenvolvimento de carcinoma mamário poderão auxiliar no entendimento dos mecanismos envolvidos no processo neoplásico.

Objetivo da Pesquisa:

Objetivo Primário:
Este projeto tem como objetivo geral a identificação de variantes genéticas e o desenvolvimento de carcinoma de mama.
Objetivo Secundário:
Investigar a frequência dos polimorfismos nos genes MTHFR (C677T e A1298C), MTR (A2756G), MTRR (A66G), GST(M1 e T1), CYP1A1*2A e CYP1A1*2C em pacientes com câncer de mama e comparando-a com aquela observada em indivíduos sem história de neoplasia;
Avaliar a associação dos polimorfismos com o desenvolvimento de tumores de mama e fatores de risco (idade, aspectos endócrinos e genéticos);
Comparar as freqüências dos polimorfismos com os diferentes tipos de sítios de ocorrência, extensão, comprometimento e agressividade do tumor.

Avaliação dos Riscos e Benefícios:

Os riscos serão mínimos, apenas relacionados a coleta de sangue que pode causar um hematoma e ficar dolorido. Benefícios: A associação dos polimorfismos investigados em carcinoma mamário e também a relação destes com os dados clínicos/epidemiológicos poderão contribuir para o diagnóstico, prognóstico e possíveis tratamentos personalizados.

Endereço: BRIGADEIRO FARIA LIMA
Bairro: VILA SAO JOAO **CEP:** 15.090-000
UF: SP **Município:** SAO JOSE DO RIO PRETO
Telefone: 1732-0158 **Fax:** 1732-0158 **E-mail:** analucialima@famerp.br

FACULDADE DE MEDICINA DE SÃO JOSE DO RIO PRETO- FAMERP - SP

Comentários e Considerações sobre a Pesquisa:

O estudo poderá elucidar mecanismos genéticos envolvidos na metilação do DNA associado a deficiência do folato, cuja baixa concentração destes pode resultar em alterações malignas, devido a mudança de função e expressão de certos genes. Daí a necessidade de se investigar a associação destes polimorfismos com os parâmetros clínicos/epidemiológicos. A identificação de associação desses polimorfismos com o desenvolvimento de carcinoma mamário poderá auxiliar no entendimento dos mecanismos envolvidos no processo neoplásico.

Considerações sobre os Termos de apresentação obrigatória:

O projeto apresenta todos os requisitos legalmente exigidos.
O termo de consentimento é adequado e contém todas as informações necessárias e cumpre as solicitações da Resolução 196/96, no entanto, deverá ser readequado.
O protocolo de pesquisa, as condições e a experiência do pesquisador são compatíveis com o proposto.

Recomendações:

Pendências no TCLE supridas, portanto, dentro das normas especificadas.

Conclusões ou Pendências e Lista de Inadequações:

Todas as recomendações foram acatadas e adequadas pelo pesquisador.
Assim, concluo que com a utilização de marcadores envolvidos nos mecanismos genéticos associados a fisiopatogenia destas neoplasias, possibilitará que a doença seja prevenida pela profilaxia ou pelo tratamento preventivo dirigido pelo monitoramento genético, bem como permitirá fazer prognósticos mais favoráveis para as pacientes.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

SAO JOSE DO RIO PRETO, 29 de Agosto de 2012

Assinado por:
Fernando Batigália

Endereço: BRIGADEIRO FARIA LIMA
Bairro: VILA SAO JOAO **CEP:** 15.090-000
UF: SP **Município:** SAO JOSE DO RIO PRETO
Telefone: 1732-0158 **Fax:** 1732-0158 **E-mail:** analucialima@famerp.br