**Faculdade de Medicina de São José do Rio Preto**
**Programa de Pós-graduação em Ciências da Saúde**

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# AVALIAÇÃO DE POLIMORFISMOS NOS GENES *MTHFR*, *MTR*, *RFC1* E *CßS* ENVOLVIDOS NO METABOLISMO DO FOLATO EM PACIENTES COM CÂNCER DE TIREOIDE

**São José do Rio Preto**
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Tairine Zara Lopes

# Avaliação de Polimorfismos nos Genes *MTHFR*, *MTR*, *RFC1* e *CßS* envolvidos no Metabolismo do Folato em Pacientes com Câncer de Tireoide

Dissertação apresentada à Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Mestre no Curso de Pós-Graduação em Ciências da Saúde. Eixo Temático: Medicina e Ciências Correlatas.

Orientadora: Prof[a]. Dr[a]. Eny Maria Goloni Bertollo

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## SUMÁRIO

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*"Tudo tem o seu tempo determinado, e há tempo para todo propósito debaixo do céu. Há tempo de nascer e tempo de morrer; tempo de plantar, e tempo de arrancar o que se plantou..."*

***Eclesiastes 3:1-2***

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## Introdução

# Lista de Tabelas e Quadros

## Artigo I



## Artigo II

## Lista de Abreviaturas e Símbolos

| | |
|---|---|
| BMI | Índice de massa corpórea (*Body-mass index)* |
| Bp | Pares de base *(Base pair)* |
| CAPES | Coordenação de Aperfeiçoamento de Pessoal de Nível Superior *(Coordination for the Improvement of Higher Level -or Education-Personnel)* |
| CI 95% | Intervalo de confiança 95% *(Confidence interval)* |
| CNPq | Conselho Nacional de Desenvolvimento Científico e Tecnológico *(National Council for Scientific and Technological Development)* |
| *CßS* | Cistationina ß-sintase *(cystathionine ß-syntase)* |
| DNA | Ácido desoxirribonicléico *(desoxirribonucleic acid)* |
| EDTA | Ácido etilenodiamino tetra-acético *(Ethylenediamine tetraacetic acid)* |
| FAMERP | Faculdade de Medicina de São José do Rio Preto *(São José do Rio Preto Medical School)* |
| FAPESP | Fundação de Amparo à Pesquisa do Estado de São Paulo *(São Paulo State Research Foundation)* |
| HWE | Equilibrio de Hardy-Weinberg *( Hardy-Weinberg equilibrium)* |
| IMC | Índice de massa corpórea *(Body-mass index)* |
| INCA | Instituto Nacional do Câncer *(Brazilian National Cancer Institute)* |
| M | Metástase à distância *(distant metastasis)* |

| | |
|---|---|
| *MTHFR* | Metilenotetrahidrofolato redutase (*methylenetetrahydrofolate reductase)* |
| *MTR* | Metionina sintase *(methionine synthase)* |
| N | Envolvimento de linfonodos regionais *(Regional lymphnode involvement)* |
| PCR | Reação em cadeia da Polimerase *(Polymerase chain reaction)* |
| PCR-RFLP | Reação em cadeia da Polimerase - Polimorfismo de tamanho do fragmento de restrição *(Polymerase chain reaction-restriction fragment lenght polymorphism* |
| RDC | Resolução da diretoria colegiada *(board resolution)* |
| *RFC1* | Carregador de folato reduzido 1 *(reduced folate carrier 1)* |
| SAM | S-adenosilmetionina *(S-adenosylmethionine)* |
| SNPs | Polimorfismo de nucleotídeo único *(single-nucleotide polymorphism)* |
| T | Tamanho de Tumor *(tumor extension)* |
| T3 | Tri-iodotironina *(triiodothyronine)* |
| T4 | Tetraiodotironina *(tetraiodothyronine)* |
| TC | Câncer de Tireoide *(Thyroid cancer)* |
| TNM | Classificação dos Tumores *(TNM classification)* |
| TSH | Hormônio estimulante da tireoide *(thyroid stimulating hormone)* |
| UICC | União de Controle Contra o Câncer *(International Union of Cancer Control)* |
| UPGEM | Unidade de Pesquisa em Genética e Biologia Molecular *(Genetics and Molecular Biology Reasearch Unit)* |

**Resumo**

**Introdução:** O câncer de tireoide é a neoplasia maligna mais comum do sistema endócrino e vem apresentando contínuo aumento nos últimos anos. Estudos sugerem que a deficiência de folato no organismo diminui a reparação do DNA, resultando em alterações celulares malignas que modulam a expressão gênica, podendo levar ao desenvolvimento de vários tipos de câncer. Polimorfismos em genes envolvidos na via do folato têm sido investigados como fatores de risco para suscetibilidade ao câncer, entre eles, polimorfismos nos genes *MTHFR, MTR, RFC1* e *CßS.* **Objetivos:** Investigar a associação dos polimorfismos nos genes *MTHFR* (C677T), *MTR* (A2756G), *RFC1* (A80G) e *CßS* (844ins68) no risco de câncer de tireoide em um estudo caso-controle; Avaliar a associação dos polimorfismos com o gênero, idade, consumo de álcool e tabaco, índice de massa corpórea (IMC) no desenvolvimento do câncer de tireoide; Avaliar a associação entre os polimorfismos e os parâmetros clínico-histopatológicos do câncer de tireoide. **Casuística e Método:** Este estudo incluiu 462 indivíduos (151 pacientes com câncer de tireoide e 311 indivíduos controles). Foi coletado sangue periférico e extraído o DNA genômico. Os polimorfismos *MTHFR* (C677T), *MTR (*A2756G) e *RFC1* (A80G) foram avaliados por meio da PCR-RFLP e o polimorfismo *CßS* (844ins68) foi analisado por PCR convencional sem corte enzimático. Para análise estatística utilizou-se o teste do qui-quadrado e regressão logística múltipla. **Resultados:** Os resultados mostraram que os polimorfismos *MTHFR* C677T (OR=2.87, 95% IC=1.50-5.48, p< 0.01, modelo codominante), (OR=1.76, 95% IC=1.18-2.64, p< 0.01, modelo dominante), (OR=2.37, 95% IC=1.28-4.39, p< 0.01, modelo recessivo) e *RFC1* A80G (OR: 1.55; 95% IC: 1.02-2.38; p=0.04, modelo recessivo) estão associados

ao câncer de tireoide. O consumo de álcool (OR=1.56, 95% IC=1.36-1.89, p< 0.01) e tabaco (OR=1.97, 95% IC=1.28-3.04, p< 0.01) foram estatisticamente significantes, sendo associados ao aumento do risco. O polimorfismo *MTR* A2756G está associado à extensão do tumor (OR=2.69, 95% IC=1.27-5.71, p< 0.01) e à agressividade (OR= 4.51, 95% IC=1.67-12.1, p< 0.01). **Conclusões:** Os polimorfismos *MTHFR* (C677T) e *RFC1* (A80G) estão envolvidos no risco de câncer de tireoide. Adicionalmente, o consumo de álcool e tabaco aumenta o risco de desenvolvimento da doença.

**Palavras-chave:** Câncer de tireoide, Fatores de risco; Polimorfismo genético

**Abstract**

**Introduction:** Thyroid cancer is the most common malignancy of the endocrine system and has been presenting continuous increase in the last years. Studies suggest that folate deficiency in the body decrease DNA repair, resulting in malignant cells changes that alter expression of genes, and may induce several kinds of cancer development. Polymorphisms in genes involved in folate pathway have been investigated as risk factors for susceptibility to cancer, among them *MTHFR*, *MTR*, *RFC1* and *CßS*. **Objectives:** To investigate association of polymorphisms in the *MTHFR* (C677T), *MTR (*A2756G), *RFC1* (A80G) and *CßS* (844ins68) genes in risk thyroid cancer in a case-control study; to evaluate the association of polymorphisms with gender, age, alcohol and tobacco consumption, body-mass index in thyroid cancer development; and to evaluated the association between polymorphisms and clinical-histopathological parameters. **Methods:** This study included 462 individuals (151 patients with thyroid cancer and 311 controls). The peripheral blood was collected and genomic DNA was extracted. The *MTHFR* (C677T), *MTR (*A2756G) and *RFC1* (A80G) were evaluated by PCR-RFLP and *CßS* (844ins68) by conventional PCR without enzymatic digestion. For statistical analysis chi-square and multiple logistic regression were used. **Results:** The results showed that *MTHFR* C677T (OR=2.87, 95% CI=1.50-5.48, p< 0.01, codominant model), (OR=1.76, 95% CI=1.18-2.64, p< 0.01, dominant model), (OR=2.37, 95% CI=1.28-4.39, p< 0.01, recessive model) and *RFC1* A80G (OR: 1.55; 95% CI: 1.02-2.38; p=0.04, recessive model) were associated with thyroid cancer. The alcohol (OR=1.56, 95% CI=1.36-1.89, p< 0.01) and tobacco consumption (OR=1.97, 95% CI=1.28-3.04, p< 0.01) were statistically significant, being associated with increased risk. The *MTR* A2756G is associated

with tumor extension (OR=2.69, 95% CI=1.27-5.71, p< 0.01) and aggressiveness (OR= 4.51, 95% CI=1.67-12.1, p< 0.01). **Conclusions:** The *MTHFR* (C677T) and *RFC1* (A80G) polymorphisms were involved in risk for thyroid cancer. Additionally, alcohol and tobacco consumption increase risk for disease development.

**Key words:** Thyroid Cancer, Risk Factors, Genetic Polymorphism.

# ***1. INTRODUÇÃO***

## 1. INTRODUÇÃO

A glândula tireoide é considerada uma das maiores glândulas endócrinas, é capaz de secretar seu produto diretamente na corrente sanguínea. Formada por dois lobos divididos pelo istmo, está localizada na região cervical anterior à laringe e se desenvolve a partir de uma invaginação do epitélio parafaríngeo. O peso da glândula é influenciado pela ingestão de iodo, idade e peso corporal, que varia de 10 a 20 g em indivíduos adultos normais. (1)

Histologicamente é formada por dois tipos de células: as células foliculares, composta por epitélio simples, dão origem aos folículos tireoidianos, unidades de estruturas esféricas que armazenam coloide e produzem os hormônios T3 (tri-iodotironina) e T4 (tetraiodotironina); e as células parafoliculares ou células C que são capazes de sintetizar o hormônio calcitonina, relacionado com a redução do nível de cálcio no plasma. (2)

A função da glândula tireoide consiste em produzir, armazenar e secretar os hormônios T3 e T4 que, por sua vez, regulam o metabolismo corporal e o funcionamento dos órgãos. A produção dos hormônios tireoideos é estimulada pelo hormônio TSH (hormônio tireoestimulante), liberado pela hipófise. Quando a produção dos hormônios tireoideos é reduzida ou elevada, caracteriza-se por hipotireoidismo e hipertireoidismo, respectivamente. (3)

O funcionamento inadequado da glândula tireoide provoca algumas anormalidades como o bócio, que é o aumento da glândula, as doenças autoimunes e até nódulos. O nódulo de origem maligna dá origem ao carcinoma, que constitui a neoplasia maligna mais frequente do sistema endócrino, embora represente cerca de 1-1,5% de

todas as neoplasias humanas. [4] Além disso, vários estudos tem relatado contínuo aumento deste tipo de câncer. [5, 6, 7]

No Brasil, as estimativas do Instituto Nacional do Câncer (INCA) apontam para o biênio 2014/2015 uma incidência de 9.200 casos novos, sendo 8.050 em mulheres e 1.150 em homens. Apresenta-se como o quinto tipo de câncer mais incidente em mulheres, principalmente na faixa etária dos 25 aos 65 anos de idade. A razão de casos entre os gêneros feminino e masculino é de 4:1. [8, 9]

O câncer de tireoide é classificado de acordo com o tipo histológico em carcinoma diferenciado, que compreende os tipos papilífero e folicular, e indiferenciado incluindo apenas o carcinoma anaplásico, por fim tem-se o carcinoma medular derivado das células parafoliculares. [10, 11, 12]

O carcinoma papilífero e folicular representam cerca de 90% dos casos e constitui uma forma menos agressiva com um bom prognóstico, a sobrevida em cinco anos pode chegar até 90%. Apesar da baixa agressividade na maioria dos casos, o acometimento de linfonodos regionais e metástases para outros órgãos podem ocorrer, levando o paciente ao óbito. [13] O carcinoma anaplásico é a forma mais rara e extremamente agressiva da doença, com uma frequência de apenas 3% acomete principalmente idosos acima de 65 anos, há crescimento celular muito acelerado formando uma massa tireoidiana, o prognóstico é ruim, a sobrevida é de 20% em um ano. [14] Derivado das células parafoliculares, o carcinoma medular tem uma frequência de 5% dos tumores malignos, sendo 80% de origem esporádica e 20% familiar e em cinco anos a sobrevida é de 30 a 80%. [15]

Com exceção da radiação ionizante na região da cabeça e pescoço, muitos fatores de risco para o câncer de tireoide ainda são mal compreendidos e pobremente

caracterizados. Fatores de risco como o consumo de álcool e tabaco e a obesidade ainda não estão completamente esclarecidos como fatores predisponentes para o desenvolvimento deste tipo de câncer, assim como os fatores genéticos envolvidos. [16]

Os dados da literatura são controversos, pois nas metanálises de Ma Jie *et al* [17]e Cho *et al* [18], foi relatada uma associação significante entre o consumo de tabaco e aumento do risco para o câncer de tireoide, assim como é em outros tipos de câncer. Curiosamente, muitos autores tem relatado o inverso, sendo o consumo de álcool e tabaco fatores que diminuem o risco para o desenvolvimento da doença. [19, 20, 21, 22] Assim acontece com o índice de massa corpórea (IMC) elevado, estudos tem apontado relação de risco com certa cautela, uma vez que são requeridos estudos em grande escala para melhor compreensão do envolvimento da obesidade e o câncer. [23, 24, 25]

Sabe-se que a produção dos hormônios tireóideos é estimulada pelo TSH produzido na hipófise, assim os níveis de TSH no sangue têm sido avaliados como fator de predisposição à malignidade, pois a elevação da concentração deste hormônio tem sido associada à hiperplasia da tireoide e aumento da transformação maligna. [26, 27]

Além dos fatores de risco citados acima, os hábitos alimentares tem sido investigados para o desenvolvimento de diversos tipos de câncer, devido ao grande potencial que os micronutrientes como as vitaminas B, C e E, carotenoides, entre outros, tem para proteger contra lesões oxidativas do DNA, uma vez que possuem propriedades anticarcinogênicas. [28]

O folato é um micronutriente responsável pela doação de grupos metil para as reações de metilação celular e sua ingestão inadequada pode estar associada à etiologia do câncer, pois as reações de síntese, metilação e reparo do DNA dependem de quantidades adequadas de folato no organismo. [29, 30] Os níveis desse micronutriente

podem ser alterados pela presença de polimorfismos em genes que codificam enzimas envolvidas no metabolismo do folato e assim ativam o processo de carcinogênese, pois induzem a hipometilação do DNA com subsequente ativação de proto-oncogenes e provocam erros de incorporação da uracila durante a síntese de DNA, que acarreta instabilidade genômica. [31]

**Polimorfismos genéticos envolvidos no metabolismo do folato**

O gene Metilenotetrahidrofolato redutase (*MTHFR)* apresenta-se polimórfico no nucleotídeo 677, há substituição de uma citosina por timina (C677T) e está associado à redução da atividade enzimática, pois limita a conversão de 5,10 - Metilenotetrahidrofolato para 5- Metilenotetrahidrofolato, a principal forma circulante de folato, que atua como doador de grupos metil para a remetilação da homocisteína (Hcy) para metionina. [32]

Analisando os dados da literatura, constatou-se que o polimorfismo *MTHFR* C677T é a única variante genética envolvida no metabolismo do folato avaliada em câncer de tireoide e inclui os estudos de Siraj *et al*., [33] Fard-Esfahani et *al*., [34] Ozdemir *et al.,* [35]; Sun-Seog *et al.,* [36] e a metanálise de Yang *et al.* [37]

Dentre esses estudos, Fard-Esfahani *et al*., [34] Ozdemir *et al.,* [35] e Yang *et al.,* [37] encontraram associação do polimorfismo e aumento do risco de câncer de tireoide.

Fard-Esfahani *et al*., [34] avaliaram 154 pacientes diagnosticados com carcinoma diferenciado de tireoide e 198 indivíduos saudáveis em uma população do Irã e encontrou que o genótipo 677TT aumenta o risco para a doença, em concordância com

os estudos de Ozdemir *et al.,* [35] com 60 pacientes e 50 controles na população turca e de Yang *et al.,* [37] que incluiu quatro estudos em sua metanálise.

A enzima Metionina sintase (*MTR*) cataliza a reação de remetilação da homocisteína (Hcy) para metionina, que resulta na formação de S-Adenosilmetionina (SAM) que está envolvido nas reações de metilação do DNA. [38] Um polimorfismo comum nesse gene é o *MTR* A2756G, que na posição 2756 há substituição de uma adenina por guanina, que resulta na substituição do aminoácido ácido aspártico por glicina e está relacionado com as alterações no metabolismo do folato, possivelmente influenciando no risco de desenvolvimento de câncer [31, 38] Com exceção do câncer de tireoide este polimorfismo tem sido avaliado em vários tipos de câncer como câncer de cabeça e pescoço [31], esôfago [39], sistema digestivo [40], mama [41] e câncer cervical. [42]

O gene Carreador de folato reduzido 1 (*RFC1*) é responsável pela absorção e transporte de folato intracelular. Apresenta substituição de uma adenina por guanina no nucleotídeo 80, afetando o transporte de 5-MTHFR para o interior das células, assim constitui um importante determinante das concentrações de folato intracelular, podendo ser um modulador do desenvolvimento de vários tipos de câncer. [43]

O gene Cistationina beta sintase (*CßS*) apresenta-se polimórfico quando há 68 pares de base (pb) inseridos no nucleotídeo 844 e tem sido relacionado à redução dos níveis de homocisteína no plasma. [44] Acredita-se que também esteja associado ao aumento da atividade da enzima CßS, que possivelmente diminui as concentrações de homocisteína, assim comprometendo a via de remetilação da homocisteína para metionina, consequentemente reduzindo a síntese de SAM (S-adenosylmethionine) e as reações de metilação celular. [44, 45] Contudo, esta variante ainda não foi avaliada em

câncer de tireoide. A figura 1 representa a via do folato com as principais enzimas envolvidas.

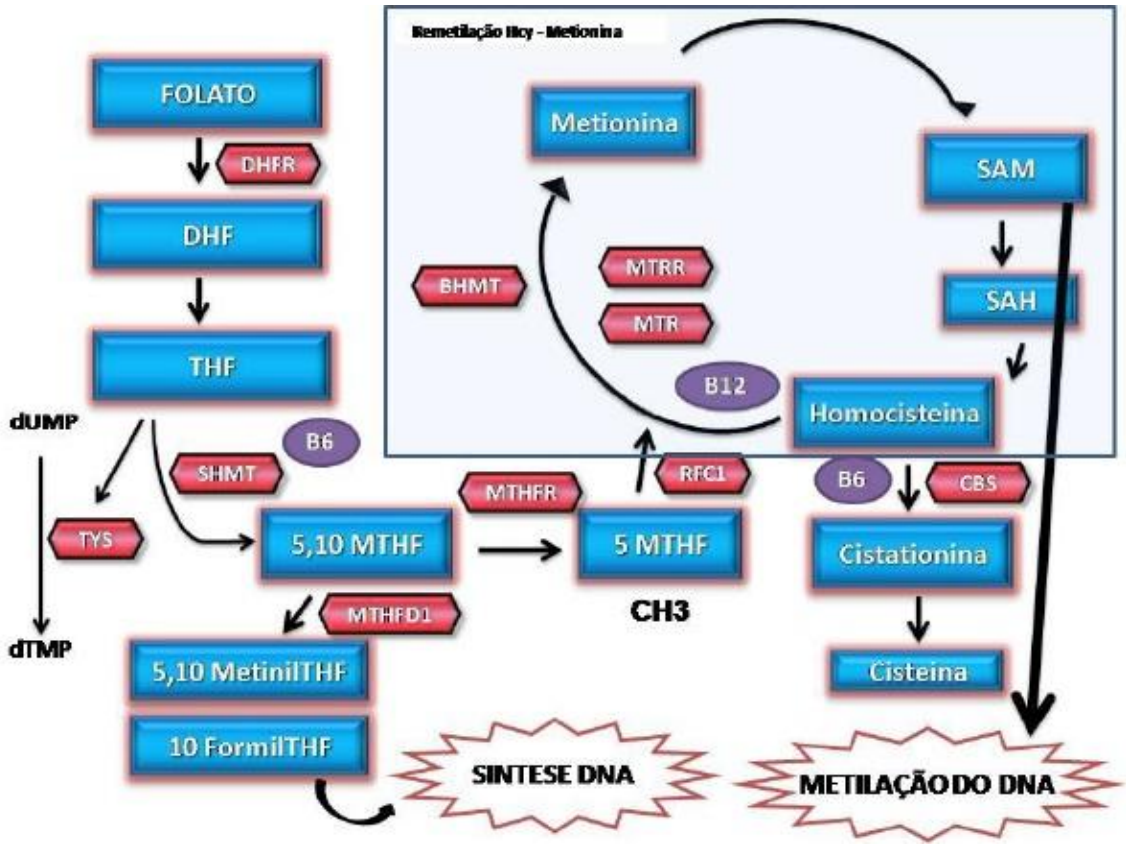


**Figura 1.** Esquema representando o metabolismo do folato com as principais enzimas envolvidas. *DHF*: Dihidrofolato; *THF*: Tetrahidrofolato; *DHFR*: Dihidrofolato redutase; *SHMT*: Serina hidroximetiltransferase; *TYS*: Timidilato sintase; *MTHFD1*: Metilenotetrahidrofolato desidrogenase 1; *MTHFR*: Metilenotetrahidrofolato redutase; *MTR:* Metionina sintase; *MTRR*: Metionina sintase redutase; *BHMT*: Betaína-homocisteína metiltransferase; *CßS*: Cistationina beta-sintase; *RFC1*: Carreador de folato reduzido 1; *SAM:* S-adenosilmetionina; *SAH*: S-adenosilhomocisteína; *dUMP*: Deoxiuridina monofosfato; *dTMP*: Timidina monofosfato (Galbiatti *et al.*, 2010) [31]

## 1.1 OBJETIVOS

Com base nos dados descritos, este estudo teve como objetivos:

1. Investigar a associação dos polimorfismos nos genes *MTHFR* (C677T), *MTR* (A2756G), *RFC1* (A80G) e *CßS* (844ins68) no risco de câncer de tireoide em um estudo caso-controle;

2. Avaliar a associação dos polimorfismos com o gênero, idade, consumo de álcool e tabaco, índice de massa corpórea (IMC), no desenvolvimento do câncer de tireoide;

3. Avaliar a associação entre os polimorfismos e os parâmetros clínico-histopatológicos do câncer de tireoide.

# ***2. ARTIGOS CIENTÍFICOS***

## 2. ARTIGOS CIENTÍFICOS

Os resultados estão apresentados em forma de artigos. No total estão apresentados dois artigos, um submetido à publicação e outro a ser submetido.

**Artigo I**

**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

**Periódico:** *Cancer Science*, submetido

**Artigo II**

**Título:** Polymorphisms in genes *MTHFR, MTR, RFC1* and *CßS* involved in folate metabolism and thyroid cancer: a case- control study

**Autores: Tairine Zara-Lopes**, Leonardo Prado Stuchi, João Armando Padovani-Júnior, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo

**Periódico:** *BMC Cancer*, a ser submetido

# ***ARTIGO CIENTÍFICO I***

# Artigo I

**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

**Periódico:** *Cancer Science*, submetido

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

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There are no conflicts of interest.

**Summary:** Folate metabolism is essential for DNA synthesis and repair. Folate deficiency is directly associated with several types of malignant neoplasms, including thyroid and breast cancer. Polymorphisms in genes coding enzymes involved in folate metabolism may cause alterations in this metabolic pathway inducing the carcinogenesis process. 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 technique was used for genotyping of the polymorphisms. Chi-square and multiple logistic regression were used for statistical analysis. An 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) were observed for the *MTHFR* C677T polymorphism. 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. Thus, it may be a possible prognostic marker for these cancers.

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

## 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 estimative of 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, alcohol and tobacco 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 types of cancer. 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 association 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 were 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 Medical School of São José do Rio Preto (FAMERP) Research Ethics Committee (Thyroid cancer REC approval: 20187413.8.0000.5415; Breast Cancer REC approval: 04069612.1.0000.5415).

**Genotyping**

Peripheral blood samples were collected from all the subjects using EDTA. 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.

**2. Statistical Analysis**

The Hardy-Weinberg equilibrium was evaluated by chi-square test using BioEstat 5.4 computer program. Multiple logistic regression 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 obeses. [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 2 more heterozygote *versus* 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 of *MTHFR* polymorphism 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 ($\chi^2$=2.02, p=0.15) and control group ($\chi^2$=0.28, p=0.59) for *MTHFR* C677T polymorphism. For *MTR* A2756G polymorphism, the equilibrium was only the control group ($\chi^2$=0.11, p=0.73); the case group presented disequilibrium ($\chi^2$=4.38, p=0.03) (Table 1). In Breast Cancer and controls individuals both polymorphisms were in equilibrium (*MTHFR* C677T case group: $\chi^2$=0.006, p=0.93 and control group: $\chi^2$=0.28, p=0.59; *MTR* A2756G case group: $\chi^2$=1.56, p=0.21 and control group: $\chi^2$=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 factors for thyroid cancer. On the other hand, patients 49 and over, as well as alcohol drinking was found no statistically significant. Patients 50 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 breast 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 $\geq 0.05$).

## 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 $\geq$ 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, a 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 met 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 these polymorphisms 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] Guignard 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, time of sample collection 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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**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 (90.9) | 1.0 (ref) | |
| | | T/T | 19 (19) | 13 (9.04) | 2.50 (1.15-5.46) | **0.02*** |
| | | | | 79 (54.9) | | |
| | *Overdominant* | C/C-T/T | 59 (59) | 65 (45.1) | 1.0 (ref) | |
| | | C/T | 41 (41) | --- | 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 (95.8) | 1.0 (ref) | |
| | | G/G | 9 (9) | 6 (4.17) | 1.93 (0.65-5.76) | 0.23 |
| | | | | 95 (66) | | |
| | *Overdominant* | A/A-G/G | 72 (72) | 49 (34) | 1.0 (ref) | |
| | | A/G | 28 (28) | --- | 0.78 (0.44-1.38) | 0.40 |
| | *Aditive* | --- | --- | | 1.07 (0.70-1.64) | 0.76 |

OR, odds Ratio; Adjusted for age, alcohol and tobacco 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* |
|---|---|---|---|---|---|---|
| | *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) | | |
| *MTHFR C677T* | | *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 (90.9) | 1.00 (ref) | |
| | | T/T | 17 (17) | 13 (9.04) | 2.53 (1.08-5.93) | **0.03*** |
| | | | | 79 (54.9) | | |
| | *Overdominant* | C/C-T/T | 52 (52) | 65 (45.1) | 1.0 (ref) | |
| | | C/T | 48 (48) | --- | 0.86 (0.49-1.53) | 0.61 |
| | *Aditive* | --- | --- | | 1.46 (0.96-2.23) | 0.07 |
| | *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) | | |
| *MTR A2756G* | | *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 (95.8) | 1.0 (ref) | |
| | | G/G | 01 (01) | 6 (4.17) | 0.24 (0.03-2.15) | 0.15 |
| | | | | 94 (65.3) | | |
| | *Overdominant* | A/A-G/G | 69 (69) | 50 (34.7) | 1.0 (ref) | |
| | | A/G | 31 (31) | --- | 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 tobacco 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 tobacco consumption, BMI (Body-mass index) and polymorphisms in the recessive model; **p* values significant.

**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

# ***ARTIGO CIENTÍFICO II***

## Artigo II

**Título:** Polymorphisms in genes *MTHFR, MTR, RFC1* and *CßS* involved in folate metabolism and thyroid cancer: a case- control study

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# Polymorphisms in genes *MTHFR, MTR, RFC1* and *CßS* involved in folate metabolism and thyroid cancer: a case- control study

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There are no conflicts of interest.

**ABSTRACT**

Polymorphisms in genes coding enzymes involved in folate metabolism may cause alterations in this metabolic pathway and contribute in carcinogenesis process, because folate is essential for DNA synthesis, methylation and repair. The association of *MTHFR* C677T (rs1801133), *MTR* A2756G (rs1805087), *RFC1* A80G (rs1051266) and *CßS* 844ins68 (rs5745905) polymorphisms was investigated between thyroid cancer (TC) patients and individuals without history of neoplasias. The association these polymorphisms with risk factors and clinical histopathological parameters was also evaluated. A total of 462 individuals (151 patients and 311 controls) were included in the study. The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism technique was used to genotyping. Chi-square and multiple logistic regression were used for statistical analysis. Polymorphism analysis revealed an association between the *MTHFR* C677T polymorphism (OR=2.87, 95% CI=1.50-5.48, p< 0.01, codominant model), (OR=1.76, 95% CI=1.18-2.64, p< 0.01, dominant model), (OR=2.37, 95% CI=1.28-4.39, p< 0.01, recessive model) and *RFC1* A80G (OR: 1.55; 95% CI: 1.02-2.38; p=0.04, recessive model) and the TC. Furthermore, alcohol (OR=1.56, 95% CI=1.36-1.89, p< 0.01) and tobacco consumption (OR=1.97, 95% CI=1.28-3.04, p< 0.01) were associated with increased risk for TC. The *MTR* A2756G polymorphism was showed statistically significant for tumour extension (OR=2.69, 95% CI=1.27-5.71, p< 0.01) and aggressiveness (OR=4.51, 95% CI=1.67-12.1, p< 0.01). In conclusion, our results demonstrate the influence of these polymorphic alleles in the development of TC in the studied population. In addition, smokers and drinkers are more susceptible to TC development.

**Keywords:** Folate; genes; genetic polymorphism; thyroid cancer

## INTRODUCTION

Thyroid Cancer (TC) is most common malignancy of the endocrine system. There are four main types: papillary, follicular, medullary and anaplastic. In Brazil the estimate for the years 2014/2015 were about 9.200 new cases, this cancer constitute the fifth most common type in women [1].

Some risk factors are being evaluated as predictors for the TC development, such as gender, age, hormonal factors, family history of cancer, alcohol and tobacco consumption and obesity [1, 2, 3]. Moreover, genetic polymorphisms involved in folate metabolism are related to carcinogenesis process which leads development several types of cancer [4, 5, 6]. However, results in literature are still controversial, furthermore, researches addressing the folate pathways are poorly studied in TC, and further studies are required in this area [7, 8].

The folate metabolism is involved in process of synthesis, methylation and DNA repair, and several genes including methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), reduced folate carrier 1 (*RFC1*) and cystathionine β-synthase (*CßS*) regulate this metabolism. The genetic polymorphisms change activity enzymatic, whereas leads the DNA hypomethylation and genomic instability [9, 10, 11].

The Reduced folate carrier 1 (*RFC1*) is responsible by process of absorption and intracellular transport of folate, besides to transport of 5-MTHFR to the interior of variety cells, being an important determinant of folate intracellularly concentrations. It is polymorphic in exon 2, with substitution of adenine for guanine at nucleotide 80 (A80G) (rs 1051266), affecting plasma folate and homocysteine levels [12, 13].

The MTHFR enzyme, encoded by *MTHFR* gene is responsible for catalyzes the irreversible reaction of 5, 10- methylenetetrahydrofolate to 5-methyltetrahydrofolate, which interferes in DNA synthesis and methylation process. There is substitution of citosine for timine at nucleotide 677 (C677T) (rs1801133), and may be associated to carcinogenesis. This molecule supplies methyl group for methylation of homocysteine and producing methionine [14].

The homocysteine remethylation to methionine is catalyzed by *MTR* enzyme, this reaction is essential to adequately maintain normal methionine and intracellular homocysteine concentrations. There is transition of adenine to guanine at position 2756 (A2756G) (rs1805087), this polymorphism is related to increase homocysteine in the plasma and DNA hypomethylation, thus possibly influencing the risk of cancer [15].

The CßS gene encodes an enzyme that catalyzes the transsulfuration of homocysteine (removes Hcy from the methionine) and serine to cystathionine. It is polymorphic in exon 8 with an insertion of 68 base pairs at nucleotide 844 (844ins68) (rs5745905). This polymorphism has been associated with reduction of Hcy levels and changes in DNA methylation because of the low availability of S-adenosylmethionine, the main methyl donor for methylation reactions, and consequently DNA hypomethylation and carcinogenesis may occur [16].

The aims of the present study were to investigate associations between *MTHFR* C677T; *MTR* A2756G, *RFC1* A80G and *CßS* 8444ins68 polymorphisms in thyroid cancer patients, to compare the results with subjects without cancer, and to evaluate association between polymorphisms with risk factors (gender, age, alcohol and tobacco consumption, Body-mass index – BMI) and clinical histopathological parameters.

## MATERIALS AND METHODS

### Subjects

This study protocol was approved by the Ethics Research Committee (20187413.8.0000.5415). All individuals who agreed to participate in the study signed an informed consent form. A total of 462 individuals (151 patients and 311 controls) were evaluated in this case-control study. The case group consisted 151 patients who were diagnosed with thyroid cancer (125 papillary and 26 follicular) at Hospital de Base, São José do Rio Preto, SP, Brazil. The definitive diagnosis is made through examining the results of imaging studies, histopathological analysis, and biopsies. The exclusion criteria were patients with other neoplasms. The tumors were classified based on three criteria of Union of Cancer Control (UICC) 2010 [17]: tumor extent (T), presence of regional lymph node involvement (N) and presence of distant metastasis (M). The clinical stage (TNM) was used to analyze aggressiveness, being stage I and II (non-aggressive); stage III and IV (aggressive). The presence or absence of extrathyroid extension was also evaluated.

The control group included 311 healthy blood donors from the Hemoterapy Center of the city of São José do Rio Preto. Individuals were excluded if they presented with family history of cancer, other neoplasms, and chronic diseases described in Resolution RDC 34 [18] of the National Health Surveillance Agency/Brazil.

### Genotyping

Genomic DNA was extracted peripheral blood leukocytes by the method described by Miller *et al*. [19] with modifications. The genotyping of *CßS* 844ins68

(rs5745905) polymorphisms were determined by PCR. The PCR-RFLP assay was used to identify the *MTHFR* C677T (rs1801133), *MTR* A2756G (rs1805087) and RFC1 (rs 1051266) polymorphisms with *Hinf I, Hae III* and *Hha I* enzymes, respectively. The genotyping confirmation was accomplished in 10% random samples of each group, and we observed 100% of the concordance. The primers sequences used for amplification of the region presenting these polymorphisms are described in Box 1.

**Box 1:** Description of the primers sequences.

| Polymorphisms | Sequence of primers |
|---|---|
| ***MTHFR* C677T** | |
| sense | 5'- TGA AGG AGA AGG TGT CTG CGG GA 3' |
| antisense | 5'- AGG ACG GTG CGG TGA GAG TG 3' |
| ***MTR* A2756G** | |
| sense | 5'- CCA GGG TGC CAG GTA TAC AG 3' |
| antisense | 5'- GCC TTT TAC ACT CCT CAA AAC 3' |
| ***RFC1* A80G** | |
| sense | 5'- AGT GTC ACC TTC GTC CC 3' |
| antisense | 5'- TCC CGC GTG AAG TTC TTG 3' |
| ***CßS* 844ins68** | |
| sense | 5'- GTT GTT AAC GGC GGT ATT GG 3' |
| antisense | 5'- GTT GTC TGC TCC GTC TGG TT 3' |

**Statistical Analysis**

The Hardy-Weinberg equilibrium (HWE) was assessed using the chi-square test using the program version BioEstat 5.4. for available the distribution of genotypes in case and controls groups. Multiple regression logistic test was performed by Minitab/Version 14.0 computer program, adjusting for gender (reference: male), age (reference: <50 years), alcohol consumption (reference: not consume alcohol), tobacco consumption (reference: nonsmoking), BMI (reference: <24.9), *MTHFR* C677T (reference: genotype CC), *MTR* A2756G (reference: genotype AA), *RFC1* A80G (reference: genotype AA) and *CßS* 844ins68 (reference: homozygous without

insertion). In this study, we considered smokers, those who smoked >100 cigarettes in their lifetime and drinkers 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. The subjects with BMI ≥ 25.0 Kg/m³ were obeses. In the case group was evaluated blood of levels TSH (reference: up to 3.0 mIU/L). The clinical histopathological parameters also was evaluated by multiple logistic regression.

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 2 more heterozygote *versus* homozygous wild-type).

SNPstat online computer program was used to investigate the interaction of studied polymorphisms with alcohol and tobacco consumption and BMI on the risk TC. The results were presented in odds ratio (OR), confidence interval 95% (CI – 95%) and value of $p$ <0.05 was considered significant.

**RESULTS**

Table 1 show association of *MTHFR* C677T, *MTR* A2756G, *RFC1* A80G and *CßS* 844ins68 polymorphisms to TC according to heritage models. For *MTHFR* C677T polymorphism was observed association with increased risk in models codominant,

dominant and recessive ($p < 0.01$). The 80GG genotype was statistically significant for *RFC1* A80G polymorphism (OR: 1.55; 95% CI: 1.02-2.38; $p$=0.04). The *MTR* A2756G and *CßS* 844ins68 polymorphisms were not associated with the TC.

The Hardy-Weinberg equilibrium analysis showed that the genotypic frequencies of *MTHFR* C677T, *MTR* A2756G, *RFC1* A80G and *CßS* 844ins68 polymorphisms are in equilibrium in the patients (*MTHFR* C677T: $\chi^2 = 1.79$, $p = 0.17$; *MTR* A2756G: $\chi^2 = 1.66$, $p = 1.19$; *RFC1* A80G: $\chi^2 = 2.89$, $p = 0.08$; *CßS* 844ins68: $\chi^2 = 0.49$, $p = 0.94$). In the control group, the *MTHFR* C677T ($\chi^2 = 0.51$, $p = 0.47$), *MTR* A2756G ($\chi^2 = 0.08$, $p= 0.77$) and *CßS* 844ins68 polymorphisms ($\chi^2 = 0.18$, $p = 0.66$) were in equilibrium. For the *RFC1* A80G polymorphisms the control group showed disequilibrium ($\chi^2 = 24.71$, $p < 0.001$).

The multiple logistic regression analysis (adjusted for gender, age, alcohol and tobacco consumption, BMI and polymorphisms) showed that alcohol consumption (OR: 1.56; 95% CI= 1.36-1.89; $p < 0.001$) and tobacco consumption (OR: 1.97; 95% CI= 1.28-3.04; $p < 0.001$) were predictors for the disease. However, there was not an association between gender (OR: 1.07; 95% CI= 0.55-2.10; $p = 0.84$), age $\geq$ 50 years (OR: 1.21; 95% CI= 0.80-1.81; $p = 0.36$) and BMI (OR: 1.24; 95% CI= 0.79-1.94; $p = 0.35$) for TC (Table 2). Regarding blood levels of TSH 21.19% presented levels <0.3 mIU/L; 53.64% were between 0.3 – 3.0 mIU/L and 25.17% presented levels of TSH >3.0 mIU/L.

In the present study, was not evidenced a potential for significant interaction for the presence of polymorphisms and alcohol and tobacco consumption and BMI on the risk for TC.

Regarding clinical histopathological parameters of TC, the Tables 3 show the results of polymorphisms association analysis with these parameters. The polymorphism *MTR* A2756G is associated with tumor extension (OR: 2.69; 95% CI= 1.27-5.71; $p$ = 0.01) and aggressiveness (OR: 4.51; 95% CI= 1.67-12.1; $p$ = 0.01). The other polymorphisms it is possible to observe that there was no association between tumor extension (T), regional lymph node involvement (N) and aggressiveness (Table 3). There was no association with extrathyroid extension for polymorphisms evaluated in TC (Table 4).

**DISCUSSION**

In the present study, we evaluated the association of *MTHFR* C677T, *MTR* A2756G, *RFC1* A80G and *CßS* 844ins68 polymorphisms involved in folate metabolism in TC. The association these polymorphisms with risk factors and clinical histopathological parameters was also performed. We found an association of the *MTHFR* C677T and *RFC1* A80G polymorphisms and increased risk for the TC. Alcohol and tobacco consumption were associated with development this desease.

In addition, we have not observed the HWE equilibrium in control group for *RFC1* A80G polymorphism. Case-controls studies with polymorphisms analysis has observed HWE disequilibrium in patients and controls. This is due to random selection samples, model, and complexity disease. Probably there is a significant contribution of biological effects and genetic features [20].

When folate levels are altered by polymorphisms, the synthesis of purines and pyrimidine, DNA methylation and repair is directly affected, because folate is a relevant

precursor substance for cell normal metabolism. The methylation, in turn, is responsible by gene expression control, chromatin structure and genomic stability [10, 21, 22].

In the present study, we found association with *MTHFR* C677T and increased risk for TC. The studies in the literature are controversial; a study involved Saudi Arabian population reported no association with this genetic variant [23]. Other study performed with papillary carcinoma, also no found evidence supporting an association with this polymorphism [22]. In other hand, a study performed in Turkey involving 60 cases and 50 controls also found increased risk for TC [24], as well as the study by Fard-Esfahani et *al*. [25], conducted with Iranian population, according our current results. A meta-analysis involving four studies showed a significant association with *MTHFR* C677T polymorphism [8].

Our results also revealed increase risk for genotype GG of *RFC1* polymorphism. Not found in the literature studies in TC, this is the first molecular epidemiological study of *RFC1* A80G polymorphism in TC, however, was studied in others kinds of cancers. In cervical cancer, Di *et al* [26] was found increase the risk for variant 80GG in Chinese population. Galbiatti *et al* [13] showed association with head and neck cancer in males with age > 50 years. Wang *et al* [27] and De Jonge *et al* [28] also found association between this polymorphism in gastroesophageal cancer and pediatric acute lymphoblastic leukemia, respectively. In other hand, studies performed with colorectal cancer [29] and breast cancer [12] did not found association with this polymorphism.

In this study, the *MTR* A2756G and *CßS* 844ins68 polymorphisms were not statistically significant; both never still were evaluated in this kind of cancer. In breast cancer, two studies not found association with this polymorphism [11, 21]. Zhou *et al* [30]

also not found increased risk for colorectal cancer. In meta-analysis by Zhao *et al* [31] was highlighted no association with *MTR* A2756G polymorphism and digestive system cancer development. Unlike to study case-control performed with Brazilian population that was found association of *MTR* A2756G polymorphism and head and neck cancer [32]. The elevation of homocysteine level and DNA hypomethylation due to decrease *MTR* enzyme is induced by *MTR* A2756G polymorphism [33]. Regarding *CßS* 844ins68, a study with Mexican population showed increased risk for breast cancer [16], differently of the study with head and neck cancer that was did not found an association with this polymorphism [34].

In the present study, alcohol consumption (OR: 1.56; 95% CI= 1.36-1.89; $p < 0.001$) was associated for TC development, in according with studies performed that also found this association [2, 35]. The tobacco consumption (OR: 1.97; 95% CI= 1.28-3.04; $p < 0.001$) also was significant in this study. In concordance, the meta-analysis by Jie Ma *et al* [36] and Cho *et al.* [37] concluded that tobacco consumption increase the risk for TC.

The gender, age ≥ 50 years and BMI ≥ 25.0 Kg/m³ was not associated with TC. The study performed with European population showed association with obesity and TC risk only women [38]. Several others studies also showed increased risk for TC in subjects with excess weight [36, 39].

In the present study, was not evidenced a potential interaction for the presence of polymorphisms with alcohol and tobacco consumption and BMI on the risk for TC, as well as in study by Sun-Seog *et al.* (22) performed with Korean population.

Regarding clinical histopathological parameters, the *MTR* A2756G polymorphism influence in tumor extension (T) (OR: 2.69; 95% CI= 1.27-5.71; p <

0.01) and aggressiveness (OR: 4.51; 95% CI= 1.67-12.1; $p < 0.01$). In addition, there has not been a significant result for regional lymph node involvement (N), extrathyroid extension. Moreover, there are not previous studies in TC evaluating these clinical variables and polymorphisms in genes involved in folate metabolism.

Our study may be limited by sample size and possibly time of sample collection was relatively short, however, studies with polymorphisms involved folate pathway and TC are still scarce in the literature, emphasizing the importance of evaluate these molecular biomarkers for better comprehension and understanding, once the *MTR* A2756G, *CßS* 844ins68 and *RFC1* A80G polymorphisms still not been evaluated in TC.

In conclusion our data demonstrate the influence of *MTHFR* C677T and *RFC1* 80GG polymorphisms in developing the TC in the population studied. In addition, alcohol and tobacco consumption are related with increased risk to this disorder. The tumor extension and aggressiveness may be influenced by *MTR* A2756G polymorphism. Thereby, studies with other enzymes involved folate metabolism could contribute to a better understanding etiology of thyroid cancer.

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**Table 1:** Association between *MTHFR* C677T, *MTR* A2756G, *RFC1* A80G and C*ßS*844ins68 polymorphisms and thyroid cancer.

| Model | Genotype | Control n(%) | Case n(%) | OR[+] (95% CI) | p-value | Genotype | Control n(%) | Case n(%) | OR[+] (95% CI) | p-value |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***MTHFR* C677T** | | | | | ***MTR* A2756G** | | | | |
| Codominant | C/C | 174 (56) | 63 (41.7) | 1.00 | | A/A | 197 (63.3) | 89 (58.9) | 1.00 | |
| | C/T | 114 (36.7) | 63 (41.7) | 1.53 (0.99-2.35) | **<0.01**[+] | A/G | 100 (32.1) | 50 (33.1) | 1.13 (0.74-1.74) | 0.4 |
| | T/T | 23 (7.4) | 25 (16.6) | **2.87 (1.50-5.48)** | | G/G | 14 (4.5) | 12 (8) | 1.75 (0.77-4.02) | |
| Dominant | C/C | 174 (56) | 63 (41.7) | 1.00 | | A/A | 197 (63.3) | 89 (58.9) | 1.00 | |
| | C/T-T/T | 137 (44) | 88 (58.3) | **1.76 (1.18-2.64)** | **<0.01**[+] | A/G-G/G | 114 (36.7) | 62 (41.1) | 1.21 (0.81-1.82) | 0.35 |
| Recessive | C/C-C/T | 288 (92.6) | 126 (83.4) | 1.00 | | A/A-A/G | 297 (95.5) | 139 (92) | 1.00 | |
| | T/T | 23 (7.4) | 25 (16.6) | **2.37 (1.28-4.39)** | **<0.01**[+] | G/G | 14 (4.5) | 12 (8) | 1.68 (0.74-3.80) | 0.22 |
| Overdominant | C/C-T/T | 197 (63.3) | 88 (58.3) | 1.00 | | A/A-G/G | 211 (67.8) | 101 (66.9) | 1.00 | |
| | C/T | 114 (36.7) | 63 (41.7) | 1.24 (0.83-1.87) | 0.3 | A/G | 100 (32.1) | 50 (33.1) | 1.08 (0.70-1.64) | 0.74 |
| Aditive | --- | --- | --- | 1.64 (0.88-1.90) | 0.3 | --- | --- | --- | 1.23 (0.89-1.70) | 0.22 |
| | ***RFC1* A80G** | | | | | ***CßS 844ins68*** | | | | |
| Codominant | A/A | 125 (40.2) | 45 (29.8) | 1.00 | | W/W | 251 (80.7) | 119 (78.8) | 1.00 | |
| | A/G | 109 (35) | 65 (43) | 1.71 (1.07-2.74) | 0.07 | W/Ins | 56 (18) | 30 (19.9) | 1.17 (0.71-1.94) | 0.83 |
| | G/G | 77 (24.8) | 41 (27.1) | 1.35 (0.80-2.27) | | Ins/Ins | 4 (1.3) | 2 (1.3) | 1.07 (0.19-6.11) | |
| Dominant | A/A | 125 (40.2) | 45 (29.8) | 1.00 | | W/W | 251 (80.7) | 119 (78.8) | 1.00 | |
| | A/G-G/G | 186 (59.8) | 106 (70.2) | 1.02 (0.65-1.60) | 0.94 | W/Ins - Ins/Ins | 60 (19.3) | 32 (21.2) | 1.16 (0.71-1.90) | 0.55 |
| Recessive | A/A-A/G | 234 (75.2) | 110 (72.8) | 1.00 | | W/W - W/Ins | 307 (98.7) | 149 (98.7) | 1.00 | |
| | G/G | 77 (24.8) | 41 (27.1) | **1.55 (1.02-2.38)** | **0.04**[+] | Ins/Ins | 4 (1.3) | 2 (1.3) | 1.04 (0.18-5.92) | 0.97 |
| Overdominant | A/A-G/G | 202 (65) | 86 (57) | 1.00 | | W/W - Ins/Ins | 255 (82) | 121 (80.1) | 1.00 | |
| | A/G | 109 (35) | 65 (43) | 1.51 (1.00-2.27) | 0.05 | W/Ins | 56 (18) | 30 (19.9) | 1.17 (0.71-1.93) | 0.55 |
| Aditive | --- | --- | --- | 1.18 (0.92-1.53) | 0.19 | --- | --- | --- | 1.13 (0.73-1.77) | 0.58 |

+ Odds Ratio (OR) adjusted for gender, age, alcohol and tobacco consumption and BMI (Body-mass index).

W/W (homozygous wild without insertion 68pb); W/Ins (heterozygous genotype); Ins/Ins (homozygous polymorphic with insertion 68pb)

**Table 2:** Distribution of demographic data and risk factors of patients with thyroid cancer and control individuals.

| Variable | Patients (n=151) n (%) | Controls (*n=311*) *n* (%) | OR (95% CI) | *p value* |
|---|---|---|---|---|
| **Gender** | | | | |
| Male | 15 (9.94) | 37 (11.90) | 1.00 (ref) | |
| Female | 136 (90.06) | 274 (88.10) | 1.07 (0.55-2.10) | 0.84 |
| **Age (years)** | | | | |
| < 50 | 73 (48.34) | 174 (55.94) | 1.00 (ref) | |
| ≥ 50 | 78 (51.66) | 137 (44.66) | 1.21 (0.80-1.81) | 0.36 |
| **Alcohol consumption** | | | | |
| No | 107 (70.87) | 191 (61.42) | 1.00 (ref) | |
| Yes | 44 (29.13) | 120 (38.58) | 1.56 (1.36-1.89) | **<0.001*** |
| **Tobacco consumption** | 89 (58.95) | 222 (71.38) | 1.00 (ref) | |
| No | 62 (41.05) | 89 (28.62) | 1.97 (1.28-3.04) | **<0.001*** |
| Yes | | | | |
| **BMI** | | | | |
| < 25.0 Kg/m³ | 40 (26.49) | 99 (31.83) | 1.00 (ref) | |
| ≥ 25.0Kg/m³ | 111 (73.51) | 212 (68.17) | 1.24 (0.79-1.94) | 0.35 |

+ Odds Ratio (OR) adjusted for gender, age, alcohol and tobacco consumption, BMI (Body-mass index) and polymorphisms.

+ p values significant at p <0.05.

**Table 3:** Distribution of the clinical histopathological parameters in relation to *MTHFR* C677T, *MTR* A2756G, *RFC1* A80G and *CßS* 844ins68 polymorphisms in patients with thyroid cancer.

| | **Tumor extension** | | | | **Regional lymphnode involvement** | | | | | | **Aggressiveness (TNM)** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **T1/T2** | **T3/T4** | | | **N=0** | | **N≥1** | | | | **Non-aggressive** | **Aggressive** | | |
| | n(%) | n(%) | O.R. $^{+}$ (I.C.95%) | p-value | n(%) | | n(%) | | O.R.$^{+}$ (I.C.95%) | p-value | n(%) | n(%) | O.R.$^{+}$ (I.C.95%) | p-value |
| | 108 (71.52) | 43 (28.48) | | | 125 (82.78) | | 26 (17.22) | | | | 118 (78.1) | 33 (21.86) | | |
| ***MTHFR*** | | | | | | | | | | | | | | |
| C/C | 44 (40.74) | 19 (44.18) | 1.00 | | 50 | (40.00) | 13 | (50.00) | 1.00 | | 52 (44.06) | 11 (33.34) | 1.00 | |
| C/T - T/T | 64 (59.26) | 24 (55.82) | 0.73 (0.34-1.56) | 0.41 | 75 | (60.00) | 13 | (50.00) | 0.72 (0.29-1.75) | 0.46 | 66 (55.94) | 22 (66.66) | 0.98 (0.37-2.63) | 0.97 |
| ***MTR*** | | | | | | | | | | | | | | |
| A/A | 69 (63.88) | 20 (46.51) | 1.00 | | 74 | (59.20) | 15 | (57.69) | 1.00 | | 76 (64.40) | 13 (39.40) | 1.00 | |
| A/G - G/G | 39 (36.12) | 23 (53.49) | 2.69 (1.27-5.71) | **0.01**$^{+}$ | 51 | (40.80) | 11 | (42.31) | 1.07 (0.44-2.65) | 0.87 | 42 (35.60) | 20 (60.60) | 4.51(1.67-12.1) | **<0.01**$^{+}$ |
| ***RFC1*** | | | | | | | | | | | | | | |
| A/A | 33 (30.55) | 12 (27.90) | 1.00 | | 38 | (30.40) | 07 | (26.92) | 1.00 | | 38 (32.20) | 07 (21.22) | 1.00 | |
| A/G - G/G | 75 (69.45) | 31 (72.10) | 0.83 (0.36-1.90) | 0.65 | 87 | (69.60) | 19 | (73.08) | 1.63 (0.58-4.56) | 0.35 | 80 (67.80) | 26 (78.78) | 1.08 (0.36-3.26) | 0.89 |
| ***CßS*** | | | | | | | | | | | | | | |
| W/W | 83 (76.85) | 36 (83.72) | 1.00 | | 101 | (80.80) | 18 | (69.23) | 1.00 | | 91 (77.11) | 28 (84.84) | 1.00 | |
| W/Ins - Ins/ Ins | 25 (23.15) | 07 (16.28) | 0.68 (0.26-1.75) | 0.42 | 24 | (19.20) | 08 | (30.77) | 1.95 (0.72-5.28) | 0.19 | 27 (22.89) | 05 (15.16) | 0.64 (0.19-2.19) | 0.18 |

+ Odds Ratio (OR) adjusted for gender, age, alcohol and tobacco consumption. * W/W (homozygous wild without insertion 68pb); W/Ins (heterozygous genotype); Ins/Ins (homozygous polymorphic with insertion 68pb)

++ p values significant at p <0.05.

**Table 4:** Association between *MTHFR* C677T, *MTR* A2756G, *RFC1* A80G and C*ßS* 844ins68 polymorphisms and extrathyroid extension.

| | **Absence n (%)** | **Presence n (%)** | **O.R (95% CI)** | ***p value*** |
|---|---|---|---|---|
| ***MTHFR* C677T** | | | | |
| C/C | 49 (41.88) | 14 (41.17) | 1.00 | |
| C/T – T/T | 68 (58.12) | 20 (58.83) | 0.85 (0.37-1.94) | 0.69 |
| ***MTR* A2756G** | | | | |
| A/A | 72 (61.53) | 17 (50.00) | 1.00 | |
| A/G – G/G | 45 (38.47) | 17 (50.00) | 1.72 (0.77-3.87) | 0.18 |
| ***RFC1* A80G** | | | | |
| A/A | 37 (31.62) | 08 (23.52) | 1.00 | |
| A/G – G/G | 80 (68.38) | 26 (76.48) | 1.43 (0.56-3.68) | 0.45 |
| ***CßS* 844ins68** | | | | |
| W/W | 91 (77.77) | 28 (82.35) | 1.00 | |
| W/Ins - Ins/Ins | 26 (22.23) | 06 (17.65) | 0.77 (0.28-2.15) | 0.62 |

+ Odds Ratio (OR) adjusted for gender, age, alcohol and tobacco consumption, BMI (Body-mass index) and polymorphisms.

+ p values significant at $p < 0.05$.

# ***CONCLUSÕES***

## 3. Conclusões

1. Os genótipos *MTHFR* 677CT ou TT e *RFC1* 80GG estão associados com o aumento do risco de câncer de tireoide.

2. O consumo de álcool e tabaco está associado ao aumento do risco de desenvolvimento da doença.

3. Há evidências de associação do polimorfismo *MTR* (A2756G) com o tamanho e agressividade tumoral.

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# ***APÊNDICES***

## MATERIAL E MÉTODOS

### Casuística

Foram analisadas 452 amostras no total, 151 pacientes com diagnóstico de câncer de tireoide, procedentes dos Ambulatórios de Cirurgia Geral e de Otorrinolaringologia e Cirurgia de Cabeça e Pescoço do Hospital de Base/ Faculdade de Medicina de São José do Rio Preto – FAMERP. Também foram avaliadas amostras de sangue de 311 indivíduos sem história de neoplasia, saudáveis, obtidos no Hemocentro do Hospital de Base/Faculdade de Medicina de São José do Rio Preto. Os indivíduos foram incluídos no estudo após o convite, esclarecimento da pesquisa e assinatura do Termo de Consentimento Livre e Esclarecido (TCLE) e todas as informações foram obtidas por meio de questionário padronizado e mantidas em sigilo, (codificadas) e armazenadas na Unidade de Pesquisa em Genética e Biologia Molecular da Faculdade de Medicina de São José do Rio Preto.

Os pacientes foram incluídos no estudo após o diagnóstico de câncer de tireoide. O diagnóstico definitivo é realizado por meio de exames histopatológicos após procedimento cirúrgico, sob a responsabilidade dos médicos do serviço e da patologia do Hospital de Base.

### Critérios de Inclusão

Foram incluídos no estudo 151 pacientes com diagnóstico de câncer de tireoide (Grupo Caso), procedentes dos Ambulatórios de Cirurgia Geral e de Otorrinolaringologia e Cirurgia de Cabeça e Pescoço do Hospital de Base**.** Também foram incluídas amostras de sangue de 311 indivíduos sem história de neoplasia (Grupo Controle), saudáveis, obtidos no Hemocentro do Hospital de Base.

### Critérios de Exclusão

Foram excluídos do grupo caso pacientes portadores de outras neoplasias e do grupo controle indivíduos com histórico pessoal e familiar de neoplasia.

**Análise dos dados demográficos**

As variáveis analisadas incluíram gênero, idade, consumo de álcool e tabaco e IMC. Informações sobre o tabagismo e etilismo foram limitadas quanto ao uso ou não de álcool e tabaco. Foram considerados tabagistas indivíduos que consumiram mais de 100 cigarros durante toda a vida e etilistas aqueles que bebem mais que quatro drinques semanalmente.

**Análise dos dados clínicos**

A análise dos tumores bem como o estadiamento foi realizada de acordo com os parâmetros da International Union of Cancer Control (UICC), em três critérios: tamanho do tumor (T), envolvimento de linfonodos regionais (N) e presença de metástase à distância (M). Também foi avaliada a presença ou ausência de invasão extratiroidal.

**Extração do DNA**

O DNA genômico foi extraído a partir de leucócitos de sangue periférico de acordo com a técnica de Miller *et al*. (1988) com modificações, conforme padronizado na UPGEM.

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). 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 adicionada solução salina (PBS) até completar o volume de 15 ml. O tubo foi centrifugado novamente a 1200 rpm por 15 minutos. O sobrenadante foi descartado e 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 protéica, foi

adicionada 1mL de NaCl 6M, agitada a solução e em seguida foi colocada no gelo por 15 minutos. Após este período, a solução foi homogeneizada e centrifugada a 2000 rpm por 15 minutos. O sobrenadante transferido para um novo tudo de 15 mL, descartando-se o *pellet.* Após a adição de etanol 100% gelado, o tubo foi fechado e misturado por inversão. O DNA precipitado foi removido para um tubo eppendorf contendo 500uL etanol 70%. Este foi centrifugado a 12000 rpm por cinco minutos. O DNA foi seco a temperatura ambiente e ressuspendido em 200 uL de tampão de eluição.

**Análise dos Polimorfismos**

**Polimorfismo C677T no gene *MTHFR***

A investigação da variante *MTHFR C677T* foi realizada por PCR-RFLP (Reação em Cadeia da Polimerase – Polimorfismos de Comprimentos de Fragmentos de Restrição). Segue abaixo o protocolo:

**Água** → 16,55 µl
**Glicerol 50%** → 2,5 µl
**Tampão** → 2,5 µl
**MgCl2** → 2,0 µl
**Sense** → 0,5 µl
**Anti-sense** → 0,5 µl
**dNTP** → 2,0 µl
**Taq** → 0,2 µl
**DNA amostra** → 2,0 µl

**Ciclagem:**
94º C – 4 minutos
94º C – 1 minuto
59º C – 50 segundos } 30x
72º C – 50 segundos
72º C – 10 minutos
4º C - hold

O produto da amplificação foi submetido à digestão enzimática utilizando:

**Água** → 7,5 µl
**Tampão** → 2,0 µl
**Enzima *Hinf I*** → 0,5 µl

**Ciclagem:**
37º → 2 horas

Após a digestão enzimática foram gerados fragmentos de 198 pb e 23 pb para o genótipo selvagem (CC), 175 e 23 pb para o genótipo homozigoto polimórfico (TT) e

198 pb, 175 pb e 23 pb para o genótipo heterozigoto (CT). A visualização dos fragmentos foi por meio de eletroforese em gel de agarose 2% corado com Brometo de Etídio.

**Polimorfismo A2756G no gene da *MTR***

A investigação da variante *MTR A2756G* foi realizada por PCR-RFLP (Reação em Cadeia da Polimerase – Polimorfismos de Comprimentos de Fragmentos de Restrição). Segue abaixo o protocolo:

**Água →** 16,55 µl
**Tampão →** 2,5 µl
**MgCl2 →** 0,75 µl
**Sense →** 0,5 µl
**Anti-sense →** 0,5 µl
**dNTP →** 2,0 µl
**Taq →** 0,2 µl
**DNA amostra →** 2,0 µl

**Ciclagem:**
94º C – 4 minutos
94º C – 1 minuto
56º C – 1 minuto
72º C – 1 minuto
} 30x
72º C – 10 minutos
4º C - hold

O produto da amplificação foi submetido à digestão enzimática utilizando:

**Água →** 3,0 µl
**Tampão →** 1,5 µl
**Enzima *Hae III* →** 0,5 µl

**Ciclagem:**
37º → 2 horas

Após a digestão enzimática obteve-se fragmentos de 413 e 85 pb para o genótipo selvagem (AA), 390, 123 e 85 pb para o genótipo homozigoto polimórfico (GG) e para o genótipo heterozigoto (AG) fragmentos de 413, 390, 123 e 85 pb. A visualização dos fragmentos foi por meio de eletroforese em gel de agarose 2% corado com Brometo de Etídio.

**Polimorfismo *RFC1* A80G**

A investigação da variante *RFC1 A80G* foi realizada por PCR-RFLP (Reação em Cadeia da Polimerase – Polimorfismos de Comprimentos de Fragmentos de Restrição). Segue abaixo o protocolo:

**Água →** 12,7 µl
**Glicerol 50% →** 2,5 µl
**Tampão →** 2,5 µl
**MgCl2 →** 2,0 µl
**Sense →** 0,5 µl
**Anti-sense →** 0,5 µl
**dNTP →** 2,0 µl
**Taq →** 0,3 µl
**DNA amostra →** 2,0 µl

**Ciclagem:**
94º C – 2 minutos
94º C – 30 segundos
58º C – 30 segundos
72º C – 45 segundos
} 35x
72º C – 7 minutos
4º C - hold

O produto da amplificação foi submetido à digestão enzimática utilizando:

**Água →** 7,5 µl
**Tampão →** 2,0 µl
**Enzima *Hha I* →** 0,5 µl

**Ciclagem:**
37º → 2 horas

Após a digestão enzimática obteve-se fragmentos de 162 e 68 pb para o genótipo selvagem (AA), 125 e 68 pb para o genótipo homozigoto polimórfico (GG) e para o genótipo heterozigoto (AG) fragmentos de 162, 125 e 68 pb. A visualização dos fragmentos foi por meio de eletroforese em gel de agarose 2% corado com Brometo de Etídio.

**Polimorfismo *CβS* 844ins68**

A investigação da variante *CβS* 844ins68 foi realiizada por PCR (Reação em Cadeia da Polimerase). Segue abaixo o protocolo:

**Água →** 11,7 µl
**Glicerol 50% →** 2,5 µl
**Tampão →** 2,5 µl
**MgCl2 →** 2,0 µl
**Sense →** 1,0 µl
**Anti-sense →** 1,0 µl
**dNTP →** 2,0 µl
**Taq →** 0,3 µl

**Ciclagem:**
94º C – 4 minutos
94º C – 1 minuto
62º C – 1 minuto
72º C – 1 minuto
} 30x
72º C – 5 minutos
4º C - hold

**DNA amostra →** 2,0 µl

Após a amplificação obteve-se um fragmento de 171 pb para o genótipo selvagem (-/-), 239 pb para o genótipo homozigoto polimórfico (+/+) e para o genótipo heterozigoto (-/+) fragmentos de 239 e 171 pb. A visualização dos fragmentos foi por meio de eletroforese em gel de agarose 1,5% corado com Brometo de Etídio.

**Análise Estatística**

Para a análise estatística foram utilizados os programas computacionais Minitab versão 14.0, BioEstat versão 5.4 e Snpstats (online). O equilíbrio de Hardy-Weinberg (HWE) foi realizado pelo teste do qui-quadrado. Os modelos de regressão logística múltipla foram utilizados para determinar a associação das variáveis analisadas em câncer de tireoide. Os modelos incluíram idade (referência: mediana grupo caso), gênero (referência: masculino), consumo de álcool (referência: não etilistas), consumo de tabaco (referência: não fumantes) e índice de massa corpórea (referência: <24.9 $Kg/m^2$). A análise dos modelos de herança (codominante, dominante, recessivo, overdominante e aditivo) foi realizada pelo programa Snpstats (online). As características clínico-patológicas também foram analisadas por regressão logística múltipla. A classificação T foi dividida em tumores com pequena extensão (T1, T2) e com grande extensão (T3, T4). A classificação N foi dicotomizada em comprometimento de linfonodos negativo (N0) e positivo (N1, N2, N3). Os estadios foram divididos em não agressivos (estadios I, II) e agressivos (estadios III e IV). Todos os resultados foram apresentados em odds ratio (OR) e intervalo de confiança de 95%. Foram considerados significantes valores $p<0,05$.

# TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

*(Conselho Nacional de Saúde, resolução 466/12)*

## Título da Pesquisa: Avaliação dos Polimorfismos envolvidos no metabolismo do folato em pacientes com câncer de tireoide

**Pesquisadora Responsável:** Eny Maria Goloni Bertollo – UPGEM: Unidade de Pesquisa em Genética e Biologia Molecular

A) o Sr (a) está sendo convidado (a) a participar desta pesquisa que visa obter maior conhecimento dos mecanismos envolvidos no desenvolvimento do câncer de tireoide (glândula localizada no pescoço), que poderá melhorar o nosso conhecimento sobre esse tumor e, portanto oferecer novas possibilidades de diagnóstico e de melhora no tratamento e consequentemente na qualidade de vida;
B) este estudo tem como objetivos: 1) Coletar informações da história e obter dados clínicos dos prontuários médicos dos pacientes com câncer de tireoide atendidos no Serviço de Atendimento Ambulatorial vinculado ao Departamento de Otorrinolaringologia e de Cirurgia Geral do Hospital de Base / Faculdade de Medicina de São José do Rio Preto – FAMERP. 2) Analisar alterações em genes (material hereditário) com a finalidade de esclarecer o papel de fatores genéticos no desenvolvimento do tumor;
C) para este estudo serão utilizados dois grupos de pessoas: 1) pacientes com câncer de tireoide; 2) indivíduos do grupo controle, não portadores do tumor e de outras doenças crônicas;
D) o estudo será feito utilizando-se sangue, que será colhido com seringa descartável por enfermeiro treinado e o risco da coleta pode incluir inchaço e vermelhidão no local, sem qualquer outro risco para minha saúde;
E) o material genético, ou seja, hereditário, extraído do sangue será utilizado para esta pesquisa e armazenado na Unidade de Pesquisa em Genética e Biologia Molecular, todas as informações serão mantidas em sigilo (codificadas). Para novos projetos, haverá nova submissão para avaliação do Comitê de Ética e Pesquisa (CEP).
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) O resultado individual não tem significado para o paciente. Trata-se de uma variante populacional que trará benefícios apenas em estudos de grandes amostras realizadas em diferentes países. No futuro poderá ser considerado um marcador específico para o tipo de câncer estudado. Os resultados serão divulgados em conjunto para todos os indivíduos após o término da pesquisa a ser aguardado com o médico responsável pelo paciente.
H) 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;
I) 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

*Em caso de dúvidas contatar a Secretaria do Comitê de Ética em Pesquisa da Faculdade de Medicina de São José do Rio Preto, Av. Brigadeiro Faria Lima, nº 5416, Telefone: (0xx17) 3201-5700, Ramal 5813.*

# Questionário

**I. IDENTIFICAÇÃO**

Nome: _____
Prontuário: _____
Data de nascimento: _____/_____/_____ Idade: _____
Sexo: ( ) Etnia: ( ) Branco ( ) Não-branco [pardo/negro] ( ) Asiático
Endereço: Rua _____ Nº: ___ Fone: _____
Bairro: _____ Cidade: _____
CEP: _____ Estado: _____
Profissão atual: _____

**II. DADOS DO TUMOR**

Data de diagnóstico: _____/_____/_____
TNM: Clínico: T ( ) N ( ) M ( )
Tumor primário: ( ) Sim ( ) Não Local: _____
Recidiva: ( ) Sim ( ) Não Local: _____
Cirurgia: ( ) Sim ( ) Não Tipo: _____ Data: _____/_____/_____

**III. FATORES DE RISCO AMBIENTAL**

Exposição ao tabaco: ( ) Sim ( ) Não ( ) Ex-fumante
Tipo: _____
Início:_____ Término:_____ Duração: _____ Consumo diário:_____
Consumo de álcool: ( ) Sim ( ) Não ( ) Ex-etilista
Tipo: _____
Início: _____ Término: _____ Duração: _____ Consumo semanal:_____

Histórico de Câncer na família: ( )Sim ( )Não
Parentesco: _____ Local: _____

PESO:_____

ALTURA:_____

IMC:_____

**Observações:**

Data:_____/_____/_____

Responsável pela entrevista: _____

# ***ANEXOS***

Cancer Science

# Submission Confirmation

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

| | |
|---|---|
| Manuscript ID: | CAS-OA-0551-2015 |
| Title: | Role of MTHFR C677T and MTR A2756G polymorphisms in thyroid and brea cancer development |
| Authors: | Zara-Lopes, Tairine<br>Gimenez-Martins, Ana Paula<br>Nascimento-Filho, Carlos Henrique<br>Castanhole-Nunes, Márcia Maria<br>Galbiatti, Ana Lívia<br>Pavarino, Érika<br>Goloni-Bertollo, Eny |
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Comitê de Ética em
Pesquisa em Seres Humanos
CEP/FAMERP

**Parecer n.° 389.380**

**COMITÊ DE ÉTICA EM PESQUISA**

O projeto de pesquisa CAAE **20187413.8.0000.5415** sob a responsabilidade de **Eny Maria Goloni-Bertollo** com o título "Avaliação dos Polimorfismos Envolvidos no Metabolismo do Folato em Pacientes com Câncer de Tireóide" está de acordo com a resolução do CNS 466/12 e foi **aprovado por esse CEP.**

Lembramos ao senhor(a) pesquisador(a) que, no cumprimento da Resolução 251/97, o Comitê de Ética em Pesquisa em Seres Humanos (CEP) **deverá receber relatórios semestrais sobre o andamento do Estudo**, bem como a qualquer tempo e a critério do pesquisador nos casos de relevância, além do envio dos relatos de eventos adversos, com certeza para conhecimento deste Comitê. **Salientamos ainda, a necessidade de relatório completo ao final do Estudo.**

São José do Rio Preto, 10 de setembro de 2013.

**Profª. Drª. Maria Rita Rodrigues Vieira**
**Vice-Presidente do CEP/FAMERP**

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