ESTUDO GENÉTICO E EPIGENÉTICO DE FATORES DE RISCO MATERNO PARA A SÍNDROME DE DOWN

São José do Rio Preto
2017
Cristiani Cortez Mendes

Estudo genético e epigenético de fatores de risco materno para a síndrome de Down

Tese apresentada à Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Doutor no Curso de Pós-graduação em Ciências da Saúde, Área de Concentração: Medicina e Ciências Correlatas.

Orientadora: Profª. Drª. Érika Cristina Pavarino

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Dedicatória

Aos meus pais, pelo incentivo e apoio em todas as minhas escolhas.
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“Por vezes, sentimos que aquilo que fazemos não é, senão, uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota”

Madre Teresa de Calcutá
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DHF Dihidrofolato (dihydrofolate)
DHFR Dihidrofolato redutase (dihydrofolate reductase)
DNA Ácido desoxirribonucléico (desoxirribonucleic acid)
DNMT1 DNA methyltransferase 1
DNMT3A DNA methyltransferase 3A
DNMT3B DNA metiltransferase 3B (DNA methyltransferase 3B)
DNMTs DNA metiltransferases (DNA methyltransferases)
DS Down syndrome
dTMP Deoxitimidina monofosfato (deoxythymydine monophosphate)
dUMP Deoxiuridina monofosfato (deoxyuridine monophosphate)
FAMERP Faculdade de Medicina de São José do Rio Preto
FAPESP Fundação de Amparo à Pesquisa do Estado de São Paulo
FUNFARME Fundação Faculdade Regional de Medicina de São José do Rio Preto
G Guanina (guanine)
Hcy Homocisteína (homocysteine)
HW Hardy-Weinberg Equilibrium
Kb Kilobases
LD Linkage Disequilibrium
LINE Elemento nuclear intercalante longo (long interspersed elements)
max Maximum
min Minimum / minutes
MMA Ácido metilmalônico (methylmalonic acid)
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RESUMO

**Introdução:** A síndrome de Down (SD) resulta de falhas na segregação cromossômica durante a meiose materna em cerca de 90-95 % dos casos. Embora a idade materna seja um fator de risco bem estabelecido, o nascimento de indivíduos com SD de mães jovens indica a existência de outros fatores etiológicos. Estudos sugerem que o metabolismo anormal do folato pode causar hipometilação do DNA pericentromérico, favorecendo a não disjunção cromossômica. Além disso, polimorfismos em genes envolvidos no metabolismo do folato tem sido apontados como fatores de risco materno para a SD. **Objetivos:** Comparar a metilação global do DNA entre mães de indivíduos com SD e mães controle; avaliar a influência de 18 polimorfismos em genes envolvidos no metabolismo do folato e das concentrações de folato, homocisteína (Hcy) e ácido metilmalônico (MMA) na metilação do DNA; investigar a contribuição dos polimorfismos timidilato sintase (*TYMS*) repetição 28 pb, *TYMS* 1494del6, DNA metiltransferase 3B (*DNMT3B*) -149C>T, *DNMT3B* -283T>C e *DNMT3B* -579G>T na modulação do risco materno para a SD e a associação entre esses polimorfismos e as concentrações de folato, Hcy e MMA. **Casuística e Métodos:** Foram incluídas 105 mães de indivíduos com trissomia livre do cromossomo 21 e 185 mães de indivíduos sem a síndrome. A metilação das sequências LINE-1 e Alu foram quantificadas por meio de pirosequenciamento. A análise do polimorfismo *TYMS* repetição 28 pb foi realizada por meio da reação em cadeia da polimerase (PCR) por diferença de tamanho de fragmentos; os polimorfismos *TYMS* 1494del6 e *DNMT3B* -579G>T foram analisados por PCR seguida de digestão enzimática; e a PCR em tempo real foi utilizada para a genotipagem dos polimorfismos *DNMT3B* -149C>T e *DNMT3B* -283T>C. Os dados dos demais polimorfismos foram obtidos de artigos publicados.
previamente pelo grupo de pesquisa. O folato sérico foi quantificado por quimioluminescência, e Hcy e MMA plasmáticos foram determinados por cromatografia líquida/espectrometria de massas sequencial. **Resultados:** A metilação da sequência LINE-1 foi menor em mães de indivíduos com SD quando comparadas com as mães controle. Os genótipos TCN2 776 CG e GG foram associados com elevada metilação da sequência Alu, enquanto baixa metilação dessa sequência foram observadas em mães com os genótipos BHMT 742 GA e AA. O folato foi um preditor da metilação da sequência LINE-1. Mães com os genótipos TYMS 3R/3R ou DNMT3B -149TT/-283TC apresentaram maior risco de ter um filho com SD. Em relação aos metabólitos, baixa concentração de Hcy foi observada em mães com os genótipos DNMT3B -149CT/-283CC quando comparados com os demais genótipos combinados.

**Conclusões:** A metilação reduzida da sequência LINE-1 é um fator de risco materno para a SD, assim como o genótipo TYMS 3R/3R e os genótipos combinados DNMT3B -149TT/-283TC. Os genótipos TCN2 776 CG e GG e BHMT 742 GA e AA modulam a metilação da sequência Alu. O folato sérico é um preditor da metilação da sequência LINE-1 e a concentração de Hcy é modulada pelos genótipos combinados DNMT3B -149CT/-283CC na população estudada.

**Palavras chave:** Síndrome de Down, Metilação do DNA, Polimorfismo Genético, Fatores de Risco.
ABSTRACT

Introduction: Down syndrome (DS) results from failure in chromosomal segregation during maternal meiosis in about 90-95% of the cases. The advanced maternal age at conception is considered the major risk factor for DS, however, many mothers of DS individuals are young, suggesting the existence of other etiological factors. Studies suggest that abnormal folate metabolism may cause hypomethylation of DNA pericentromeric, resulting in chromosomal nondisjunction. Moreover, genetic polymorphisms involved in folate pathway have been appointed as maternal risk factors for DS. Objectives: We compared the global DNA methylation between mothers of individuals with DS and mothers of individuals without the syndrome. We also investigated the impact of 18 polymorphisms involved in folate metabolism, and folate, homocysteine (Hcy) and methylmalonic acid (MMA) concentrations on the global DNA methylation. Finally, we evaluated the influence of thymidylate synthase (TYMS) 28-base pair (bp) repeats, TYMS 1494del6, DNA methyltransferase 3B (DNMT3B) -579G>T, DNMT3B -149C>T and DNMT3B -283T>C polymorphisms as maternal risk factors for DS and the association between these polymorphisms and the concentrations of folate, Hcy and MMA. Methods: One hundred and five mothers of individuals with free trisomy 21 and 185 mothers of individuals without the syndrome were included in this study. LINE-1 and Alu methylation were quantified by pyrosequencing. The TYMS 28-bp repeats polymorphism was performed by Polymerase Chain Reaction (PCR) using difference in the size of fragments; TYMS 1494del6 and DNMT3B -579G>T polymorphisms were analyzed by PCR followed by enzymatic digestion; and real-time polymerase chain reaction allelic discrimination was used for the genotyping of DNMT3B -149C>T and -283T>C polymorphisms. Data from the other polymorphisms
were obtained from previously published articles by the research group. Plasma MMA and Hcy concentrations were determined by liquid chromatography-tandem mass spectrometry and serum folate by chemiluminescence. **Results:** LINE-1 methylation was lower in DS mothers than control mothers. Mothers with TCN2 776 CG and GG genotype present high Alu methylation and BHMT 742 GA and AA genotype were associated with low Alu methylation. Moreover, serum folate concentration was a predictor of LINE-1 methylation. Increased maternal risk for DS was associated with TYMS 3R/3R and DNMT3B -149TT/-283TC genotypes. In relation to metabolites, low Hcy concentration was observed in mothers with DNMT3B -149CT/-283CC genotypes when compared with the other combined genotypes. **Conclusions:** Reduced methylation of the LINE-1 sequence is a maternal risk factor for SD, as well as the TYMS 3R/3R genotype and the DNMT3B -149CT/-283CC combined genotypes. TCN2 776 CG and GG and BHMT 742 GA and AA genotypes modulate the Alu methylation. Serum folate is a predictor of the LINE-1 methylation and Hcy concentration is modulated by DNMT3B -149CT/-283CC combined genotypes in the studied population.

**Key words:** Down Syndrome, DNA Methylation, Genetic Polymorphism, Risk Factors.
1. INTRODUÇÃO
1. INTRODUÇÃO

A síndrome de Down é uma desordem de origem genética que causa deficiência intelectual e afeta um em cada 1000 nascidos vivos.\(^{(1)}\) Os indivíduos com a síndrome apresentam características específicas, como hipotonia muscular, prega palmar única, braquicefalia, prega epicântica, ponte nasal achatada, entre outras,\(^{(1)}\) além de maior suscetibilidade ao desenvolvimento de algumas doenças, como cardiopatias, problemas oftalmológicos e de audição, doenças respiratórias e gastrointestinais.\(^{(1-4)}\)

A trissomia livre do cromossomo 21, caracterizada pela presença de uma cópia extra deste cromossomo, é responsável por cerca de 90-95 % dos casos de síndrome de Down e resulta de falhas na segregação cromossômica durante a meiose. A não disjunção cromossômica pode ocorrer tanto na formação do óvulo quanto do espermatozoide, mas a origem materna é a principal causa, ocorrendo, predominantemente, durante a primeira divisão meiótica. Somente 10 % da trissomia livre do cromossomo 21 apresentam origem paterna.\(^{(5-8)}\) A trissomia parcial do cromossomo 21 causada por translocação Robertsonianas envolvendo, principalmente, os cromossomos 14 e 21 é a causa de cerca de 3 % dos casos.\(^{(9)}\) O restante dos casos é resultante de mosaicismo e caracteriza-se pela presença de células com arranjo cromossômico normal e células trissômicas (contendo um cromossomo 21 extra).\(^{(10)}\)

A idade materna avançada é um fator de risco bem estabelecido para a síndrome de Down e esse risco aumenta proporcionalmente com a idade. Enquanto a prevalência de síndrome de Down em mães com vinte anos é de 1:1476 nascidos vivos, com trinta e cinco anos, aumenta para 1:352 e, com cinquenta anos, para 1:25 nascidos vivos.\(^{(11)}\) Na espécie humana, os oócitos primários entram em meiose I entre a décima e décima terceira semanas de gestação e permanecem na prófase I por vários anos até a
ovulação.\textsuperscript{(12)} Embora os mecanismos celulares e moleculares que associam a idade materna avançada com a não disjunção cromossômica ainda não estejam totalmente esclarecidos, este risco pode estar associado com a perda da eficiência do processo meiótico, causado, por exemplo, por defeitos na coesão das cromátides irmãs ou por degradação de proteínas envolvidas na formação do fuso mitótico.\textsuperscript{(12-14)} Entretanto, o nascimento de indivíduos com síndrome de Down de mães jovens sugere a existência de outros fatores etiológicos.

Estudos mostram que mães de crianças com síndrome de Down com idade inferior a 35 anos apresentam instabilidade genômica e aumento na frequência de micronúcleos.\textsuperscript{(15,16)} Os micronúcleos são originados a partir de fragmentos de cromossomo ou de cromossomos inteiros perdidos durante a divisão celular,\textsuperscript{(17)} e sua elevada frequência está associada a suscetibilidade aumentada de danos e malsegregação cromossômica.\textsuperscript{(15,19)} Recente estudo também mostrou que essas mães jovens apresentam alterações no padrão global de metilação do DNA.\textsuperscript{(19)}

A metilação do DNA é uma reação que envolve a adição de um grupo metil (CH\textsubscript{3}) na posição 5\textsuperscript{\textdegree} de resíduos de citosina localizados principalmente em dinucleotídeos citosina-fosfato-guanina (CpG), sendo considerada uma importante característica epigenética que afeta a expressão gênica, a estabilidade e a integridade do DNA.\textsuperscript{(20)}

**Metabolismo do folato e metilação global do DNA**

O folato pertence à família de vitaminas do complexo B e é encontrado em alimentos como vegetais de folhas verdes, feijão, figado, cereais, kiwi e morango.\textsuperscript{(21)} A ingestão desse nutriente é essencial para o crescimento e replicação celular, uma vez que desempenha importante papel na síntese de ácidos nucléicos, aminoácidos e S-
Introdução

A adenosilmetionina (SAM), o principal doador de grupo metil para as reações de metilação do DNA, RNA e proteínas.\(^{(20)}\) A deficiência de folato está associada ao crescimento celular anormal, alterações na metilação do DNA, aumento de mutações pontuais, danos cromossômicos e aneuploidia.\(^{(22-24)}\)

No fígado, o folato ingerido na dieta é reduzido e metilado em 5-metiltetrahidrofolato (5-metilTHF), a principal forma circulante do folato que é absorvida pelas células por meio de receptores, como, por exemplo, o RFC-1 (carreador de folato reduzido 1), também conhecido como SLC19A1.\(^{(25)}\) No interior das células, o 5-metilTHF atua como doador de grupo metil para a remetilação da homocisteína (Hcy), reação catalisada pelo complexo enzimático MTR/MTRR (metionina sintase/metionina sintase redutase) que produz metionina e tetratihdrolfolato (THF). A enzima MTRR é responsável pela manutenção do estado ativo da enzima MTR, enquanto a enzima MTR necessita da vitamina B\(_{12}\), que é transportada para o interior da célula pela enzima transcobalamina 2 (TCN2), como cofator para a reação de remetilação da Hcy.\(^{(26,27)}\) O THF, produto dessa reação, funciona como cofator da síntese de ácidos nucleicos e a metionina é convertida em SAM, que atuará nas reações de metilação catalisadas pelas enzimas DNA metiltransferases (DNMTs). Estas enzimas transferem o grupo metil, resultante da transformação do SAM em S-adenosilhomocisteína (SAH), para citosinas localizadas, predominantemente, em dinucleotídeos CpG.\(^{(28,29)}\)

Sob a ação da enzima serina hidroximetiltransferase (SHMT), o THF transforma-se em 5,10-metilenotetrahidrofolato (5,10-MTHF) ou pode sofrer a ação da enzima trifuncional metilenotetrahidrofolato desidrogenase 1 (MTHFD1), que o converte em 10-formiltetrahidrofolato (10-formilTHF), 5,10-metiniltetrahidrofolato
(5,10-metinilTHF) e 5,10-MTHF. O 5,10-MTHF é transformado em 5-metilTHF, substrato para a remetilação da Hcy em metionina, pela ação da enzima metilenotetrahidrofolato redutase (MTHFR).

Outra via de remetilação da Hcy é catalisada pela enzima betaina-homocisteína metiltransferase (BHMT), na qual o aminoácido betaina atua como doador de grupo metil para esta reação. A Hcy pode também ser transformada em cistationina em uma via denominada de transulfuração, catalisada pela enzima cistationina beta-sintase (CBS).

A enzima dihidrofolato redutase (DHFR) é responsável pela conversão de dihidrofolato (DHF) em THF, a forma metabolicamente ativa do folato no organismo humano. A enzima timidilato sintase (TYMS) catalisa a conversão de deoxiuridina monofosfato (dUMP) em deoxitimidina monofosfato (dTMP), utilizando o 5,10-MTHF como doador de grupo metil. Essa reação é essencial para o fornecimento de nucleotídeos para a síntese e reparo do DNA. O metabolismo do folato e suas enzimas estão apresentados na Figura 1.
**Figura 1.** Metabolismo do folato e as enzimas envolvidas. 10-formilTHF = 10-formiltetrahidrofolato, 5-metilTHF = 5-metiltetrahidrofolato, 5,10-metililTHF = 5,10-metileniltetrahidrofolato, 5,10-MTHF = 5,10-metilenotetrahidrofolato, B12 = vitamina B12, BHMT = betaina-homocisteína metiltransferase, CBS = cistationina beta-sintase, CH3 = grupo metil, DHF = dihidrofolato, DHFR = dihidrofolato redutase, DNMT = DNA metiltransferases, dTMP = deoxitimidina monofosfato, dUMP = deoxiuridina monofosfato, MTHFD1 = metilenotetrahidrofolato desidrogenase 1, MTHFR = metilenotetrahidrofolato redutase, MTR = metionina sintase, MTRR = metionina sintase redutase, RFC1 = carreadora de folato reduzido 1, SAM = S-adenosilmetionina, SAH = S-adenosilhomocisteína, SHMT = serina hidroximetiltransferase, TCN2 = transcobalamina 2, THF = tetrahidrofolato, TYMS = timidilato sintase.
Estudos mostram que o metabolismo anormal do folato e, consequentemente, falhas na metilação do DNA podem aumentar o risco de doenças, como cânceres, doenças cardiovasculares, defeitos de tubo neural e síndrome de Down.\(^{(19,36-38)}\)

Em 1999, James et al.\(^{(39)}\) publicaram o primeiro artigo que relacionava o metabolismo anormal do folato com o risco materno para a síndrome de Down. Os autores sugeriram que a presença do polimorfismo \textit{MTHFR} C677T poderia ser um fator de risco para o nascimento de prole com síndrome de Down, independente da idade materna.\(^{(39)}\) A presença do polimorfismo \textit{MTHFR} C677T prejudica a estabilidade da enzima MTHFR e, consequentemente, reduz a síntese de SAM, causando hipometilação do DNA.\(^{(40)}\) A formação do cinetócoro, complexo DNA-proteína que garante a divisão precisa de cromossomos entre as células-filhas por meio da ligação do centrômero aos microtúbulos do fuso mitótico, depende de padrões de metilação específicos e da ligação de proteínas sensíveis à metilação na cromatina centromérica.\(^{(13,41)}\) Assim, a presença de polimorfismos em genes envolvidos no metabolismo do folato pode causar hipometilação do DNA pericentromérico, prejudicando a formação do cinetócoro e favorecendo a não disjunção cromossômica.\(^{(39)}\)

De fato, Božović et al.\(^{(19)}\) observaram que os níveis de metilação das sequências LINE-1, um marcador de metilação global do DNA, foram menores em mães de indivíduos com síndrome de Down quando comparadas com mães de indivíduos sem a síndrome. Sequências do tipo LINE (elemento nuclear intercalante longo) são elementos repetitivos que compreendem 15 % do genoma humano.\(^{(42)}\) Esses elementos possuem cerca de 6 kilobases (Kb) de comprimento e são pobres em sequência GC, sendo a família LINE-1 a mais abundante.\(^{(43-44)}\) Outro tipo de sequência repetitiva é o elemento nuclear intercalante curto (SINE). A principal família de SINE é a Alu, ocupando cerca
As sequências Alu possuem comprimento de aproximadamente 300 pares de base (pb) e são ricas em sequências GC. Os elementos LINE-1 e Alu são fortemente metilados e estimam-se que mais de um terço da metilação do DNA ocorra nessas sequências. Assim, a análise da metilação de elementos repetitivos pode servir como um marcador para a metilação global do DNA.

Os genes MTHFR, MTRR e RFC1 são os mais investigados como fatores de risco materno para a síndrome de Down. Estudos mostram que mães portadoras do polimorfismo MTHFR C677T apresentam risco aumentado de ter um filho com síndrome de Down. Os genótipos MTHFR 677 CT e TT em pessoas com dieta pobre em folato apresentaram níveis de metilação global menores em comparação às pessoas com genótipo MTHFR 677CC e dieta rica em folato, reforçando a influência desse polimorfismo na não disjunção do cromossomo 21.

A presença do polimorfismo MTRR A66G foi associada com o aumento do risco de nascimento de prole com síndrome de Down e Ishikawa et al. mostraram a influência desse polimorfismo na hipometilação do DNA em indivíduos fumantes. Outro polimorfismo associado ao risco materno para a síndrome de Down é o RFC1 A80G. O gene RFC1 codifica a enzima responsável pelo transporte de 5-metilTHF para o interior da célula e estudo mostra que mães de indivíduos com síndrome de Down portadoras do polimorfismo RFC1 A80G apresentam concentrações reduzidas de folato sérico.

Outros polimorfismos em genes envolvidos no metabolismo do folato também tem sido investigados como fatores de risco materno para a síndrome de Down. O gene TYMS apresenta um polimorfismo de repetição em tandem de 28 pb na região promotora, contendo, principalmente, duas (2R) ou três repetições
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(3R),\(^{68}\) e a quantidade de repetições afeta a expressão gênica.\(^{69}\) Coppedê et al.\(^{59}\) analizaram o polimorfismo TYMS repetição 28 pb como fator de risco materno para a síndrome de Down na população italiana, mas nenhuma associação foi observada. Por outro lado, neste mesmo estudo, o genótipo combinado TYMS 2R/2R / MTHFR 1298AC foi associado à diminuição do risco de prole com síndrome de Down. Em outro estudo que utilizou testes preditivos, Coppedê et al.\(^{70}\) observaram que o genótipo TYMS 2R/3R permite discriminar mães de indivíduos com síndrome de Down e mães de indivíduos sem a síndrome.

O gene TYMS também apresenta um polimorfismo de deleção de 6 pb na posição 1494 da região 3’ não traduzida, que pode alterar a estabilidade e expressão do RNAm, influenciando, assim, a atividade da enzima TYMS.\(^{71}\) Estudos que avaliaram a influência do polimorfismo TYMS 1494del6 no risco materno para a síndrome de Down não observaram associação.\(^{59,70}\)

O gene DNMT3B contém três polimorfismos (-149C>T, -283T>C e -579G>T) localizados na região promotora que podem influenciar a atividade da enzima DNMT3B na metilação do DNA, alterando a instabilidade centromérica.\(^{72-75}\) Esta enzima é essencial para a metilação de sítios anteriormente não metilados ou hemimetilados, processo denominado metilação de novo.\(^{28,76}\) Recentemente, Jaiswal et al.\(^{67}\) avaliaram os polimorfismos DNMT3B -149C>T e -579G>T como fatores de risco materno para a síndrome de Down e observaram um aumento da frequência do haplótipo T-G em mães de indivíduos com síndrome de Down na população indiana. Na Itália, Coppedê et al.\(^{63}\) verificaram que o genótipo DNMT3B -579GT e o genótipo combinado DNMT3B -149CC/-579GG foram associados com diminuição do risco materno para a síndrome de
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Down. Não há estudos que avaliem a associação entre o polimorfismo *DNMT3B*-283T>C e a síndrome de Down.

Considerando a importância da metilação do DNA na não disjunção cromossômica, a investigação de fatores de risco materno para a síndrome de Down relacionados ao metabolismo do folato torna-se relevante.

1.1. OBJETIVOS

1. Detectar e comparar a metilação global do DNA, refletida nas sequências LINE-1 e Alu, entre mães de indivíduos com síndrome de Down e mães com filhos sem a síndrome;


3. Investigar a contribuição dos polimorfismos *TYMS* repetição 28 pb, *TYMS* 1494del6, *DNMT3B*-149C>T, *DNMT3B*-283T>C e *DNMT3B*-579G>T na modulação do risco materno para a síndrome de Down e a associação entre esses polimorfismos e as concentrações de folato sérico e Hcy e MMA plasmáticos.
2. ARTIGOS CIENTÍFICOS
2. ARTIGOS CIENTÍFICOS

Os resultados desta tese estão apresentados em três artigos que serão submetidos para publicação.

Artigo 1

Título: Global DNA methylation and Down syndrome: a case-control study in Brazilian women.

Autores: Cristiani Cortez Mendes, Lidia Maria Rebolho Batista Arantes, Matias Eliseo Melendez, Bruna Lancia Zampieri, Joice Matos Biselli-Périco, André Lopes Carvalho, Marcos Nogueira Eberlin, Maria Francesca Riccio, Hélio Vannucchi, Valdemir Melechco Carvalho, Eny Maria Goloni-Bertollo, Érika Cristina Pavarino.

Periódico: *PLoS ONE*.

Artigo 2

Título: Effect of polymorphisms in the *TYMS* and *DNMT3B* genes on the maternal risk for Down syndrome and on the concentration of metabolites of folate pathway.

Autores: Cristiani Cortez Mendes, Patrícia Yumi Barbosa, Tatiane Éster Aidar Fernandes, Daniella Balduino Victorino, Alex Dorta, Bruna Lancia Zampieri, Joice Matos Biselli-Périco, Marcos Nogueira Eberlin, Maria Francesca Riccio, Hélio Vannucchi, Valdemir Melechco Carvalho, Eny Maria Goloni-Bertollo, Érika Cristina Pavarino.

Periódico: *Nutrients*.
Artigo 3

Título: *DNMT3B* -149C>T and -283T>C polymorphisms as a maternal risk factor for Down syndrome.

Autores: Cristiani Cortez Mendes, Thiago Luiz Aidar Fernandes, Aline Maria Zanchetta de Aquino Raimundo, Bruna Lancia Zampieri, Joice Matos Biselli-Périco, Marcos Nogueira Eberlin, Maria Francesca Riccio, Hélio Vannucchi, Valdemir Melechco Carvalho, Eny Maria Goloni-Bertollo, Érika Cristina Pavarino.

Periódico: *Molecular Biology Reports.*
Global DNA methylation and Down syndrome: a case-control study in Brazilian women

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Abstract

This study compared the global DNA methylation, reflected in LINE-1 and Alu elements, between mothers of individuals with Down syndrome (DS) and mothers of individuals without the syndrome and investigated the impact of 18 polymorphisms involved in folate metabolism, maternal age, serum folate, plasma homocysteine (Hcy) and methylmalonic acid (MMA) concentrations on the global DNA methylation. Eighty-three mothers of children with free trisomy 21 and 147 mothers without syndrome were included in this study. LINE-1 and Alu methylation were quantified by pyrosequencing. The thymidylate synthase (TYMS) 28-bp repeats polymorphism was performed by Polymerase Chain Reaction (PCR) using difference in the size of fragments; TYMS 1494del6 and DNA methyltransferase (DNMT3B) -579G>T polymorphisms were analyzed by PCR followed by enzymatic digestion; and real-time polymerase chain reaction allelic discrimination was used for the genotyping of DNMT3B -149C>T and -283T>C polymorphisms. Data from the other polymorphisms were obtained from previously published studies by the research group. Plasma MMA and Hcy concentrations were determined by liquid chromatography-tandem mass spectrometry and serum folate by chemiluminescence. The results showed that LINE-1 methylation was lower in DS mothers than control mothers. Moreover, mothers with transcobalamin 2 (TCN2) 776 CG or GG genotype present high Alu methylation and betaine-homocysteine methyltransferase (BHMT) 742 GA or AA genotype were associated with low Alu methylation. Serum folate concentration was a predictor of LINE-1 methylation. We concluded that reduced methylation of the LINE-1 sequence is a maternal risk factor for DS. In addition, TCN2 776 CG and GG and BHMT 742 GA
and AA genotypes modulate the Alu methylation and serum folate is a predictor of the LINE-1 methylation.

**Keywords:** Down syndrome, DNA methylation, genetic polymorphism.

**Introduction**

Down syndrome (DS) is the most studied human chromosomal abnormality, although little is known about the molecular mechanisms responsible for the nondisjunction. In 1999, James et al. [1] suggested that impaired folate metabolism due to polymorphisms in genes involved in this pathway might be maternal risk factor for DS. It is known that polymorphisms in genes involved in the folate metabolism modulate the maternal risk for DS [1-15] This relationship could be explained because of the role of this metabolism in DNA methylation reactions and thus in maintaining the structure of chromatin and chromosome segregation [16].

Folate is essential for the biosynthesis of nucleotides and S-adenosylmethionine (SAM), the primary methyl donor for DNA methylation (Figure 1), which regulates gene expression and prevents chromosomal fragility in specific regions such as the centromere [17-20]. Folate deficiency reduces SAM synthesis causing DNA hypomethylation [21-24]. Pericentromeric hypomethylation could impair the heterochromatin formation and kinetochore establishment, resulting in chromosomal nondisjunction [1]. This could happen because the a stable centromeric DNA chromatin depends on the epigenetic inheritance of specific centromeric methylation patterns and on the binding of specific methyl-sensitive proteins in order to maintain the higher-order DNA architecture necessary for kinetochore assembly [25,26].
Interspersed repeated DNA sequences are generally found in centromeres or centromere-adjacent heterochromatin, and contain numerous CpG dinucleotides [27]. These repetitive elements comprise approximately 45% of the human genome [28] and include long interspersed nucleotide elements (LINE), responsible for 15% of human genome with half a million copies, and Alu sequences, the most prevalent human short interspersed elements (SINE), occupying 10% of the genome with approximately 1.4 million sequences [28]. LINE are 6–8 kb long and GC-poor sequences, being LINE-1 the most abundant [29,30]. Alu-repetitive elements are short, with approximately 300 base pair (bp) in length and GC-rich [31,32]. The LINE-1 and Alu elements are heavily methylated, and it is estimated that more than one-third of DNA methylation occurs in the repetitive sequences [31,33]. Thus, analyzing the methylation of repetitive elements can serve as a marker for global genomic DNA methylation. Although more data exist at the tissue level, several studies of global white blood cell DNA methylation have shown a relation between methylation levels and human diseases [34-39].

In recent study, Božović et al. [40] observed lower levels of global DNA methylation, evaluated as LINE-1 methylation, in mothers of DS children when compared to control mothers, confirming the influence of global methylation on maternal risk for DS. Moreover, the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism was associated with reduced levels of global DNA methylation.

The aim of this study was to compare the global DNA methylation, reflected in LINE-1 and Alu elements, between mothers of individuals with DS and mothers of individuals without the syndrome. In addition, we investigated the impact of 18 polymorphisms involved in folate metabolism, maternal age, serum folate, plasma...
homocysteine (Hcy) and methylmalonic acid (MMA) concentrations on the global DNA methylation.

**Materials and methods**

**Subjects**

This case-control study included 83 mothers of DS children with karyotypically confirmed free trisomy 21 (case group) and 147 mothers with healthy offspring and no experience of miscarriages (control group). Mothers with DS offspring presenting translocation or mosaicism were not included in the case group. The study protocol was approved by the Research Ethics Committee of São José do Rio Preto Medical School (CEP-FAMERP, 222/2011), and all participants were enrolled after an informed written consent. The case group presented median maternal age of 30.17 years (min-max: 12.90-46.90) and control group of 29.16 years (15.40-57.90).

**DNA extraction and genotyping of polymorphisms**

Genomic DNA was extracted from peripheral blood lymphocytes according to Miller et al. [41] or using the *GFX*™ *Genomic Blood DNA Purification Kit* (GE Healthcare, USA). The genotyping of thymidylate synthase (*TYMS*) 28-bp repeats polymorphism was performed by PCR using difference in the size of fragments [42]. *TYMS* 1494del6 and DNA methyltransferase 3B (*DNMT3B*) -579G>T polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [43,44]. *DNMT3B* -149C>T was detected using *Taqman SNP Genotyping Assays* (C__25620192_20) and *DNMT3B* -283T>C was genotyped using TaqMan® probes and primer sequences described by Jung et al. [45].
Other 13 polymorphisms (\textit{MTHFR} C677T, \textit{MTHFR} A1298C, methionine synthase (\textit{MTR}) A2756G, methionine synthase reductase (\textit{MTRR}) A66G, reduced folate carrier type 1 (\textit{RFC1}) A80G, cystathionine beta-synthase (\textit{CBS}) 844ins68, \textit{CBS} T833C, transcobalamin 2 (\textit{TCN2}) C776G, \textit{TCN2} A67G, betaine-homocysteine methyltransferase (\textit{BHMT}) G742A, methylenetetrahydrofolate dehydrogenase 1 (\textit{MTHFD1}) G1958A, dihydrofolate reductase (\textit{DHFR}) del 19 pb, serine hydroxymethyltransferase (\textit{SHMT}) C1420T) were genotyped previously by our group [4,6,7] and the results were used in the present study.

\textit{Bisulfite treatment}

DNA samples previously extracted were treated with sodium bisulfite, which converts unmethylated cytosines to uracil, while the methylated ones remain as cytosines. Sodium-bisulfite conversion of 1 µg of DNA was performed through a bisulfite treatment kit (\textit{EpiTect® Bisulfite Kit}; \textit{Qiagen}, Valencia, CA), following manufacturer’s recommendations.

\textit{Quantitative Pyrosequencing Methylation Assays – PMA}

LINE-1 and Alu pyrosequencing assays were performed according to Bediaga et al. [46] and Bollati et al. [47], respectively, with some modifications. Briefly, hot-start PCR was carried out using 3 µL of bisulfite-treated DNA template in each reaction. Primer sequences and PCR conditions are available in Table 1. Confirmation of PCR product quality and lack of contamination was established on 2 % agarose gels using \textit{GelRedTM} (\textit{Uniscience}). Pyrosequencing was performed using the PSQ96ID pyrosequencer (\textit{Qiagen}, Valencia, CA) with the \textit{PyroMark Gold Q96 Reagent Kit} (\textit{Qiagen}, Valencia, CA). PCR products were denatured in sodium hydroxide (NaOH) and washed before a sequencing primer was annealed. The pyrosequencing reaction
starts from the 3’-end of the sequencing primer. Nucleotides (A, T, C, and G) were
dispensed into each sample well, one at a time. Whenever a base complementary to the
base in the PCR product is added, it is incorporated into the growing DNA strand,
resulting in an enzymatic cascade and production of light. The light intensity is
measured at each dispensation and presented graphically in a pyrogram. The
dispensation order was generated automatically by the *Pyromark CpG Software*. A
mean methylation index (MtI) was calculated from the mean of methylation percentages
for the CpG sites evaluated in the *Pyromark Software*. The results were analyzed using
default software settings. No samples had a zero methylation index for sequences
evaluated by pyrosequencing and low-level methylation (0 – 15 %) were considered to
represent background ‘noise’ and were calculated as unmethylated [48-50].

*Folate, Hcy and MMA concentrations*

Serum folate was measured by chemiluminescence (*Immulate Kit, DPC Medlab,*
Brazil), as per the manufacturer’s instructions. Hcy and MMA were measured from
plasma by liquid chromatography-tandem mass spectrometry, as previously described
[51-53].

*Statistical analysis*

For comparison of the levels of DNA methylation between the case and control
groups, the Mann-Whitney test was performed. Stepwise forward multivariate logistic
regression analysis was performed to access the influence of the polymorphisms on the
global DNA methylation. The genotype data used in the stepwise forward multivariate
logistic regression were analyzed in two different ways: considering either (1) the
dominant model (mutant homozygous + heterozygous *versus* wild-type homozygous);
or (2) the recessive model (wild-type homozygous + heterozygous *versus* mutant
homozygous). Maternal age was categorized in two groups: <35 years and ≥35 years. The association between metabolites concentrations, age and DNA methylation was investigated by linear regression test. *Minitab for Windows program (Release 14)* was used to analyze the data and p values < 0.05 was taken to be significant.

**Results**

LINE-1 and Alu elements were quantified as markers for global DNA methylation. LINE-1 methylation was lower in DS mothers (median: 66.00 %; min-max: 63.00 %-69.00 %) than control mothers (median: 67.00 %; min-max: 57.00 %-72.00 %) (P < 0.001) (Figure 2). Alu methylation did not differ between groups (median: 69.00 %; min-max: 59.00 %-80.00 %; median: 70.00 %; min-max: 59.00 %-80.00 %, case and control groups respectively; P = 0.15).

The analysis of the impact of 18 polymorphisms on the global DNA methylation showed that LINE-1 methylation was not influenced by these polymorphisms in the dominant and recessive models. However, in the dominant model, mothers with *TCN2 776 CG or GG genotypes present high Alu methylation (P = 0.01) and *BHMT 742 GA or AA genotypes were associated with low Alu methylation (P < 0.01). Levels of global DNA methylation according to genotype in the dominant model are presented in Table 2. In the recessive model, we observed no association between Alu methylation and polymorphisms. All statistical tests were adjusting for age.

Maternal age was also associated with Alu methylation. On both dominant and recessive models, mothers ≥35 years had significantly lower levels of methylation compared to mothers <35 years (P = 0.01 and P = 0.003, respectively). The data are shown in Table 2.
Among all studied mothers, serum folate concentration was a predictor of LINE-1 methylation (coefficient 0.03, \( P = 0.02 \)). Other metabolites were not associated with global DNA methylation (data not shown).

**Discussion**

Folate is a nutrient required for the synthesis of the major DNA-methylating agent, the SAM (Figure 1). It is known that stable centromeric DNA chromatin might depend on the epigenetic inheritance of specific centromeric methylation patterns and on the binding of specific methyl-sensitive proteins in order to maintain the higher-order DNA architecture necessary for kinetochore assembly [25,26]. In 1999, it was hypothesized that pericentromeric hypomethylation, resulting from impaired folate metabolism, could impair the formation of the kinetochore, resulting in chromosomal nondisjunction [1]. Although the global methylation is widely assumed to be a maternal risk factor for DS, it is poorly quantified at this time.

Our study compared the global DNA methylation, reflected in LINE-1 and Alu elements, between mothers of DS individuals and mothers of individuals without the syndrome in Brazilian population. The results showed a reduction of the LINE-1 methylation in DS mothers. Although the obtained values of LINE-1 methylation have been close (case group = 66.00 %, control group = 67.00 %), the minimum and maximum values and the difference in the number of case and control mothers may explain the significance of the statistical tests. Alu methylation did not differ between groups. To our knowledge, this is the first study that analyzed the global DNA methylation and its relationship with 18 polymorphisms in the Brazilian population.
LINE-1 and Alu are repetitive elements in the human genome that are normally heavily methylated. These sequences are found mainly in centromeres heterochromatin [27] and serve as maker for global DNA methylation [31,33]. In this study, the LINE-1 and Alu methylation were measured by pyrosequencing, a methodology highly reproducible that provides an accurate at measuring small changes in DNA methylation [54].

Our results corroborates with the only study that quantified the global DNA methylation in mothers of DS individuals and compared with control mothers [40]. In this study carried out in the Croatia, 94 mothers of children with full trisomy 21 and 100 control mothers were enrolled and the results showed that global DNA methylation, reflected in LINE-1 sequences, was lower in DS mothers (median: 95.45 %; min-max: 79.13 %-99.90 %) than in control mothers (median: 97.99 %; min-max: 88.61 %-99.78 %) [40]. They also observed that MTHFR C677T genotype/diet combination was predictor of global DNA methylation. Mothers with MTHFR 677 CT or TT genotype and low-folate diet presented lower levels of LINE-1 methylation than mothers with MTHFR 677 CC [40].

The analysis of influence of 18 polymorphisms in genes involved in folate metabolism on global DNA methylation showed that the TCN2 776 CG and GG genotypes are associated with high Alu methylation and low Alu methylation was observed in the BHMT 742 GA and AA genotypes. The presence of genetic polymorphisms that modify the functionality of enzymes involved in the folate metabolism can predispose to abnormal DNA methylation. Thus, our results show that the TCN2 C776G and BHMT G742A polymorphisms modulate the levels of Alu methylation.
TCN2, a cobalamin-transporting protein, plays an important role in vitamin B₁₂ cellular uptake and metabolism, cofactor for methionine synthase (MTR) enzyme, which transfers a methyl group from 5-methyltetrahydrofolate (5-methylTHF) to Hcy forming methionine [55]. TCN2 C776G polymorphism can affect the function of the enzyme and influence folate metabolism altering the DNA methylation reactions [55]. In fact, Tajuddin et al. [56] showed that this polymorphism is associated with LINE-1 methylation. They evaluated 892 control participants from the Spanish Bladder Cancer/EPICURO Study and observed a positive association between TCN2 C776G polymorphism and LINE-1 methylation in the recessive model [56]. On the other hand, Hazra et al. [57] analyzed 172 individuals with colorectal cancer and showed that TCN2 C776G polymorphism is not associated with LINE-1 methylation. Our results contradict previous study that evaluated the TCN2 C776G polymorphism as maternal risk factor for DS. Zampieri et al. [7] observed that the TCN2 776 GG genotype increase the maternal risk for DS suggesting that mothers with this genotype present low levels of global DNA methylation.

BHMT catalyzes the transfer of a methyl group from betaine to Hcy to produce methionine. Methionine is then converted to SAM, the primary methyl donor for DNA methylation [58]. BHMT G742A polymorphism can alter the affinity of the enzyme to Hcy [59], and, consequently, influences the DNA methylation reactions. However, the only study that evaluated the influence of this polymorphism on global DNA methylation, reflected in LINE-1 sequences, showed no association between them [57]. Our results also contradict studies that analyzed the association between BHMT 742G>A polymorphism and maternal risk for DS. These studies showed that the BHMT
742G>A polymorphism presents a protective effect for bearing a DS child suggesting that it is associated with high levels of global DNA methylation. [7,8,60].

In this study, reduced levels of Alu methylation were observed in mothers ≥35 years. This result corroborates with the literature that suggests that global DNA methylation reduces with increased age [61]. Indeed, studies show that advanced age is associated with low Alu methylation [62,63]. Regarding the metabolites, serum folate was a predictor of LINE-1 methylation. Although this association has not been studied, Božović et al. [40] showed that 4.5% of global DNA methylation can be predicted by the diet combined with MTHFR C677T genotype in mothers of individuals with DS.

In conclusion, reduced methylation of the LINE-1 sequence is a maternal risk factor for DS. Moreover, TCN2 776 CG and GG and BHMT 742 GA and AA genotypes modulate the Alu methylation and serum folate is a predictor of the LINE-1 methylation.

Acknowledgments

This research was financially supported by Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (grants no 2010/10771-6) and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (grants no 302157/2008-5). The authors are grateful to the participants in this study and to the Faculdade de Medicina de São José do Rio Preto (FAMERP)/Fundação Faculdade Regional de Medicina (FUNFARME) for their collaboration in this work.
References


Figure 1. Folate metabolism. 10-formylTHF = 10-formyltetrahydrofolate; 5,10-methylnilTHF = 5,10-methylniltetrahydrofolate, 5,10-MTHF = 5,10-methylenetetrahydrofolate; 5-methylTHF = 5-methyltetrahydrofolate; B12 = vitamin B12; BHMT = betaine-homocysteine methyltransferase; CBS = cystathionine beta-synthase; DHF = dihydrofolate; DHFR = dihydrofolate reductase; DNMT = DNA methyltransferases; dTMP = thymidine monophosphate; dUMP = uridine monophosphate; MTHFD1 = methylenetetrahydrofolate dehydrogenase 1; MTHFR = methylenetetrahydrofolate reductase; MTR = methionine synthase; MTRR = methionine synthase reductase; RFC1 = reduced folate carrier type 1; SAH = S-adenosylhomocysteine; SAM = S-adenosylmethionine; SHMT = serine hydroxymethyltransferase; TCN2 = transcobalamin 2; THF = tetrahydrofolate; TYMS = thymidylate synthase.
**Figure 2.** LINE-1 methylation in mothers of DS individuals and control mothers. Data are reported as median and range.
### Table 1. Primers sequences and PCR conditions used for pirosequencing.

<table>
<thead>
<tr>
<th>Forward 5’-3’ (primer)</th>
<th>Reverse 5’-3’ (primer)</th>
<th>Sequencing 5’-3’ (primer)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LINE-1</strong></td>
<td>TAGGGAGTGTAGATAGTGG</td>
<td>TTAGATAGTGGGTATAGGT</td>
<td>95°C 14:30 min</td>
</tr>
<tr>
<td></td>
<td>Biotin-AACTCCCTAACCTTAC</td>
<td></td>
<td>42°C 45s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C 45s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>x 40 cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C 10 min</td>
</tr>
<tr>
<td><strong>Alu</strong></td>
<td>Biotin-TTTTTATTAAAAATATAATT</td>
<td>AATAACTAAATTACAAACA</td>
<td>95°C 14:30 min</td>
</tr>
<tr>
<td></td>
<td>CCCAAACTAAATAACAAATAA</td>
<td></td>
<td>43°C 60s</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>72°C 120s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>x 10 cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C 10 min</td>
</tr>
</tbody>
</table>
Table 2. Percentage of LINE-1 and Alu methylation according to genotype and maternal age.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>LINE-1 (%)</th>
<th>Alu (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MTHFR C677T</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>66.00 [57.00-72.00]</td>
<td>70.00 [59.00-80.00]</td>
</tr>
<tr>
<td>CT or TT</td>
<td>67.00 [57.00-70.00]</td>
<td>70.00 [60.00-77.00]</td>
</tr>
<tr>
<td><strong>MTHFR A1298C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>67.00 [57.00-72.00]</td>
<td>69.00 [59.00-76.00]</td>
</tr>
<tr>
<td>AC or CC</td>
<td>66.00 [57.00-69.00]</td>
<td>70.00 [59.00-80.00]</td>
</tr>
<tr>
<td><strong>MTR A2756G</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>67.00 [57.00-72.00]</td>
<td>69.00 [62.00-80.00]</td>
</tr>
<tr>
<td>AG or GG</td>
<td>66.00 [57.00-69.00]</td>
<td>70.00 [59.00-76.00]</td>
</tr>
<tr>
<td><strong>MTRR A66G</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>66.00 [57.00-72.00]</td>
<td>69.00 [59.00-78.00]</td>
</tr>
<tr>
<td>AG or GG</td>
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<td>70.00 [59.00-80.00]</td>
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<tr>
<td><strong>RFC1 A80G</strong></td>
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<td>AA</td>
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<td>70.00 [63.00-80.00]</td>
</tr>
<tr>
<td>AG or GG</td>
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<td>70.00 [59.00-80.00]</td>
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<tr>
<td><strong>TCN2 A67G</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>67.00 [57.00-72.00]</td>
<td>69.50 [60.00-76.00]</td>
</tr>
<tr>
<td>AG or GG</td>
<td>66.00 [64.00-70.00]</td>
<td>70.00 [59.00-80.00]</td>
</tr>
<tr>
<td><strong>TCN2 C776G</strong></td>
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<td></td>
</tr>
<tr>
<td>CC</td>
<td>66.00 [57.00-69.00]</td>
<td>69.00 [59.00-80.00]</td>
</tr>
<tr>
<td>CG or GG</td>
<td>67.00 [57.00-72.00]</td>
<td>70.00 [62.00-80.00]</td>
</tr>
<tr>
<td><strong>CBS 844ins68</strong></td>
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<td></td>
</tr>
<tr>
<td>WW</td>
<td>66.00 [57.00-70.00]</td>
<td>70.00 [59.00-80.00]</td>
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<td>69.00 [62.00-75.00]</td>
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<tr>
<td><strong>CBS T833C</strong></td>
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<tr>
<td>TT</td>
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<td>70.00 [59.00-80.00]</td>
</tr>
<tr>
<td>TC or CC</td>
<td>67.00 [63.00-69.00]</td>
<td>69.00 [62.00-75.00]</td>
</tr>
<tr>
<td>Gene/Mutation</td>
<td>Genotype/Allele</td>
<td>Median (Range) 67.00</td>
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<td>-------------------------------</td>
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<tr>
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<td>GA or AA</td>
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<tr>
<td><strong>BHMT G742A</strong></td>
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<td>GA or AA</td>
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<td><strong>SHMT C1420T</strong></td>
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<td>66.00 [57.00-69.00]</td>
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<tr>
<td></td>
<td>CT or TT</td>
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</tr>
<tr>
<td><strong>DHFR 19-bp deletion</strong></td>
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<td>66.00 [64.00-72.00]</td>
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<tr>
<td></td>
<td>ins/del or del/del</td>
<td>67.00 [57.00-70.00]</td>
</tr>
<tr>
<td><strong>DNMT3B -149C&gt;T</strong></td>
<td>CC</td>
<td>66.00 [57.00-69.00]</td>
</tr>
<tr>
<td></td>
<td>CT or TT</td>
<td>67.00 [58.00-72.00]</td>
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<tr>
<td><strong>DNMT3B -283T&gt;C</strong></td>
<td>TT</td>
<td>67.00 [63.00-70.00]</td>
</tr>
<tr>
<td></td>
<td>TC or CC</td>
<td>66.00 [57.00-72.00]</td>
</tr>
<tr>
<td><strong>DNMT3B -579G&gt;T</strong></td>
<td>GG</td>
<td>67.00 [63.00-70.00]</td>
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<tr>
<td></td>
<td>GT or TT</td>
<td>66.00 [57.00-72.00]</td>
</tr>
<tr>
<td><strong>TYMS 28-bp repeats</strong></td>
<td>2R/2R</td>
<td>66.00 [57.00-72.00]</td>
</tr>
<tr>
<td></td>
<td>2R/3R or 3R/3R</td>
<td>67.00 [57.00-69.00]</td>
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<td><strong>TYMS 1494del6</strong></td>
<td>+6bp/+6bp</td>
<td>66.00 [63.00-69.00]</td>
</tr>
<tr>
<td></td>
<td>+6bp/-6bp or -6bp/-6bp</td>
<td>67.00 [57.00-72.00]</td>
</tr>
<tr>
<td><strong>Maternal age</strong></td>
<td>&lt;35 years</td>
<td>67.00 (57.00-72.00)</td>
</tr>
<tr>
<td></td>
<td>≥35 years</td>
<td>66.00 (64.00-69.00)</td>
</tr>
</tbody>
</table>

Data are reported as median and range.
Effect of polymorphisms in the *TYMS* and *DNMT3B* genes on the maternal risk for Down syndrome and on the concentration of metabolites of folate pathway

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Abstract

Down syndrome (DS) is a genetic disorder that results from failure of chromosome 21 segregation. Studies suggest that the occurrence of DS is associated with centromeric DNA hypomethylation as consequence of abnormal folate metabolism. Moreover, genetic polymorphisms of genes involved on this pathway have been associated with maternal risk for this syndrome. The present study evaluated the association between thymidylate synthase (TYMS) 28-base pair (bp) repeats, TYMS 1494del6 and DNA methyltransferase 3B (DNMT3B) -579G>T polymorphisms and the maternal risk for DS and the influence of these polymorphisms on the concentrations of serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA). The investigation of the TYMS 28-bp repeats polymorphism was performed by Polymerase Chain Reaction (PCR) using difference in the size of fragments, and TYMS 1494del6 and DNMT3B -579G>T polymorphisms were analyzed by PCR followed by enzymatic digestion. MMA and Hcy concentrations were determined by liquid chromatography-tandem mass spectrometry and folate by chemiluminescence. Increased maternal risk for DS was associated with TYMS 3R/3R genotype. However, the TYMS 1494del6 and DNMT3B -579G>T polymorphisms were not associated with this risk. No difference was observed in the distribution of metabolites concentrations between the genotypes. Our study suggests that the TYMS 28-bp repeats polymorphism can module the DS risk in Brazilian mothers.
1. Introduction

Down syndrome (DS) is a genetic disorder resulting from the failure of chromosome 21 segregation during maternal meiosis [1-3]. The only well-known risk factor for DS is the advanced maternal age at the time of conception [1,4-6]. However, the birth of children with DS from young mothers suggests the existence of other etiological factors.

Folate is essential for prevention of chromosome breakage and DNA hypomethylation. This important micronutrient is required for the synthesis of methionine and S-adenosylmethionine (SAM), the common methyl donor required for the maintenance of DNA methylation patterns that determine gene expression and DNA conformation [7-10]. Deficiencies in cellular folate are associated with aberrant DNA methylation, point mutations, chromosome breakage, defective chromosome recombination and aneuploidy [11]. A stable centromeric DNA chromatin depends on the epigenetic inheritance of specific centromeric methylation patterns and on the binding of specific methyl-sensitive proteins in order to maintain the higher-order DNA architecture necessary for kinetochore assembly, thus ensuring normal chromosome segregation [12,13].

James et al. [14] suggested that impaired folate metabolism due to polymorphisms in genes involved in this pathway is a risk factor for DS in young mothers. In that study, mothers of children with DS were found to have increased plasma homocysteine (Hcy) concentrations and a 2.6-fold higher risk of having a child with DS in the presence of the methylenetetrahydrofolate reductase (MTHFR) 677C>T polymorphism compared with control mothers (CI 95 % = 1.2–5.8). That paper was followed by many other studies in several genes involved in folate metabolism. Despite
considerable researches in different populations, results are often conflicting or inconclusive and the question is still unsolved [14-33].

The genomic DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which includes the DNMT3B enzyme, specialized in methylation of CpG dinucleotides within repeated sequences of the pericentromeric regions of chromosomes [34]. The \( DNMT3B \) gene, located on chromosome 20q11.2, contains a single \( G>T \) nucleotide polymorphism in the transcription start site of the promoter region (position -579) and studies suggest that mutations within the \( DNMT3B \) gene can be associated with centromere instability [35,36]. Coppedè et al. [19] showed that the \( DNMT3B -579T \) allele and \( DNMT3B -579GT \) genotype are associated with reduced risk of having a child with DS in Italian population. On the other hand, in a recent study performed in Indian women, Jaiswal et al. [33] observed no significant differences in the genotypes and alleles frequency distributions between DS and control mothers.

Other key enzyme involved in the folate metabolism is thymidylate synthase (TYMS), responsible for the conversion of deoxyuridine monophosphate (dUMP) to deoxythymydine monophosphate (dTMP) using the 5,10-methylenetetrahydrofolate as methyl donor. This reaction is the \textit{de novo} source of cellular thymidine, which is essential for the supply of nucleotides required for DNA synthesis and repair [37]. A tandem-repeats polymorphism has been identified in the promoter enhancer region of the \( TYMS \) gene, located on chromosome 18p11.32. It is a 28-base pair (bp) tandem repeats sequence within the 5'-untranslated region, and the vast majority of individuals show one of three genotypes: two tandem repeats (2R/2R), three tandem repeats (3R/3R) or a heterozygous (2R/3R) genotype [38]. Quadruple repeat (4R) and quintuple repeat (5R) are rare, but can also be observed [39-41]. Coppedè et al. [42] analyzed the
effect of this polymorphism on the maternal risk for DS and observed that combined
*TYMS* 2R/2R/*MTHFR* 1298AC genotype decreases this risk in Italian mothers.

The gene *TYMS* can also present a deletion polymorphism of 6-bp at the 1494 position in the 3'-untranslated region [43]. This polymorphism has been associated with decreased mRNA stability *in vitro* and lower gene expression *in vivo* [44]. In semantic connectivity map with data from their previous studies in Italy, Coppedè et al. [45] showed, using a mathematical approach, that the *TYMS* 1494 6bp +/+ genotype is connected with the *TYMS* 28bp 2R/2R one, and the *TYMS* 1494 6bp -/- genotype with the TYMS 28bp 3R/3R, demonstrating linkage disequilibrium between these two polymorphisms. Furthermore, in their study, the *TYMS* 1494 +6bp/-6bp genotype resulted to be the variable that had the maximal connections with other polymorphisms in genes involved on the folate metabolic pathway (principal hub of the system). Overall, the *TYMS* 28-bp 2R/3R genotype resulted to be associated to the status of being a DS mother and the *TYMS* 28-bp 3R/3R genotype was connected to the condition of being a control mother.

We carry out the present case control study to evaluate the association between *TYMS* 28-bp repeats (rs34743033), *TYMS* 1494del6 (rs16430) and *DNMT3B* -579G>T (rs569686) polymorphisms and the maternal risk for DS, along with the influence of these polymorphisms on the concentrations of serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA).

2. Experimental section

2.1. Subjects

This case-control study was composed by 94 mothers of DS children with karyotypically confirmed free trisomy 21 (mean age at conception = 31.1 ± 8.0) and a
control group of 181 mothers with healthy offspring and no reported experience of miscarriages (mean age at conception = 27.0 ± 5.3). Informed consent was obtained from all volunteers, according to the research protocol approved by the Research Ethics Committee of the São José do Rio Preto Medical School (CEP-FAMERP 222/2011).

2.2. Molecular and biochemical analysis

Fasting blood samples were collected for molecular and biochemical analysis (serum folate and plasma Hcy and MMA). Genomic DNA was extracted from peripheral blood mononuclear cells as previously described by Miller et al. [46] or using the GFX™ Genomic Blood DNA Purification Kit (GE Healthcare, USA). The TYMS 28-bp repeats polymorphism was analyzed by PCR using difference in the size of fragments [47], and TYMS 1494del6 and DNMT3B -579G>T polymorphisms were determined by PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism) [40,48]. Folate was quantified by chemiluminescence (Immulite Kit, DPC Medlab) and liquid chromatography-tandem mass spectrometry was used to determine the plasma Hcy and MMA concentrations, as previously described [49-51].

2.3. Statistical analysis

Hardy-Weinberg (HW) equilibrium was tested by the chi-square test using the BioEstat program (version 5.0), and genotype frequencies in case and control mothers were compared by the likelihood ratio. To evaluate if the polymorphisms modulate the maternal risk for DS, logistic regression test was performed. The genotype data used in the logistic regression model were analyzed in three different ways: (1) co-dominant model (wild-type homozygous versus heterozygous versus mutant homozygous); (2) dominant model (mutant homozygous + heterozygous versus wild-type homozygous);
or (3) recessive model (wild-type homozygous + heterozygous versus mutant homozygous).

The association between polymorphisms and metabolites concentrations was investigated by linear regression test. Folate, Hcy and MMA data did not present normal distribution and were analyzed in the logarithmic scale. The computer-assisted statistical analyses were carried out using the *Minitab for Windows program (Release 14)*. P values ≤ 0.05 were considered statistically significant.

### 3. Results

HW analysis showed that the allele frequencies for *TYMS* 28-bp repeats polymorphism were in equilibrium in the case group ($\chi^2 = 0.58; P = 0.45$), but in disequilibrium in the control group ($\chi^2 = 12.35; P < 0.01$). As observed for *TYMS* 28-bp repeats, *DNMT3B* -579G>T alleles were also in equilibrium in the case group ($\chi^2 = 0.16; P = 0.69$), but in disequilibrium in the control group ($\chi^2 = 5.74; P = 0.02$). Regarding *TYMS* 1494del6 polymorphism, both the case and control groups were in HW equilibrium ($\chi^2 = 1.05; P = 0.31; \chi^2 = 0.13; P = 0.72$, respectively). According to the likelihood ratio test, the genotype frequencies for *TYMS* 28-bp repeats polymorphism differed between the groups, as presented in Table 1. However, the genotype frequencies for *TYMS* 1494del6 and *DNMT3B* -579G>T polymorphisms were not different between DS and control mothers.

In the co-dominant and recessive models, the logistic regression analysis showed that *TYMS* 3R/3R genotype is associated with increased maternal risk for DS with OR = 3.99 and 3.80, respectively (95 % CI = 1.36 – 11.68; P = 0.01 and 95 % CI = 1.36 – 10.65; P = 0.01). On the other hand, *TYMS* 1494del6 and *DNMT3B* -579G>T
polymorphisms were not associated with the risk of having a child with DS. Results from logistic regression test are presented in Table 2.

When we analyzed the influence of these polymorphisms on the concentrations of folate, Hcy and MMA, no significant differences were observed in the concentration of these metabolites between the genotypes in the case and control groups (Table 3).

4. Discussion

Folate is an essential nutrient required for the one-carbon biosynthetic and epigenetic processes and its deficiency has been reported to result in aberrant DNA methylation, point mutations, chromosome breakage, defective chromosome recombination and aneuploidy [13]. Studies show that, independently of maternal age, DS can be associated with abnormal folate metabolism, and it is likely that some polymorphisms in genes involved in this pathway might impair DNA methylation and consequently chromosome segregation resulting in an increase in DS risk [14-17,19-22,24,26,28,30-33].

The TYMS 28-bp repeats polymorphism, investigated in this study, was associated with increased level of TYMS expression [38,52,53]. The TYMS enzyme catalyzes the conversion of dUMP to dTMP and is essential in the regulation of the balanced supply of the four precursors for the normal replication of DNA [37]. Studies on enzyme expression show that the 3R/3R genotype is associated with higher TYMS activity compared to 2R/2R and 2R/3R genotypes. In relation to the deletion polymorphism of 6-bp, Mandola et al. [44] observed its association with decreased mRNA stability in vitro and lower gene expression in vivo. In other study, the wild-type
genotype (+6bp/+6bp) was associated with increase in the mRNA level compared with the other genotypes [54].

In the present study, although TYMS 1494del6 polymorphism was not associated with maternal risk for DS, a significantly increased risk for the TYMS 3R/3R genotype was observed. TYMS enzyme competes with MTHFR enzyme for the 5,10-MTHF, a substrate used in DNA synthesis and methylation. Polymorphisms increasing TYMS enzyme activity might shift pools of 5,10-MTHF from DNA methylation toward DNA synthesis, thus resulting in DNA hypomethylation and consequent chromosome nondisjunction. Contrary to our findings, Coppedè et al. [42] observed no significant differences between 82 Italian mothers of DS individuals and 107 control mothers considering the isolated analysis of TYMS 28-bp repeats polymorphism. However, they observed that the combined TYMS 2R/2R /MTHFR 1298AC genotype resulted in decreased DS risk. In other study, using data from previous studies, Coppedè et al. [45] assessed the predictive capacity of artificial neural networks and built a semantic connectivity map to offer some insight regarding the complex biological connections with variables and the condition of being a DS or a control mother. The results showed that the TYMS 2R/3R genotype is a maternal risk factor for DS and that the TYMS 3R/3R genotype is associated with the condition of being a control mother. Moreover, although they showed the TYMS 1494del6 polymorphism as the principal hub of the system, it was not associated with the condition of being a DS or control mother [45].

Regarding the DNMT3B -579G>T polymorphism, our results show that it was not associated with maternal risk for DS. We observed that this polymorphism is in HW disequilibrium in the control group what could be a result of random selection or a small sample size. Although Lee et al. [48] had shown that this polymorphism does not affect
the transcriptional activity of the *DNMT3B* promoter, the *DNMT3B -579G>T* polymorphism has been associated with susceptibility to cancer [55-61]. Recently, its role has also been investigated in the maternal risk for the birth of a child with DS. Similar results to our findings were observed by Jaiswal et al. [33] in Indian population; genotypes and alleles frequencies for *DNMT3B -579G>T* polymorphism were not different between DS (n = 150) and control mothers (n = 172). On the other hand, the haplotype analysis of *DNMT3B -579G>T* and *DNMT3B -149C>T* polymorphisms, performed by Jaiswal et al. [33], showed that the G-T haplotype is a maternal risk factor for DS in their population. It is known that the DNMT3B enzyme plays an important role in DNA methylation at the centromere [34], thus it is expected that polymorphisms in *DNMT3B* gene might affect chromosome segregation and play a role in DS risk. A study performed in Italy analyzed 172 DS mothers and 157 control mothers and observed a decreased risk for DS in mothers with the *DNMT3B -579T* allele and the *DNMT3B -579GT* genotype [19]. As pointed by Jaiswal et al. [33] the discrepancy between the two studies might be due to health, nutritional and socio-economic indicators that are better in the Italian population compared to the Indian population, along with the ethnic variability between two populations.

We also evaluated the influence of the *TYMS* 28-bp repeats, *TYMS* 1494del6 and *DNMT3B -579G>T* polymorphisms on the concentrations of folate, Hcy and MMA. Although it has been shown that the *TYMS* 28-bp repeats polymorphism modulates folate and Hcy concentrations [39,62,63], we observed no difference in the distribution of the metabolites concentrations between the genotypes. Summers et al. [63] and Kumudini et al. [64] also observed no effect of this polymorphism on red blood cell and plasma folate and plasma Hcy concentrations.
TYMS enzyme competes with MTHFR for the 5,10-MTHF as the substrate for intracellular conversion of dUMP to dTMP, limiting DNA synthesis rate \[39,65\]. Despite this competition, Trinh et al. \[39\] concluded that TYMS polymorphisms are genetic determinant of folate metabolites, independent of MTHFR genotype. Chen et al. \[62\] verified a higher plasma folate concentration in individuals with TYMS 3R/3R genotype. They suggested that an overexpressed TYMS may remove its feedback inhibition on MTHFR leading to a enhanced synthesis of 5-methylTHF (5-methyltetrahydrofolate), the circulating form of folate. In contrast, Trinh et al. \[39\] showed that the TYMS 3R/3R genotype is associated with reduced plasma folate concentration and that plasma Hcy concentration was elevated in individuals with low dietary folate intake. An association between TYMS 2R allele and increased ratio of red blood cell folate derivatives (5-methylTHF:THF) was also observed \[63\].

The association between TYMS 1494del6 polymorphism and folate and Hcy concentrations remains also controversial. Kealey et al. \[66\] observed that individuals with TYMS -6bp/-6bp genotype present higher red blood cell folate and lower plasma Hcy concentration compared to the other genotypes. In other study, this genotype was associated with high plasma Hcy and 5-methylTHF concentrations \[63\] and Chen et al. \[62\] showed no effect of this polymorphism on plasma folate and Hcy concentrations. In relation to MMA, an indicator of vitamin B\(_{12}\) status, there are not studies that evaluate the impact of the TYMS 28-bp repeats and 1494del6 polymorphisms on this metabolite. Recent study verified the association between DNMT3B -579G>T polymorphism and plasma folate concentration and observed no statistically significant difference \[67\].
This is the first study evaluating the role of TYMS 28-bp repeats, TYMS 1494del6 and DNMT3B -579G>T polymorphisms in mothers of DS children in a Brazilian population. although our results did not support an association between TYMS 1494del6 and DNMT3B -579G>T polymorphisms and the risk for DS, significant evidence of an association between increased maternal risk for DS and TYMS 28-bp repeats was observed in the Brazilian population.

5. Acknowledgments

This research was financially supported by Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (grants n° 2010/10771-6) and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (grants n° 149714/2011-4; 149661/2011-8; 302157/2008-5). The authors are grateful to the participants in this study and to the Faculdade de Medicina de São José do Rio Preto (FAMERP)/Fundação Faculdade Regional de Medicina (FUNFARME) for their collaboration in this work.

6. References


[10] Palou, M.; Picó, C.; McKay, J.A.; Sánchez, J.; Priego, T.; Mathers, J.C. Protective effects of leptin during the suckling period against later obesity may be associated


syndrome: increased susceptibility in women carriers of the MTHFR 677T allele. 


the endothelial dysfunction evoked in the early stages of hyperhomocysteinemia.  


Table 1. Genotype distribution of thymidylate synthase (TYMS) 28-base pair (bp) repeats, TYMS 1494del6 and DNA methyltransferase 3B (DNMT3B) -579G>T polymorphisms in the case and control groups.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Case mothers</th>
<th>Control mothers</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYMS 28-bp repeats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2R/2R</td>
<td>34 (37.4)</td>
<td>74 (43.0)</td>
<td></td>
</tr>
<tr>
<td>2R/3R</td>
<td>46 (50.5)</td>
<td>92 (53.5)</td>
<td>0.03*</td>
</tr>
<tr>
<td>3R/3R</td>
<td>11 (12.1)</td>
<td>6 (3.5)</td>
<td></td>
</tr>
<tr>
<td>TYMS 1494del6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+6bp/+6bp</td>
<td>16 (19.7)</td>
<td>49 (29.3)</td>
<td></td>
</tr>
<tr>
<td>+6bp/-6bp</td>
<td>45 (55.6)</td>
<td>85 (50.9)</td>
<td>0.24</td>
</tr>
<tr>
<td>-6bp/-6bp</td>
<td>20 (24.7)</td>
<td>33 (19.8)</td>
<td></td>
</tr>
<tr>
<td>DNMT3B -579G&gt;T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>26 (27.6)</td>
<td>60 (33.1)</td>
<td></td>
</tr>
<tr>
<td>GT</td>
<td>45 (47.9)</td>
<td>74 (40.9)</td>
<td>0.51</td>
</tr>
<tr>
<td>TT</td>
<td>23 (24.5)</td>
<td>47 (26.0)</td>
<td></td>
</tr>
</tbody>
</table>

* χ² test.
Table 2. Association between thymidylate synthase (TYMS) 28-base pair (bp) repeats, TYMS 1494del6 and DNA methyltransferase 3B (DNMT3B) -579G>T polymorphisms and Down syndrome maternal risk according to multiple logistic regression analyses.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>OR</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TYMS 28-bp repeats</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-dominant model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2R/2R</td>
<td>1.00</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>2R/3R</td>
<td>1.09</td>
<td>0.64-1.86</td>
<td>0.76</td>
</tr>
<tr>
<td>3R/3R</td>
<td>3.99</td>
<td>1.36-11.68</td>
<td>0.01*</td>
</tr>
<tr>
<td>Dominant model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2R/2R</td>
<td>1.00</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>2R/3R and 3R/3R</td>
<td>1.27</td>
<td>0.75-2.13</td>
<td>0.38</td>
</tr>
<tr>
<td>Recessive model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2R/2R and 2R/3R</td>
<td>1.00</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>3R/3R</td>
<td>3.8</td>
<td>1.36-10.65</td>
<td>0.01*</td>
</tr>
<tr>
<td><strong>TYMS 1494del6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-dominant model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+6bp/+6bp</td>
<td>1.00</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>+6bp/-6bp</td>
<td>1.62</td>
<td>0.83-3.17</td>
<td>0.16</td>
</tr>
<tr>
<td>-6bp/-6bp</td>
<td>1.86</td>
<td>0.84-4.10</td>
<td>0.13</td>
</tr>
<tr>
<td>Dominant model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+6bp/+6bp</td>
<td>1.00</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>+6bp/-6bp and -6bp/-6bp</td>
<td>1.69</td>
<td>0.89-3.20</td>
<td>0.11</td>
</tr>
<tr>
<td>Recessive model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+6bp/+6bp and +6bp/-6bp</td>
<td>1.00</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>-6bp/-6bp</td>
<td>1.33</td>
<td>0.71-2.51</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>DNMT3B -579G&gt;T</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-dominant model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>1.00</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>GT</td>
<td>1.40</td>
<td>0.78-2.53</td>
<td>0.26</td>
</tr>
<tr>
<td>TT</td>
<td>1.13</td>
<td>0.57-2.23</td>
<td>0.73</td>
</tr>
<tr>
<td>Dominant model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>1.00</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>GT and TT</td>
<td>1.30</td>
<td>0.75-2.24</td>
<td>0.35</td>
</tr>
<tr>
<td>Recessive model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG and GT</td>
<td>1.00</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>0.92</td>
<td>0.52-1.64</td>
<td>0.79</td>
</tr>
</tbody>
</table>

OR = odds ratio; CI = confidence interval. *Statistically significant.
Table 3. Distribution of serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA) concentrations according to genotypes in the dominant and recessive models.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Folate (ng/mL)</th>
<th>Hcy (µmol/L)</th>
<th>MMA (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TYMS 28-bp repeats</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dominant model</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2R/2R</td>
<td>14.30 (5.60-74.00)</td>
<td>7.52 (1.09-36.23)</td>
<td>0.15 (0.05-1.46)</td>
</tr>
<tr>
<td>2R/3R and 3R/3R</td>
<td>13.70 (3.70-57.00)</td>
<td>7.91 (1.73-39.60)</td>
<td>0.16 (0.05-1.46)</td>
</tr>
<tr>
<td><strong>Recessive model</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2R/2R and 2R/3R</td>
<td>13.80 (3.70-74.00)</td>
<td>7.79 (1.09-39.60)</td>
<td>0.16 (0.05-1.46)</td>
</tr>
<tr>
<td>3R/3R</td>
<td>14.10 (6.00-57.00)</td>
<td>8.08 (3.57-20.03)</td>
<td>0.15 (0.09-0.46)</td>
</tr>
<tr>
<td><strong>TYMS 1494del6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dominant model</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+6bp/+6bp</td>
<td>15.35 (5.00-41.00)</td>
<td>6.87 (1.09-36.23)</td>
<td>0.15 (0.05-0.48)</td>
</tr>
<tr>
<td>+6bp/-6bp and -6bp/-6bp</td>
<td>13.80 (6.30-74.00)</td>
<td>8.01 (1.09-36.23)</td>
<td>0.16 (0.05-1.46)</td>
</tr>
<tr>
<td><strong>Recessive model</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+6bp/+6bp and +6bp/-6bp</td>
<td>14.00 (5.00-74.00)</td>
<td>7.75 (1.09-36.23)</td>
<td>0.16 (0.05-1.46)</td>
</tr>
<tr>
<td>-6bp/-6bp</td>
<td>14.30 (6.30-74.00)</td>
<td>8.08 (1.09-36.23)</td>
<td>0.15 (0.05-1.46)</td>
</tr>
<tr>
<td><strong>DNMT3B -579G&gt;T</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dominant model</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>13.80 (3.70-36.50)</td>
<td>8.11 (2.09-36.23)</td>
<td>0.15 (0.05-1.46)</td>
</tr>
<tr>
<td>GT and TT</td>
<td>14.00 (5.00-74.00)</td>
<td>7.61 (1.09-39.60)</td>
<td>0.16 (0.05-1.46)</td>
</tr>
<tr>
<td><strong>Recessive model</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG and GT</td>
<td>13.80 (3.70-74.00)</td>
<td>7.99 (1.09-39.60)</td>
<td>0.16 (0.05-0.52)</td>
</tr>
<tr>
<td>TT</td>
<td>14.20 (6.90-57.00)</td>
<td>7.43 (1.73-21.30)</td>
<td>0.16 (0.06-1.46)</td>
</tr>
</tbody>
</table>

Data are reported as median and range.
**DNMT3B** -149C>T and -283T>C polymorphisms as maternal risk factor for Down syndrome

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Abstract

Down syndrome (DS) results from failure in chromosomal segregation during maternal meiosis in about 90 % of the cases. Many mothers of DS individuals are young and studies show that polymorphisms in genes involved in folate metabolism might modulate this risk. We investigated the influence of DNA methyltransferase 3B (DNMT3B) -149C>T and -283T>C polymorphisms as maternal risk factors for DS and the association between these polymorphisms and the concentrations of folate, homocysteine (Hcy) and methylmalonic acid (MMA) in Brazilian population. One hundred and five mothers of DS individuals and 185 control mothers were studied. Molecular analysis of the DNMT3B -149C>T and -283T>C polymorphisms was performed by real-time polymerase chain reaction allelic discrimination. Serum folate was quantified by chemiluminescence, and plasma Hcy and MMA by liquid chromatography-tandem mass spectrometry. The DNMT3B -149TT/-283TC combined genotypes were associated with increased maternal risk for DS. In relation to the metabolites, low Hcy concentration was observed in mothers with DNMT3B -149CT/-283CC genotypes. This study provided evidence that DNMT3B polymorphisms can be associated with the maternal risk for DS and with the modulation of Hcy in the studied population.
1. Introduction

Down syndrome (DS) is characterized by free trisomy of chromosome 21, resulting from failure in chromosomal segregation during maternal meiosis in about 90% of the cases [1-3]. However, the molecular mechanisms responsible for meiotic nondisjunction are still poorly understood. The advanced maternal age at conception is considered the major risk factor for trisomy 21 [1,4-6], nonetheless, many mothers of DS individuals are young, suggesting the existence of other etiological factors.

James et al. [7] suggested that abnormal folate metabolism and DNA hypomethylation might increase the risk of chromosome nondisjunction. Studies show that folate deficiency reduces the production of S-adenosylmethionine (SAM), altering the pericentromeric DNA methylation [8-11]. This alteration could impair the heterochromatin formation and kinetochore establishment, resulting in chromosomal nondisjunction [7].

DNA methylation is mediated by DNA methyltransferases (DNMTs) enzymes, specifically DNMT1, DNMT3A and DNMT3B. Although DNMTs act cooperatively in order to achieve the establishment and maintenance of a genomic methylation pattern, DNMT1 is responsible for maintaining pre-existing methylation patterns due to its ability to preferentially methylate hemimethylated DNA during DNA replication. DNMT3A and DNMT3B act as de novo methyltransferases, which methylate unmethylated and hemimethylated DNA with equal efficiencies after replication [12-14]. The DNMT3B is specialized in the methylation of CpG dinucleotides within repeated sequences of the pericentromeric regions of chromosomes. Mutations within the $DNMT3B$ gene can be associated with centromere instability [15,16].
Conflicting results have been observed when evaluating polymorphism as independent DS risk factors. Studies have suggested that some polymorphisms in genes involved in the folate metabolism modulate the maternal risk for DS [7,17-30], while others show that there is no association [31-37]. The relationship between the presence of genetic polymorphisms involved in folate metabolism and maternal risk for DS is probably due to their influence in the DNA methylation reactions and thus in maintaining the structure of chromatin and chromosome segregation [38]. Considering the importance of the *DNMT3B* gene in DNA methylation reactions, investigation of polymorphisms in this gene as maternal risk factor for DS becomes relevant.

The *DNMT3B* gene, located on chromosome 20q11.2, contains a C>T transition polymorphism at -149 base pair (bp) (rs2424913) from the transcription start site, which confers 30 % increase in promoter activity [39,40]. Other variant, a T to C transition at position -283 (rs6087990) from the exon 1A transcription start site has been shown to decrease the promoter activity in 50 % [41]. Coppèdè et al. [20] showed that the *DNMT3B* -149CC genotype, combined with *DNMT3B* -579GG wild genotype, was associated with decreased DS maternal risk. On the other hand, Jaiswal et al. [30] observed no association between *DNMT3B* -149C>T and maternal risk for DS. There are no studies that evaluated the association between *DNMT3B* -283T>C polymorphism and DS.

In this study, we investigated the influence of *DNMT3B* -149C>T and *DNMT3B* -283T>C polymorphisms as maternal risk for DS and evaluated the association between these polymorphisms and the concentrations of serum folate and plasma Hcy and MMA in the Brazilian population.
2. Materials and Methods

2.1. Subjects

We enrolled 105 mothers of DS children with karyotypically confirmed free trisomy 21 and 185 mothers with healthy offspring and no experience of miscarriages. The exclusion criteria of the case group was offspring presenting translocation or mosaicism. All participants provided informed consent according to the research protocols approved by the institutional Ethics Committees.

2.2. Molecular and biochemical analysis

Fasting blood samples were collected for molecular and biochemical analysis (serum folate and plasma Hcy and MMA). DNA extraction was performed as previously described by Miller et al. [42] and the polymorphisms in DNMT3B gene were analyzed by real-time polymerase chain reaction (PCR) allelic discrimination. DNMT3B -149C>T was detected using Taqman SNP Genotyping Assays (C__25620192_20) and DNMT3B -283T>C was genotyped using TaqMan® probes and primer sequences described by Jung et al. [43].

The serum folate concentrations was quantified by chemiluminescence (Immulite Kit, DPC Medlab, Brazil) and the plasma Hcy and MMA concentrations were determined by liquid chromatography-tandem mass spectrometry, as previously described [44-46].

2.3. Statistical analysis

Allele and genotype frequencies were compared using the likelihood ratio and logistic regression tests and chi-square test was used to assess the Hardy-Weinberg equilibrium. The haplotypes frequencies of DNMT3B gene were inferred by the Haploview program (version 4.0).
Association between $DNMT3B$ polymorphisms and metabolites concentrations was investigated by linear regression test. Folate, Hcy and MMA data did not present normal distribution and were analyzed in the logarithmic scale. Data were analyzed with *Minitab for Windows program (Release 14)*. P values $\leq 0.05$ were considered to indicate a statistically significant difference.

### 3. Results

Allele frequencies were in Hardy-Weinberg equilibrium and no differences in allele and genotype frequencies was observed between DS mothers and control group. The genotype distribution of the polymorphisms is presented in Table 1.

The results of haplotype analysis showed that the variants at positions -149 and -283 of the $DNMT3B$ gene are strongly linked (LOD = 43.55; $D’ = 0.77$). However, there was no difference in the haplotype frequencies between the groups (Table 2).

The influence of $DNMT3B$ -149C>T and -283T>C polymorphisms on maternal risk for DS and the association between these polymorphisms and folate, Hcy and MMA concentrations were evaluated both alone and in combination. The logistic regression analysis showed that $DNMT3B$ -149C>T and -283T>C polymorphisms were not associated with the risk of having a child with DS, on the other hand, $DNMT3B$ -149TT/-283TC combined genotypes were associated with increased maternal risk for DS with OR = 4.61 (CI 95 % = 1.35 – 15.79; $P = 0.02$). The combined genotypes and respective OR values are presented in Table 3.

The analysis of combined genotypes showed that mothers with $DNMT3B$ -149CT/-283CC genotype presented lower plasma Hcy concentration ($P = 0.04$) compared to the other combined genotypes in the studied population (case and control
mothers). The distribution of the folate, Hcy and MMA concentrations according to the combined genotypes are presented in Table 4.

4. Discussion

Folate is an essential vitamin for DNA synthesis and epigenetic processes [47]. Its deficiency can cause problems such as aberrant DNA methylation, point mutations, chromosome breakage, defective chromosome recombination and aneuploidy [8-11]. Considering that impairments in folate metabolism due to genetic polymorphisms can change the risk of having an infant with DS [7,17-30], this study evaluated the influence of DNMT3B -149C>T and -283T>C polymorphisms on maternal risk for DS and on the concentrations of folate pathway metabolites.

Our results show that DNMT3B -149TT/-283TC combined genotypes increase the risk of having a child with DS. DNMT3B enzyme is responsible for methylation of cytosine to 5-methylcytosine after replication [48]. It is known that DNMT3B -149C>T and -283T>C polymorphisms affect enzyme activity, resulting in changes in the DNA methylation of centromeric and pericentromeric regions [39-41]. Thus, these polymorphisms might affect chromosome segregation and increase the risk for DS. In Italy, Coppedè et al. [23] analyzed 172 DS mothers and 157 control mothers and observed that DNMT3B -149CC/-579GG combined genotypes is associated with decreased DS maternal risk (OR = 0.22; 95 %, CI = 0.08–0.64, P = 0.003). In contrast, in India, although Jaiswal et al. [30] showed that DNMT3B -149C>T polymorphism is not an independent risk factor for DS, the haplotype analysis of DNMT3B -149C>T and -579G>T polymorphisms showed an increased frequency of T-G alleles and a decreased frequency of C-G allelic combinations in DS mothers compared with controls.
Regarding our haplotype analysis, although there was evidence of Linkage Disequilibrium (LD) between the $DNMT3B$ -149C>T and -283T>C polymorphisms, no haplotype frequency was associated with maternal risk for DS.

We observed that mothers with the combined $DNMT3B$ -149CT/-283CC genotypes presented reduced Hcy concentrations compared to other combined genotypes. The DNMTs enzymes catalyze the methylation reaction, in which the SAM donates methyl group to the DNA. This reaction results in 5-methylcytosine and S-adenosyl-L-homocysteine (SAH), which is converted into Hcy (Figure 1). It is possible that the $DNMT3B$ -149C>T polymorphism, responsible for the increased enzymatic activity [39,40], reduces the amount of SAM and, consequently, the conversion of SAH to Hcy. On the other hand, the $DNMT3B$ -283T>C polymorphism was associated with decreased enzymatic activity [41]. Thus, further studies are needed to clarify the association of the combined $DNMT3B$ -149C>T and -283T>C genotypes with Hcy concentrations.

In conclusion, the results of the present study provide evidence that $DNMT3B$ polymorphisms can be associated with the maternal risk for DS and with the modulation of Hcy in the studied population. A larger study is of interest to confirm these findings.

5. Acknowledgements

This research was financially supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - grants no. 2009/04304-9; 2010/10771-6), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – grants no. 302157/2008-5). The authors are grateful to the participants in this study and to the
Faculdade de Medicina de São José do Rio Preto (FAMERP)/Fundação Faculdade Regional de Medicina (FUNFARME) for their collaboration in this work.

6. References


Figure 1. Folate metabolism. 5,10-MTHF = 5,10-methylenetetrahydrofolate; 5-methylTHF = 5-methyltetrahydrofolate; B₁₂ = vitamin B₁₂; CH₃ = methyl; DHF = dihydrofolate; DNMTs = DNA methyltransferases; dTMP = deoxythymidine monophosphate; dUMP = deoxyuridine monophosphate; MTHFR = methylenetetrahydrofolate reductase; MTR = methionine synthase; SAH = S-adenosylhomocysteine; SAM = S-adenosylmethionine; THF = tetrahydrofolate.
Table 1. Genotype distribution of DNA methyltransferase 3B (DNMT3B) -149C>T and DNMT3B -283T>C polymorphisms between the case and control groups.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Case mothers</th>
<th>Control mothers</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td><strong>DNMT3B -149C&gt;T</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>16 (19.0)</td>
<td>43 (24.3)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>44 (52.4)</td>
<td>91 (51.4)</td>
<td>0.57</td>
</tr>
<tr>
<td>TT</td>
<td>24 (28.6)</td>
<td>43 (24.3)</td>
<td></td>
</tr>
<tr>
<td><strong>DNMT3B -283T&gt;C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>23 (22.1)</td>
<td>53 (28.7)</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>59 (56.7)</td>
<td>87 (47.0)</td>
<td>0.30</td>
</tr>
<tr>
<td>CC</td>
<td>22 (21.2)</td>
<td>45 (24.3)</td>
<td></td>
</tr>
</tbody>
</table>

* Likelihood ratio test
Table 2. Haplotype frequencies of DNA methyltransferase 3B (\textit{DNMT3B}) -149C>T and \textit{DNMT3B} -283T>C polymorphisms in the case and control groups.

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Frequencies</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>\textit{DNMT3B} -149/-283</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>0.46</td>
<td>0.33</td>
<td>0.57</td>
</tr>
<tr>
<td>CC</td>
<td>0.41</td>
<td>0.68</td>
<td>0.41</td>
</tr>
<tr>
<td>CT</td>
<td>0.07</td>
<td>0.17</td>
<td>0.68</td>
</tr>
<tr>
<td>TC</td>
<td>0.06</td>
<td>0.098</td>
<td>0.32</td>
</tr>
</tbody>
</table>
Table 3. Association between combined genotypes and Down syndrome maternal risk according to multiple logistic regression analyses.

<table>
<thead>
<tr>
<th>Combined genotypes</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT3B -149 / -283</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC/TT</td>
<td>0.88</td>
<td>0.30-2.59</td>
<td>0.82</td>
</tr>
<tr>
<td>CT/TT</td>
<td>0.97</td>
<td>0.32-2.88</td>
<td>0.95</td>
</tr>
<tr>
<td>CC/TC</td>
<td>1.14</td>
<td>0.67-1.94</td>
<td>0.63</td>
</tr>
<tr>
<td>CT/TC</td>
<td>4.61</td>
<td>1.35-15.79</td>
<td>0.02*</td>
</tr>
<tr>
<td>TT/TC</td>
<td>1.60</td>
<td>0.43-5.96</td>
<td>0.49</td>
</tr>
<tr>
<td>CC/CC</td>
<td>1.39</td>
<td>0.66-2.92</td>
<td>0.39</td>
</tr>
<tr>
<td>TT/TT</td>
<td>0.87</td>
<td>0.45-1.68</td>
<td>0.69</td>
</tr>
<tr>
<td>TT/CC</td>
<td>1 sample</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OR = odds ratio; CI = confidence interval. *Statistically significant.
Table 4. Distribution of serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA) concentrations according to combined genotypes DNA methyltransferase 3B (DNMT3B) -149C>T/DNMT3B -283T>C.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Folate (ng/mL)</th>
<th>Hcy (µmol/L)</th>
<th>MMA (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC/TT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.20</td>
<td>6.97</td>
<td>0.27</td>
</tr>
<tr>
<td>CT/TT</td>
<td>14.10 (6.30-35.50)</td>
<td>9.29 (3.03-36.23)</td>
<td>0.15 (0.07-0.35)</td>
</tr>
<tr>
<td>CC/TC</td>
<td>13.50 (9.60-32.60)</td>
<td>8.32 (2.81-39.60)</td>
<td>0.17 (0.11-0.28)</td>
</tr>
<tr>
<td>CT/TC</td>
<td>14.35 (3.70-74.00)</td>
<td>7.86 (2.09-26.20)</td>
<td>0.15 (0.05-1.46)</td>
</tr>
<tr>
<td>TT/TC</td>
<td>13.15 (7.50-23.50)</td>
<td>7.26 (3.36-22.13)</td>
<td>0.16 (0.08-0.67)</td>
</tr>
<tr>
<td>CT/CC</td>
<td>20.85 (10.40-50.00)</td>
<td>8.68 (1.09-10.90)*</td>
<td>0.16 (0.09-1.41)</td>
</tr>
<tr>
<td>CC/CC</td>
<td>12.50 (6.90-57.00)</td>
<td>7.39 (2.01-15.13)</td>
<td>0.16 (0.06-0.72)</td>
</tr>
<tr>
<td>TT/TT</td>
<td>13.85 (5.60-36.50)</td>
<td>7.96 (2.37-15.27)</td>
<td>0.14 (0.05-0.53)</td>
</tr>
<tr>
<td>TT/CC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.60</td>
<td>8.51</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Metabolites data are reported as median and range. *Genotypes observed in one individual. *Statistically significant.
3. CONCLUSÕES
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1. Mães de indivíduos com síndrome de Down apresentam níveis reduzidos de metilação da sequência LINE-1 em relação às mães controle.

2. Os polimorfismos TCN2 C776G e BHMT G742A modulam os níveis de metilação da sequência Alu enquanto a metilação da sequência LINE-1 é modulada pelo folato sérico.

3. O polimorfismo TYMS repetição 28 pb e os genótipos combinados DNMT3B -149TT/-283TC são fatores de risco materno para a síndrome de Down e a concentração de Hcy é modulada pelos genótipos combinados DNMT3B -149CT/-283CC.
4. REFERÊNCIAS BIBLIOGRÁFICAS
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