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SÍNDROME DE DOWN E O METABOLISMO DO FOLATO: ANÁLISE GENÉTICA E METABÓLICA

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Orientadora: Prof[a]. Dr[a]. Érika Cristina Pavarino-Bertelli

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Sumário

Dedicatória

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Epígrafe

"O ponto, que ontem era invisível, é hoje o ponto de chegada. Amanha será o de partida..." Macaulay

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5-MTHF	5-metiltetrahidrofolato (5-*methyltetrahydrofolate*)
5,10-MTHF	5,10-metilenotetrahidrofolato (*5,10-methylenetetrahydrofolate*)
ANOVA	*Variance analysis*
BHMT	Betaína-homocisteína metiltransferase (*Betaine-homocysteine methyltransferase)*
BML	*Binucleated micronucleated lymphocytes*
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (*Coordination for the Improvement of Higher Education Personnel*)
Cb	Cobalamina
CβS	Cistationina β-sintase (*Cystathionine β-synthase*)
CEP	*Research Ethics Committee*
CH_3	Metil (*Methyl*)
CI	*Confidence interval*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico (*National Council for Scientific and Technological Development*)
CONEP	Comitê Nacional de Pesquisa (*National Research Commission*)
D'	*Lewontin's D'*
dATP	Desoxiadenina 5' trifosfato *(Deoxyadenosine 5'-triphosphate)*
dGTP	Desoxiguanosina 5' trifosfato (*Deoxyguanosine 5'-triphosphate)*
DNA	Ácido desoxirribonucléico (*Desoxirribonucleic acid*)
DS	*Down syndrome*

DTN	Defeito de fechamento de tubo neural
dTTP	Desoxitimidina 5' trifosfato *(Deoxythymidine 5'-triphosphate)*
FAMERP	Faculdade de Medicina de São José do Rio Preto (*São José do Rio Preto Medical School*)
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo (*São Paulo State Research Foundation*)
FUNFARME	Fundação Faculdade Regional de Medicina de São José do Rio Preto
HB	Hospital de Base
Hcy	Homocisteína (*Homocysteine*)
HW	*Hardy-Weinberg*
LC-MS/MS	Cromatografia líquida/espectrometria de massas seqüencial
LD	*Linkage disequilibrium*
L-MM-Coa mutase	L-metilmalonil coenzima A mutase (*L-methylmalonyl coenzyme A mutase*)
LOD	*Logarithm of odds*
KS	*Klinefelter syndrome*
MI	*Meiosis I*
MII	*Meiosis II*
MMA	Ácido metilmalônico (*Methylmalonic acid)*
MTHFD1	Metilenotetrahidrofolato desidrogenase 1 (*Methylenetetrahydrofolate dehidrogenase 1*)

MTHFR	Metilenotetrahidrofolato redutase (*Methylenetetrahydrofolate reductase*)
MTR	Metionina sintase (*Methionine synthase*)
MTRR	Metionina sintase redutase (*Methionine synthase reductase*)
NADPH	Nicotinamida adenina dinucleotídeo fosfato
OR	*Odds ratio*
PB	Pares de base
PCR	Reação em Cadeia da Polimerase (*Polymerase chain reaction*)
PCR-RFLP	*Polymerase chain reaction-restriction fragment length polymorphism*
QF-PCR	*Multiplex quantitative fluorescence polymerase chain reaction*
RFC1	Carregador de folato reduzido 1 (*Reduced folate carrier 1*)
RNA	Ácido ribonucléico (*Ribonucleic acid)*
SAH	S-adenosilhomocisteína (*S-adenosylhomocysteine*)
SAM	S-adenosilmetionina (*S-adenosylmethionine*)
SD	Síndrome de Down
TC2	Transcobalamina 2 *(Transcobalamin 2)*
THF	Tetrahidrofolato *(Tetrahydrofolate)*
UNICAMP	Universidade Estadual de Campinas (*State University of Campinas*)
UPGEM	Unidade de Pesquisa em Genética e Biologia Molecular (*Genetics and Molecular Biology Research Unit*)

nmol/L	Nanomol / litro (*Nanomol / liter*)
μmol/L	Micromol / litro (*Micromol / liter*)
ng/mL	Nanograma / mililitro (*Nanogram / milliliter*)

Introdução: A síndrome de Down (SD) é a cromossomopatia humana mais comum com prevalência aproximada de 1 em cada 660 nativivos e ocorre em 95% dos casos como resultado da não-disjunção cromossômica. Acredita-se que o metabolismo anormal do folato como resultado de polimorfismos genéticos possa levar à hipometilação do DNA e consequente não-disjunção cromossômica. **Objetivos:** Avaliar a influência dos polimorfismos *Betaína-homocisteína metiltransferase (BHMT)* G742A*, Cistationina β-sintase (CβS)* 844ins68 *e* T833C, *Metilenotetrahidrofolato desidrogenase 1 (MTHFD1)* G1958A, *Transcobalamina 2 (TC2)* A67G e C776G e das concentrações de homocisteína (Hcy) e ácido metilmalônico (MMA) plasmáticos e folato sérico no risco materno para a SD; investigar o impacto dos polimorfismos *BHMT* G742A, *CβS* 844ins68 e T833C, *MTHFD1* G1958A, *TC2* A67G e C776G nas concentrações de Hcy e MMA plasmáticos e folato sérico em mães caso e controle e em indivíduos com SD. **Casuística e Método:** Foram incluídas 105 mães de indivíduos com SD (grupo caso), 185 mulheres que tiveram filhos não afetados pela SD e sem história de aborto (grupo controle), e 90 indivíduos com trissomia livre do 21. As quantificações de Hcy e MMA plasmáticos foram obtidas pela técnica de cromatografia líquida/espectrometria de massas sequencial (LC-MS/MS) e a quantificação do folato sérico por quimioluminescência. A extração do DNA foi realizada a partir de leucócitos do sangue periférico para investigação do polimorfismo *CβS* 844ins68 pela técnica da reação em cadeia da polimerase (PCR), dos polimorfismos *CβS* T833C*, MTHFD1* G1958A e *TC2* C776G pela técnica de PCR seguida por digestão enzimática, e dos polimorfismos *TC2* A67G e *BHMT* G742A pela técnica de Discriminação Alélica por PCR em tempo real. **Resultados:** O genótipo *TC2* 776 GG apresentou-se mais frequente no grupo de mães caso quando comparado ao grupo controle e foi associado ao aumento

do risco materno para a SD no subgrupo de mulheres com idade materna inferior a 35 anos. Os genótipos combinados *MTHFR* 677 TC ou TT / *TC2* 776 CC, *MTHFR* 677 TC ou TT / *MTHFD1* 1958 GA ou AA e *MTR* 2756 AG ou GG / *MTHFD1* 1958 GA ou AA foram associados ao aumento do risco materno para a SD, enquanto os genótipos combinados *TC2* 67 AA / *BHMT* 742 GA ou AA apresentaram um efeito protetor. Considerando a quantificação dos metabólitos, concentrações aumentadas de MMA e concentrações reduzidas de Hcy e folato foram observadas no grupo de mães caso em comparação ao grupo controle. Concentrações aumentadas de Hcy foram observadas na presença do genótipo *BHMT* 742 GG quando comparado aos genótipos AA ou GA. Concentrações reduzidas de MMA foram associadas à presença dos genótipos *BHMT* 742 AA ou GA. Em relação ao grupo de indivíduos com SD, os polimorfismos *TC2* C776G e *BHMT* G742A mostraram-se moduladores das concentrações de Hcy plasmática, enquanto o polimorfismo *TC2* A67G afetou as concentrações de folato e os polimorfismos *CβS* T833C e 844ins68 as concentrações de MMA. **Conclusão:** Polimorfismos em genes envolvidos no metabolismo do folato exercem influência no risco materno para a SD e regulam as concentrações dos metabólitos envolvidos nesse metabolismo.

Palavras-chave: síndrome de Down, trissomia do 21, metabolismo do folato, polimorfismo genético.

Introduction: Down syndrome (DS) is the most common human chromosomal disorder with prevalence of 1 in 660 live births and occurs in 95% of the cases as a result of chromsomic nondisjunction. Abnormal folate metabolism as a consequence of genetic polymorphisms could result in DNA hypomethylation and consequent chromosomal nondisjunction. **Objetivos:** Evaluate the influence of *Betaine-homocysteine methyltransferase (BHMT)* G742A, *Cystathionine ß-synthase (CβS)* 844ins68 and T833C, *Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1)* G1958A, *Transcobalamin 2 (TC2)* A67G and C776G polymorphisms and plasma homocysteine (Hcy) and methylmalonic acid (MMA) concentrations and serum folate concentration on maternal risk factor for DS; investigate the impact of *BHMT* G742A, *CβS* 844ins68 and T833C, *MTHFD1* G1958A, *TC2* A67G and C776G polymorphisms in Hcy, MMA and folate concentrations in control and DS mothers and in individuals with DS. **Methods:** 105 mothers of DS individuals (case group), 185 mothers that had children not afected by DS and history of miscarriages (control group), and 90 individuals with free trisomy 21 were included in the study. Plasma Hcy and MMA concentrations were obtained by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and folate concentration by quimioluminescence. DNA was isolated from peripheral blood for the investigation of *CBS* 844ins68 polymorphism amplified by polymerase chain reaction (PCR), *CβS T833C, MTHFD1* G1958A and *TC2* C776G polymorphisms by PCR followed by enzyme digestion, and *BHMT* G742A and *TC2* A67G polymorphisms by the technique of Allelic Discrimination by Real Time PCR. **Results:** Genotype *TC2* 776 GG was more frequently observed in the group of case mothers compared to control and was associated with increased maternal risk for DS in the group of women with maternal age less than 35 years old. The combined genotypes *MTHFR* 677 TC or TT /

TC2 776 CC, *MTHFR* 677 TC or TT / *MTHFD1* 1958 GA or AA and *MTR* 2756 AG or GG / *MTHFD1* 1958 GA or AA were associated with increased maternal risk for DS, while the combined genotypes *TC2* 67 AA / *BHMT* 742 GA or AA showed a protective effect. Regarding quantification of metabolites, higher MMA concentration and lower Hcy and folate concentrations were observed in DS mothers compared to control mothers. Higher Hcy concentrations were associated with the presence of *BHMT* 742 GG genotype when compared to GA or AA genotypes. Lower MMA concentrations were associated with the presence of *BHMT* 742 AA or GA. Regarding individuals with DS, polymorphisms *TC2* C776G and *BHMT* G742A showed to be modulators of Hcy concentrations, while polymorphism *TC2* A67G affected folate concentration and polymorphisms *CβS* T833C and 844ins68 the MMA concentrations. **Conclusions:** Polymorphisms in genes involved in folate metabolism exert influence on maternal risk for DS and modulate the concentrations of metabolites involved in this metabolism.

Key words: Down's syndrome, 21 trisomy, folate metabolism, genetic polymorphism.

1.INTRODUÇÃO

1. Introdução

A síndrome de Down (SD) ou trissomia do 21 é a cromossomopatia humana mais comum, com prevalência aproximada de um em cada 660 nascidos vivos e seu fenótipo é complexo e variável entre os indivíduos.[1] Apesar da grande ocorrência dessa síndrome e dos avanços nas áreas celular e molecular, pouco é conhecido a respeito das causas da não-disjunção cromossômica, responsável por cerca de 95% dos casos.[2]

Dentre os fatores associados à ocorrência da síndrome, a idade materna avançada é o único fator de risco estabelecido.[3] Entretanto, o nascimento de indivíduos com SD de mães jovens sugere a existência de outros fatores etiológicos para a síndrome. Recentemente, a hipótese mais investigada para explicar a etiologia da SD, independente da idade materna, é a que relaciona a trissomia do cromossomo 21 à hipometilação do DNA como consequência do metabolismo anormal do folato.[4] Estudos mostram que a hipometilação está associada à instabilidade cromossômica, à alterações na expressão gênica e à segregação anormal dos cromossomos.[5,6]

O metabolismo do folato está envolvido em dois processos fisiológicos essenciais: síntese de purinas e pirimidinas, necessárias para a síntese e reparo do DNA; e metilação celular, que atua no controle associada com expressão gênica e na manutenção da estabilidade genômica (Figura 1).

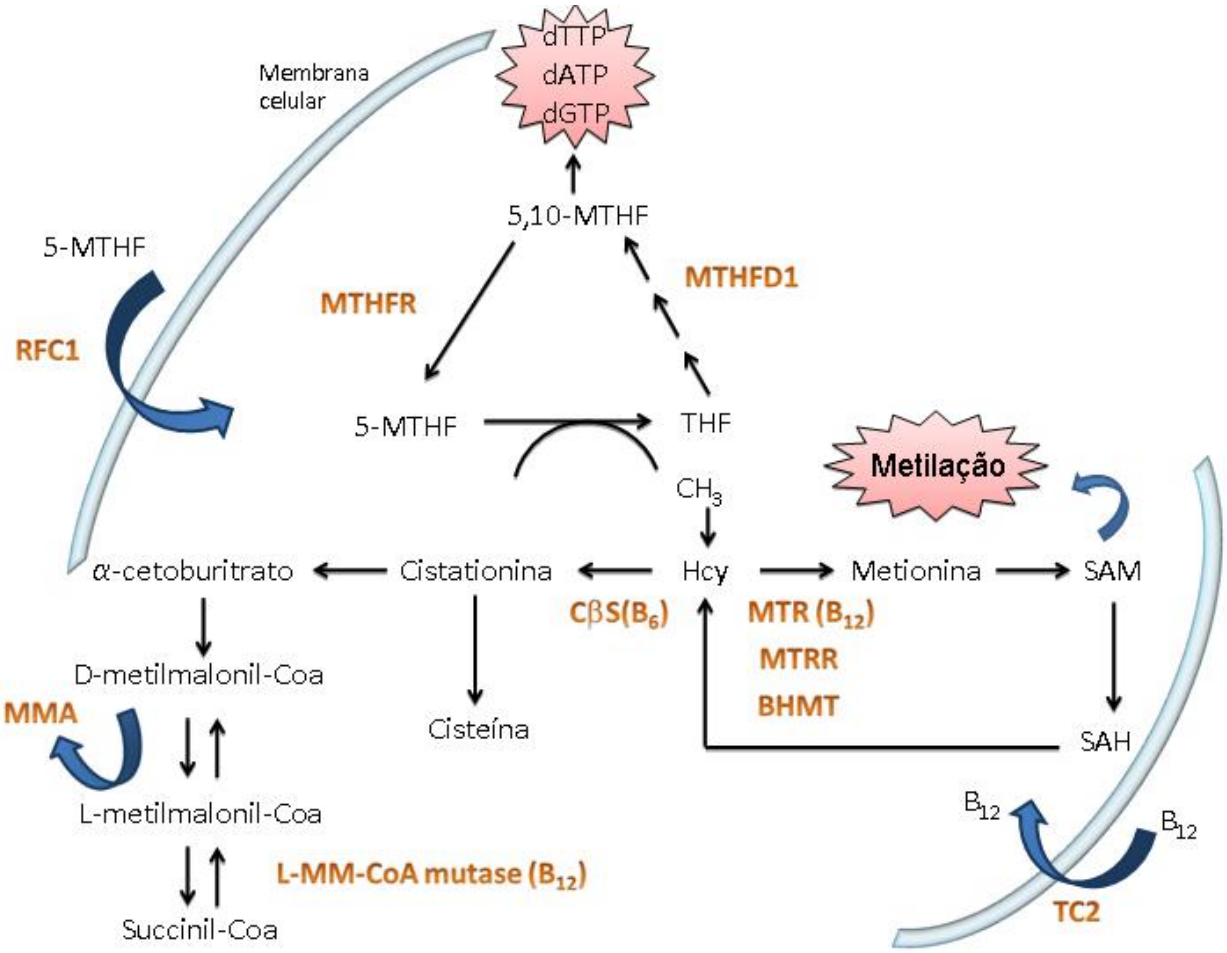

Figura 1. Esquema representando o metabolismo do folato com as principais enzimas envolvidas. BHMT = Betaína-homocisteína metiltransferase, CβS = Cistationina β-sintase, dATP = Desoxiadenosina 5'-trifosfato, dGTP = Desoxiguanosina 5'-trifosfato, dTTP = Desoxitimidina 5'-trifosfato, CH_3 = Metil, 5-MTHF = 5-metiltetrahidrofolato, 5,10-MTHF = 5,10-metilenotetrahidrofolato, Hcy = Homocisteína, L-MM-Coa mutase = L-metilmalonil coenzima A mutase, MMA= Ácido metilmalônico, MTHFD1 = Metilenotetrahidrofolato desidrogenase 1, MTHFR = Metilenotetrahidrofolato redutase, MTR = Metionina sintase, MTRR = Metionina sinstase redutase, RFC1 = Carregador de folato reduzido 1, SAH = S-adenosilhomocisteína, SAM = S-adenosilmetionina, TC2 = Transcobalamina 2, THF = Tetrahidrofolato.

Nesse metabolismo, a enzima trifuncional Metilenotetrahidrofolato desidrogenesase 1 (MTHFD1) catalisa a conversão de tetrahidrofolato (THF) para os derivados correspondentes 10-formil, 5,10-metinil e 5,10-metilenotetrahidrofolato.[7] A enzima Metilenotetrahidrofolato redutase (MTHFR) por sua vez, catalisa a conversão do 5,10-metilenotetrahidrofolato para 5-metiltetrahidrofolato (5-MTHF), a principal forma circulante de folato que atua como doador de grupos metil para a remetilação da homocisteína (Hcy) em metionina. Esta reação de remetilação é catalisada pela enzima Metionina sintase (MTR), que requer a vitamina $B_{12,}$ ou cobalamina (Cbl), como co-fator, e resulta na formação de S-adenosilmetionina (SAM),[8,9] que é desmetilada para formar S-adenosilhomocisteína (SAH) e, posteriormente, hidrolisada para adenosina e Hcy.

A enzima Metionina sintase redutase (MTRR), codificada pelo gene *MTRR*, é responsável pela manutenção do estado ativo da enzima MTR. Durante a remetilação da Hcy para metionina, catalisada pela enzima MTR, a metilcob(III)alamina atua como um doador intermediário de metil. Nesta reação, a transferência do grupo metil da metilcob(III)alamina resulta na formação de cob(I)alamina altamente reativa, a qual torna-se oxidada em cob(II)alamina, resultando na inativação da enzima MTR.[19] Neste mecanismo de inativação, é formado um complexo entre as enzimas MTR e MTRR, e elétrons derivados da oxidação de nicotinamida adenina dinucleótido fosfato (NADPH), catalisada pela enzima MTRR, são transferidos para a forma inativa de MTR. Este processo facilita a transferência de grupos metil de SAM para a enzima MTR, gerando, assim, metilcob(III)alamina, e restabelecendo a atividade da MTR.[20-22]

A enzima Betaína-homocisteína metiltransferase (BHMT) catalisa a conversão da Hcy para metionina em uma via alternativa de remetilação da Hcy, na qual o

aminoácido betaína atua como doador de grupo metil para esta reação. Quando a via de remetilação da Hcy catalisada pela enzima MTR encontra-se alterada, por fatores genéticos ou ambientais, a enzima BHMT desenvolve papel crucial na homeostase da Hcy.[10]

Na via de transsulfuração, a Hcy é convertida em cistationina pela enzima Cistationina β-sintase (CβS), dependente de vitamina B_6 e, posteriormente, em cisteína.[11] Sob condições fisiológicas, toda Hcy é remetilada para metionina ou catabolizada para formação de cistationina e não é excretada pelos rins em quantidades significativas.[12] O aumento da Hcy plasmática pode indicar alterações no metabolismo do folato e, consequentemente, nas reações de metilação celulares.

Em outra via de eliminação do excesso de Hcy, a segunda enzima dependente de vitamina B_{12}, a L-metilmalonil-coA mutase, faz a conversão de metilmalonil-coA para succinil-coA, tendo a adenosilcobalamina como cofator.[13-15] A deficiência de vitamina B_{12} impede esta reação desviando o substrato para a formação de ácido metilmalônico (MMA), resultando em níveis elevados no sangue e urina.[16-18]

Proteínas transportadoras de folato reduzido (proteína carregadora de folato reduzido 1, gene *RFC1*) e vitamina B_{12} (proteína transcobalamina II, gene *TC2*), são também importantes para o metabolismo do folato. A proteína RFC1 localiza-se na membrana das células da mucosa intestinal e participa do processo de absorção do ácido fólico, realizando o transporte do 5-MTHF para o interior de uma variedade de células, constituindo um importante determinante das concentrações de folato disponíveis no meio intracelular.[23] Por sua vez, a proteína TC2 é sintetizada no endotélio vascular da vilosidade intestinal e liga-se à vitamina B_{12} livre no fluido intersticial. A proteína TC2

ligada à vitamina B_{12} (complexo TC2- vitamina B_{12}) passa, então, a microcirculação da vilosidade intestinal e por meio da veia porta alcança a circulação sistêmica.[24]

Polimorfismos em genes envolvidos no metabolismo do folato parecem modular concentrações de metabólitos envolvidos nesse metabolismo.[25-27] O primeiro estudo a relacionar metabolismo anormal do folato, polimorfismos em genes que codificam enzimas envolvidas nesse metabolismo e o risco materno para a SD foi o de James e colaboradores (1999),[4] no qual o polimorfismo de substituição de citosina para timina na posição 677 do gene *MTHFR* (C677T), que resulta em termolabilidade e atividade enzimática reduzida, foi associado ao aumento do risco materno para a SD. Posteriormente, vários estudos em diferentes populações confirmaram essa associação [28-30] e outros demonstraram a contribuição do polimorfismo *MTHFR* C677T para o aumento das concentrações de Hcy e redução das concentrações de folato. [26,28,31-33]

Além do gene *MTHFR*, polimorfismos em outros genes envolvidos no metabolismo do folato também foram associados ao risco materno para a SD. O polimorfismo *MTR* A2756G foi associado a esse risco na presença dos genótipos AG ou GG, e em combinação com o polimorfismo *MTRR* A66G (*MTR* 2756AG/*MTRR* 66AG).[34] Ainda, em estudo recente, o alelo variante G do polimorfismo *MTRR* A66G apresentou maior frequência, tanto em homozigose quanto em heterozigose, em mães de indivíduos com SD em relação ao grupo controle.[35] Estudos também sugerem um papel para o polimorfismo *RFC1* A80G no risco materno para a SD quando combinado com outros polimorfismos da via metabólica do folato.[36,37]

Em relação ao gene *CβS*, que atua na remoção da Hcy do ciclo de remetilação, um polimorfismo de inserção de 68 pares de base (pb) na posição 844[38] parece estar associado a uma melhora da atividade da enzima CβS e consequente diminuição das

concentrações de Hcy, comprometendo a via de remetilação da Hcy para metionina, reduzindo a síntese de SAM e prejudicando as reações de metilação celulares.[39,40] Essa variante ocorre sempre em *cis* com o polimorfismo de substituição de timina para citosina no nucleotídeo 833, que resulta na troca de uma treonina por uma isoleucina na proteína produzida.[38]

O polimorfismo 844ins68 do gene *CβS* foi investigado como fator de risco materno para a SD.[28,41] Chango et al. (2005)[41] não associaram a presença deste polimorfismo ao aumento do risco, assim como para outros polimorfismos envolvidos no metabolismo do folato, em população francesa. Em estudo brasileiro, da Silva et al. (2005)[28] avaliaram cinco polimorfismos em genes do metabolismo do folato, incluindo o gene *CβS*, e observaram a presença de maior número de alelos mutados no grupo de mães de indivíduos com SD em relação ao grupo controle.

O polimorfismo G1958A do gene *MTHFD1* foi relacionado ao risco materno para prole com defeitos de fechamento de tubo neural (DTN).[42-44] Há evidências de frequência elevada de casos com SD em famílias com risco para DTN e vice-versa.[45] Segundo estudo de Gueant et al. (2003),[46] ambas as afecções são influenciadas pelos mesmos determinantes genéticos do metabolismo do folato. Assim, a investigação de variantes genéticas envolvidas no risco para DTN pode contribuir com o esclarecimento dos fatores etiológicos da SD. Para o nosso conhecimento, apenas um estudo[47] avaliou a influência desse polimorfismo no risco materno para a SD e não encontrou associação entre a presença do polimorfismo e o risco materno.

É possível que outros polimorfismos em genes que participam da via de degradação da Hcy e das reações de metilação celulares que ainda não foram estudados em SD possam exercer influência no risco materno. O gene *BHMT*, apresenta-se

polimórfico no nucleotídeo 742, levando à substituição de glicina por arginina (G→A) na proteína produzida.[48,49] Embora o efeito funcional desse polimorfismo não tenha sido comprovado até o momento, estudos apontam para um papel protetor do genótipo homozigoto polimórfico AA contra DTN[50] e doenças cardiovasculares.[49] Por outro lado, uma associação entre o genótipo AA e risco aumentado para descolamento de placenta foi observada.[25]

O gene *TC2* codifica uma proteína responsável pelo transporte de vitamina B_{12},[51] que atua como co-fator da enzima MTR na reação de remetilação da Hcy,[19] e a presença de polimorfismos nesse gene pode influenciar a quantidade de vitamina B_{12} disponível no organismo. Existem evidências de associação entre a variante *TC2* 776 G e o risco materno para DTN.[52] Ainda, concentração do complexo TC2-vitamina B_{12} significantemente mais alta foi observada na presença do polimorfismo *TC2* C776G em homozigose (GG) e concentrações médias de MMA foram significantemente mais baixas na presença dos genótipos *TC2* 776 GG e CG em relação ao genótipo CC.[53] Recentemente, o polimorfismo *TC2* C776G foi associado a variações nas concentrações de SAM em mulheres em idade reprodutiva, uma vez que mulheres com os genótipos *TC2* 776 CG ou GG apresentaram concentrações mais baixas do que mulheres com os genótipos 776 CC.[54] Considerando que SAM é o principal doador de grupos metil para as reações de metilação do DNA, é possível que o polimorfismo *TC2* C776G exerça influência no risco materno para a SD. Outra variante neste gene, *TC2* A67G, foi associada com concentrações mais baixas da proteína produzida na presença do genótipo heterozigoto *TC2* 67AG quando comparado ao genótipo tipo selvagem AA,[55] entretanto, este genótipo também foi associado à proporção maior da proteína produzida ligada à vitamina B_{12}. Estudos quanto a influência dos polimorfismos *BHMT* G742A,

TC2 C776G e *TC2* A67G no risco para a SD são ausentes, todavia considerando a importância dos mesmos no metabolismo do folato, a investigação desses genes no risco materno para a SD torna-se relevante.

1.1- Metabolismo do folato em indivíduos com SD

Um importante fator que relaciona a SD e o metabolismo do folato é o fato de o gene *CβS* estar localizado no cromossomo 21. A presença deste gene em triplicata em indivíduos com SD leva a alterações nesse metabolismo, uma vez que um aumento na conversão de Hcy em cistationina, devido à expressão elevada do gene *CβS,* reduz a quantidade de substrato (Hcy) disponível para a atuação da enzima MTR.[9,56] O decréscimo da atividade da enzima MTR promove o acúmulo de 5-MTHF, que resulta na redução de sua conversão para THF, a forma metabolicamente ativa de folato, requerida para a síntese de RNA e DNA. Como consequência deste evento conhecido como "*methyl trap*" (captura de metil), uma deficiência funcional de folato pode existir na presença de concentrações normais, ou até mesmo elevadas, de folato sérico e vitamina B_{12}. Dessa forma, a presença do gene *CβS* em triplicata em indivíduos com SD resulta em alteração no perfil plasmático de metabólitos envolvidos na via metionina / Hcy.[56] A alteração do perfil plasmático da Hcy pode comprometer a divisão e o crescimento das células, e como consequência a sobrevivência do embrião, o que pode explicar o elevado número de abortos da trissomia do 21.[57]

A presença de polimorfismos em genes localizados em outros cromossomos pode também contribuir para alterações em produtos derivados da via metabólica do folato em indivíduos com SD.[58-60] Estudos mostraram frequências elevadas dos genótipos polimórficos *MTHFR* 677TT,[60] *MTR* 2756AG e *MTR* 2756GG[34] em

indivíduos com SD em relação ao grupo controle, e a presença de algumas variantes foi associada ao aumento das concentrações de Hcy plasmática nestes indivíduos.[58,60] Ainda, a presença de certos polimorfismos na mãe, que levam ao aumento da Hcy plasmática, pode compensar a deficiência fisiológica do embrião com a trissomia. Assim diferentes combinações de genótipos maternos e fetais podem influenciar a sobrevivência do feto com SD.[53]

1.2- Objetivos

Considerando as evidências apresentadas, este estudo teve como objetivos:

1. Avaliar a influência dos polimorfismos *BHMT* G742A, *CβS* 844ins68 e T833C, *MTHFD1* G1958A, *TC2* A67G e C776G e das concentrações de Hcy e ácido metilmalônico (MMA) plasmáticos e folato sérico no risco materno para a SD;
2. Investigar o impacto dos polimorfismos *BHMT* G742A, *CβS* 844ins68 e T833C, *MTHFD1* G1958A, *TC2* A67G e C776G nas concentrações circulantes de Hcy e MMA plasmáticos e folato sérico nos grupos de mães caso e controle e em indivíduos com SD.

2. ARTIGOS CIENTÍFICOS

2. Artigos Científicos

Os resultados referentes aos objetivos dessa dissertação estão apresentados na forma de artigo juntamente com os resultados obtidos da análise dos polimorsfismos *MTHFR* C677T, *MTHFR* A1298C, *MTHFR* T1317C, *MTR* A2756G, *MTRR* A66G e *RFC1* A80G, referentes a um projeto mais amplo desenvolvido pelo grupo de pesquisa:

Artigo 1

Tílulo: Double aneuploidy (48,XXY,+21) of maternal origin in a child born to a 13-year-old mother: evaluation of the maternal folate metabolism.

Periódico: Genetic Counseling, aceito para publicação.

Artigo 2

Título: Maternal risk for Down syndrome is modulated by genes involved in folate metabolism.

Periódico: American Journal of Medical Genetics Part A, a ser submetido para publicação.

Artigo 3

Título: Folate metabolism in Brazilian individuals with Down syndrome.

Periódico: Molecular Genetics and Metabolism, a ser submetido para publicação.

Title: Double aneuploidy (48,XXY,+21) of maternal origin in a child born to a 13-year-old mother: evaluation of the maternal folate metabolism.

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Summary

The occurrence of non-mosaic double trisomy is exceptional in newborns. In this paper, a 48,XXY,+21 child, the parental origin of the extra chromosomes and the evaluation of the maternal folate metabolism are presented. The infant was born to a 13-year-old mother and presented with the typical clinical features of Down syndrome (DS). The origin of the additional chromosomes was maternal and resulted from errors during the first meiotic division. Molecular analysis of 12 genetic polymorphisms involved in the folate metabolism revealed that the mother is heterozygous for the *MTHFR* C677T and *TC2* A67G polymorphisms, and homozygous for the mutant *MTRR* A66G polymorphism. The maternal homocysteine concentration was 4.7 nmol/L, a value close to the one considered as a risk factor for DS in our previous study. Plasma methylmalonic acid and serum folate concentrations were 0.17 umol/L and 18.4 ng/mL, respectively. It is possible that the presence of allelic variants for the folate metabolism and Hcy concentration might have favored errors in chromosomal disjunction during gametogenesis in this young mother. To our knowledge, this is the first patient with non-mosaic Down-Klinefelter born to a teenage mother, resulting from a rare fertilization event combining an abnormal 25,XX,+21 oocyte and a 23,Y spermatozoon.

Key words: Aneuploidy; Down syndrome; Folic acid; Genetic Nondisjunction; Genetic Polymorphisms; Klinefelter syndrome.

Running title: Double aneuploidy (48,XXY,+21): maternal folate metabolism.

Introduction

The occurrence of single trisomy is common, being found in around 50% of the karyotyped spontaneous abortions occurred before 15 weeks of gestation [26]. However, the occurrence of double aneuploidy in the same individual is a relatively rare phenomenon, detected in about 0.7% of all miscarriages and exceptionally rare in liveborns [51].

Double aneuploidy leading to trisomy and/or monosomy of two different chromosomes arises due to two nondisjunction events, and these two aneuploidies may have the same or different parental origin [24,38], and occur during meiosis I (MI), meiosis II (MII), or during an early mitotic division in the developing zygote.

Advanced maternal age is a well-established risk factor for nondisjunction of both autosomes and sex chromosomes [8,32]. More recently, the abnormal maternal folate metabolism has been pointed as a maternal risk factor for chromosome 21 nondisjunction by several studies [7,15,18]. In addition, studies have associated the occurrence of chromosomal nondisjunction with altered patterns of meiotic recombination [35,54,55].

In this paper, we describe a 48,XXY,+21 infant with double aneuploidy of maternal origin, born to a 13-year-old mother. The parental origin of the extra chromosomes and the meiosis stage of the chromosomal nondisjunctions were determined. The association of two nondisjunction events in such a young mother provides a good opportunity for an insight into the role of folate metabolism deficiency in the etiology of chromosomal malsegregation. We analyzed 12 polymorphisms of genes involved in the folate metabolic pathway, and measured serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA), an indicator of the status of vitamin B_{12}, in the mother of

the affected child, in order to investigate possible maternal risk factors for chromosomal nondisjunction.

Materials and Methods

This study was approved by the Research Ethics Committee of the São José do Rio Preto Medical School (CEP-FAMERP), in the State of São Paulo, and by the National Research Commission (CONEP), Brazil.

Clinical report: A 3-month-old male patient was referred for chromosomal analysis to the General Outpatient Service of Hospital de Base in São José do Rio Preto, due to dysmorphic features suggestive of DS. The child was born at 32 weeks of gestation, the first child of young, nonconsanguineous parents (13-year-old mother and 24-year-old father). Physical examination showed the presence of features typical of DS, including brachycephaly, flat facial profile, flat nasal bridge, hypertelorism, simian crease, slanted palpebral fissures, epicanthal folds, sandal gap sign, low weight and stature, muscular hypotonia. The child also presented congenital heart disease (interatrial communication), as observed in a Doppler echocardiogram. No genital anomalies were present. Cytogenetic analysis showed a 48,XXY,+21 chromosome complement in all 20 metaphases analyzed by GTG banding. When he was seen again at 18 months, the child had not presented any health problems.

Parental origin and meiosis stage of chromosomal nondisjunction: DNA samples of both parents and the child were subjected to multiplex quantitative fluorescence polymerase chain reaction (QF-PCR) for the chromosome-specific short tandem repeat DNA markers D21S11, D21S226, D21S1270, D21S1411, and IFNAR, using published primer sequences [39,40,52], except for the D21S1270 reverse primer

(5'-TGAGTTTCCAGGTTGCAGGTGACA-3'). For X and Y chromosomes the markers were P39, DXS981, DYS448, DXS1187, XHPRT, amelogenine, DXS996, DXS1283E, SRY, and X22, using published primer sequences [45]. Additionally, chromosome Y haplotypes were determined by QF-PCR using the Yfiler kit (*Applied Biosystems*, USA).

Folate metabolism evaluation: Fasting blood samples were collected from the mother for plasma and serum separation. Plasma Hcy [20,25] and MMA [12] concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The reference values for Hcy were those defined by the American Society of Human Genetics [3], and MMA concentrations greater than 0.5 μmol/L defined vitamin B_{12} deficiency. Folate concentrations were measured by competitive immunoassay (*Immulite kit*, *DPC Medlab*, Brazil), according to the manufacturer's instructions, and concentrations below 3 ng/mL were considered to characterize folate deficiency.

Genomic DNA was extracted from blood leukocytes using the GFX^{TM} Genomic Blood DNA Purification Kit (*GE Healthcare*, EUA). The *methylenetetrahydrofolate reductase* (*MTHFR*) C677T, *methionine synthase* (*MTR*) A2756G, *reduced folate carrier 1* (*RFC1*) A80G, *cystathionine β-synthase* (*CβS*) T833C, *transcobalamin 2* (*TC2*) C776G and *methylenetetrahydrofolate dehydrogenase 1* (*MTHFD1*) G1958A polymorphisms were determined by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique [6,21,22,23,28,48]. The *methionine synthase reductase* (*MTRR*) A66G, *TC2* A67G and *betaine-homocysteine methyltransferase* (*BHMT*) G742A polymorphisms were investigated by Allelic Discrimination (*Applied Biosystems,* USA) using TaqMan probes (Taqman SNP

Genotyping Assays C__3068176_10; C__25967461_10 and C__11646606_20). *CβS* 844ins68 was genotyped by conventional PCR [21] and the polymorphisms *MTHFR* A1298C and T1317C by direct sequencing as described by Alvarenga et al. [19], except the purification process, performed using the enzymes Exonuclease I and Shrimp Alkaline Phosphatase (*Fermentas Life Sciences*, Brazil), according to manufacturer's instruction.

Results

Parental origin and meiosis stage of nondisjunction: By comparing the allele profiles and allele copy number for chromosome 21 and the sex chromosomes of the family trio it was possible to determine that the parental origin of the double aneuploidy was maternal (Tables 1 and 2, informative markers D21S11, DXS996 and DXS1283E) and both nondisjunction errors arose in meiosis I. No evidence of recombination in either 21q or Xp/Xq was observed. The father and the affected infant exhibited identical Y haplotypes for the 17 DNA markers genotyped (data not shown).

Folate metabolism

The genotyping results obtained for the polymorphisms of genes involved in the folate metabolism are presented in Table 3. In the mother, the allelic variants *MTHFR* C677T and *TC2* A67G were in heterozygosity, and *MTRR* A66G in homozygosity. Plasma Hcy and MMA and serum folate concentrations were 4.7 umol/L, 0.17 umol/L and 18.4 ng/mL, respectively.

Discussion

We present here an exceptional case of double autosome / sex chromosome aneuploidy in a male infant born to a 13-year-old mother. As most of the previously published cases of 48,XXY,+21 patients, this patient also presented typical features of DS only [2,4,5,16,29,33], once the characteristic features of KS are not apparent until the post-pubertal stage [50].

Few molecular studies on double trisomies are available so far, and the parental origin of the nondisjunction in those individuals is predominantly maternal [24,36,47], as in single autosomal trisomies [13,32,43,44]. Concerning the extra X chromosome, its origin is either maternal or paternal in similar proportions [37,56]. In the present study, both nondisjunction events were maternal and occurred during MI.

The occurrence of both aneuploidies in this very young woman (12-year-old at the time of conception) is in contrast to published reports indicating an increased risk of autosomal and sex-chromosomal nondisjunction, including the 21 and X trisomies, with increasing maternal age [8,32,42]. According to population studies recorded by the National Down Syndrome Cytogenetic Register in the United Kingdom [42], no case of single trisomy 21 at the maternal age of 13 has been reported, and the predicted odds (upper 95% CI) of DS live births for the maternal age of 13 years are 1 in 1,918. Although in Brazil no such register exists, it is worth noting that for the year 2005 the birth rate to mothers aged <14 years for Sao Jose do Rio Preto, the city where the affected infant was born, was 0.6% (44 in 7,195 liveborns) [10]. These data alone show that the occurrence of double aneuploidy in infants born to 13-year-old mothers is extremely rare.

The exclusion of advanced maternal age as risk factor for chromosomal nondisjunction in the present study suggests the existence of other risk factors. Significant reduction in recombination events both of autosomes and sex chromosomes was associated with the occurrence of nondisjunction in MI in several studies [35,54,55]. In the present study, no evidence of recombination was observed either within the intervals 3 to 5 of the 21q region [35] or in the Xp and Xq regions genotyped. However, since the number of markers used was limited, possible recombination events within pericentric or telomeric intervals could not have been assessed.

Several studies have shown association between polymorphisms in genes encoding enzymes involved in the folate metabolism and the maternal risk for trisomy 21 DS [7,14,15,18,30,41,57]. Folate metabolism is responsible for the synthesis of S-adenosylmethionine, the main methyl group donor for methylation reactions of the DNA. DNA methylation is important for the maintenance of centromeric chromatin and chromosomal stability and plays an important role in chromosomal segregation [17,31,53].

In the present study, the molecular analysis of the mother revealed heterozygosity for *MTHFR* C677T and *TC2* A67G and homozygosity for the mutant *MTRR* A66G polymorphism. A higher frequency of the allelic variant *MTHFR* 677T has been observed in mothers of DS individuals in comparison with control mothers [18,27,30,41,49,57]. The occurrence of the *MTHFR* 677T allele is associated with reduced enzyme activity (60-70% for homozygous TT and 59% for heterozygous CT) [58]. The impact of the *MTHFR* C677T polymorphism on protein stability and activity may lead to impaired Hcy remethylation, affecting DNA methylation and chromosomal stability [34].

The allelic variant *MTRR* 66G also occurs more frequently in DS mothers than in control mothers. Homozygosity for this allele (GG) was associated with an approximately 2.7-fold increased risk of having a DS pregnancy compared to homozygous wild-type subjects [27,57].The maternal risk for DS in the presence of a MTRR 66GG genotype is higher when combined with genotypes *MTHFR* 677CT or TT (2.98 to 6-fold) [27,46,57].

The *TC2* A67G polymorphism had not been previously investigated in mothers of DS individuals. Protein TC2 transports vitamin B_{12}, which is an important co-factor for the folate metabolism [11]. The heterozygous genotype *TC2* 67 AG was associated with lower total transcobalamin concentrations compared to the wild-type genotype *TC2* 67AA; however, it was also was associated with a higher proportion vitamin B_{12} bound to transcobalamin protein [1]. Thus, another studies on these aspects are warranted to better characterize the impact of this polymorphism on protein expression and vitamin B_{12} availability.

The maternal plasma Hcy and MMA and serum folate concentrations were normal, according to the reference values. However, the 4.7 umol/L Hcy concentration observed was very close to the value considered as a risk factor for DS in our previous study [7]. We had observed that the median Hcy concentrations were significantly higher in DS mothers (5.38 μmol/L) as compared to the control group (4.22 μmol/L), and a higher maternal risk for DS was observed when the Hcy concentrations were greater than 4.99 μmol/L. Other studies also have shown higher Hcy concentrations in DS mothers compared to control mothers [9,30], although the values were within the normal range.

In conclusion, we suggest that the presence of allelic variants of genes involved in the folate metabolism and Hcy concentration might have favored errors in chromosomal disjunction during gametogenesis in this very young mother. To our knowledge, this is the first case of non-mosaic Down-Klinefelter child born to a teenage mother, resulting from an exceptionally rare fertilization event, occurred between an abnormal double autosomal/sex-chromosomal disomic 25,XX,+21 oocyte and a normal 23,Y spermatozoon.

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Table 1. Chromosome 21 DNA marker allele profile and informative allele copy numbers established by QF-PCR for the family trio.

	ALLELE PROFILE [a]							**ALLELE COPY**[b]		
MARKER	**Mother**		**Father**		**Infant**			**Infant**		
D21S11 (21q21)	243	257	239	239	239	243	257	1	1	1
D21S226 (21q22.1)	451	459	455	459	451	459		1	2	
D21S1270 (21q21-q22.1)	293	299	299	312	293	299	312	1	1	1
D21S1411 (21q22.3)	284	292	288	292	284	292		1	2	
IFNAR (21q22.1)	384	388	388	388	384	388		1	2	

[a] Allele size in base pairs.

[b] Samples exhibiting a diallelic profile with peak area ratios 0.7 to 1.4 were considered to have a normal copy number; samples exhibiting either diallelic profiles with area ratios > 1.8 or triallelic profiles with ratios 1:1:1 were considered trisomic. All numbers were rounded to the closest integer.

Table 2. Sex chromosome DNA marker allele profile and informative allele copy numbers established by QF-PCR for the family trio.

	ALLELE PROFILE [a]					
MARKER	**Mother**		**Father**		**Infant**	
P39 (Xq28)	151	159	159		151	159
DXS981 (Xq13.1)	244	244	244		244	244
DYS448 (Yq11.2)	Absent		351		351	
DXS1187 (Xq26.2)	143	147	147		143	147
XHPRT (Xq26.1)	276	284	276		276	284
AMEL (Xp22.22/Yp11.2)	104		104	109	104 [b]	109
DXS996 (Xp22.3)	129	162	152		129	162
SRY (Yp11.2)	Absent		244		244	
DXS1283E (Xp22.3)	311	326	320		311	326
X22 (Xq28/Yq12)	204	218	204	243	204 [b]	218

[a] Allele size in base pairs.

[b] Allele copy number ratio 2:1.

Table 3. Genotype of gene polymorphisms involved in the folate metabolism in the mother.

Gene polymorphisms	Genotypes
MTHFR C677T	CT
MTHFR A1298C	AA
MTHFR T1317C	TT
MTR A2756G	AA
MTRR A66G	GG
RFC1 A80G	AA
TC2 A67G	AG
TC2 C776G	CC
CBS 833T>C	TT
CBS 844ins68 [a]	-/-
MTHFD1 G1958A	GG
BHMT G742A	GG

[a] -/- represents the absence of insertion.

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Re: GC 08/09

Leuven, March 2, 2009

Dear Dr. Pavarino Bertelli,

Your manuscript "Double aneuploidy (48,XXY,+21) of maternal origin in a child born to a 13-year-old mother: evaluation of the maternal folate metabolism" is accepted in the journal.

Included you may find the referees comments.

Please adapt the lay-out of the manuscript to the requirements of the journal (see manuscript) and send the final version at your earliest convenience.

With best regards,

yours sincerely,

Prof. Dr. J.P. FRYNS

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Title: Maternal Risk for Down syndrome is modulated by genes involved in Folate Metabolism

Running title: Maternal risk for Down syndrome and folate metabolism

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ABSTRACT

Studies have shown that the maternal risk for Down syndrome (DS) could be modulated by alterations in folate metabolism. The aim of this study was to evaluate the influence of 12 polymorphisms involved in folate metabolism and the concentrations of serum folate, plasma homocysteine (Hcy) and methylmalonic acid (MMA) as maternal risk factors for DS. In addition, the influence of the polymorphisms on folate, Hcy and MMA concentrations was also evaluated. The genotypes *MTHFR* 677 CT or TT, *MTR* 2756 AG and *TC2* 776 GG were associated with increased maternal risk for DS and some genotype combinations were also able to modulate this risk. Higher frequency of the *MTHFR* 677C-1298A-1317T haplotype was observed in the control group, indicating a protective maternal effect of these alleles against DS. Regarding the metabolites, lower folate and Hcy concentrations and higher MMA concentration were observed in DS mothers compared to control mothers. The polymorphisms *RFC1* A80G and *BHMT* G742A were associated with variations in Hcy concentrations in control mothers, while the polymorphisms *MTHFR* C677T and *BHMT* G742A modulate the folate and MMA concentrations, respectively. In conclusion, the results of the present study indicate that the polymorphisms *MTHFR* C677T, *MTR* A2756G and *TC2* C776G, and some genotype combinations, modulate maternal risk for DS. The polymorphisms *RFC1* A80G and *BHMT* G742A modulate the Hcy concentrations in control mothers, while the *MTHFR* C677T and *BHMT* G742A genotypes affect the folate and MMA concentrations, respectively. These findings are consistent with the influence of genetic polymorphisms in modulating the folate metabolism and on the maternal risk for DS.

Key words: Down syndrome, folate, homocysteine, risk

INTRODUCTION

Down syndrome (DS) is the most common genetic disorder with a prevalence of 1 in 660 live births [Jones, 2006]. The only well-established risk factor for DS is advanced maternal age [Allen et al., 2009], conversely, many DS children are born to mothers aged <35 years, suggesting other factors influencing DS etiology. James et al. (1999) proposed that the occurrence of DS independent of maternal age is associated with hypomethylation of DNA due to impairments in folate metabolism. Since then, several studies have shown that polymorphisms in genes involved in the folate pathway could modulate the maternal risk for bearing a child with DS [Bosco et al., 2003; Scala et al., 2006; Meguid et al., 2008; Wang et al., 2008] and the concentrations of metabolites involved in the folate pathway [Ananth et al. 2007; Barbosa et al., 2008; Devos et al., 2008].

Folate / homocysteine (Hcy) metabolism plays an important role in biosynthesis of nucleotides and S-adenosyl-methionine (SAM), the major methyl donor for DNA methylation reactions (Figure 1). Folate deficiency has been associated with hypomethylation of DNA, DNA damage, chromosomal instability, abnormal chromosome segregation and aneuploidy of chromosome 21 [James et al., 2003; Beetstra et al., 2005].

In the Brazilian population, Acácio et al (2005) showed that the frequency of combined heterozygotic polymorphisms C677 and A1298 in the *Methylenetetrahydrofolate reductase* (*MTHFR*) gene was significantly higher in women with children affected by trisomy 21 than in those with chromosomally normal offspring. In a larger sample, da Silva et al. (2005) studied three other polymorphisms, *Methionine synthase* (*MTR*) A2756G, *Methionine synthase reductase* (*MTRR*) A66G

and *Cystathionine beta-synthase* (*CβS*) 844ins68, in addition to *MTHFR* C677T and A1298C. They found a significantly higher frequency of the *MTHFR* 677 T allele in DS mothers compared to control mothers and observed that the mothers of children with DS tend to have a higher number of uncommon alleles than mothers with no previously affected child [da Silva et al., 2005]. In relation to metabolites of the folate pathway, da Silva et al. (2005) showed that Brazilian mothers of DS individuals presented higher Hcy concentration compared to non-DS mothers.

Recently, we obtained results similar to those found by da Silva et al. (2005) regarding the role of Hcy concentrations and the number of polymorphic alleles for the polymorphisms *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *Reduced folate carrier 1* (*RFC1*) A80G in the maternal risk for DS in our Brazilian sample [Biselli et al., 2008]. In the present study, we extend this analysis to 12 polymorphisms of genes involved in the folate pathway in the maternal risk for DS, including *MTHFR* C677T, A1298C and T1317C, *MTR* A2756G, *MTRR* A66G, *CβS* T833C and 844ins68, *RFC1* A80G, *Transcobalamin 2* (*TC2*) A67G and C776G, *Methylenetetrahydrofolate dehydrogenase 1* (*MTHFD1*) G1958A and *Betaine-homocysteine methyltransferase* (*BHMT*) G742, seven of which have never been studied in the Brazilian population. In addition, we evaluated the concentrations of serum folate, and plasma Hcy and methylmalonic acid (MMA, an indicator of the vitamin B_{12} status) in relation to the genotypes and their influence on the maternal risk for DS.

METHODS

This study protocol was approved by the Research Ethics Committee of the São José do Rio Preto Medical School (CEP-FAMERP), in the State of São Paulo, and by the National Research Commission (CONEP), Brazil. Fasting blood samples from 105

women with a liveborn child with karyotypically confirmed full trisomy 21 (case mothers) were obtained at the General Genetics Outpatient Service of Hospital de Base, São José do Rio Preto, SP, Brazil. The control group was composed of 185 mothers, who had at least one healthy child and no experience of miscarriages, enrolled at the FAMERP Campus and at the HB Clinical Analysis Laboratory. Informed consent for participation in the study was obtained from each subject.

Plasma Hcy and MMA concentrations were determined by liquid chromatography-tandem mass spectrometry as previously described [Haddad et al., 2001; de Andrade et al., 2003; Carvalho and Kok, 2008] and folate by quimioluminescence (*Immulite* Kit, *DPC Medlab,* Brazil), in overnight fasted mothers. Hcy and MMA concentrations above 15.0 and 0.5 μmol/L were considered to characterize hyperhomocysteinaemia [American Society of Human Genetics, 1998] and vitamin B_{12} deficiency, respectively [Carvalho and Kok, 2008], and folate concentrations lower than 3.0 ng/mL were considered to characterize folate deficiency, according to *Immulite* Kit manufacturer's instructions.

Genomic DNA was isolated from lymphocytes in whole blood according to Miller et al. (1988) or using the *GFX™ Genomic Blood DNA Purification* Kit (*GE Healthcare*, USA). The polymorphisms *MTHFR* C677T, *MTR* A2756G, *RFC1* A80G, *TC2* C776G, *CβS* 844ins68, *CβS* T833C and *MTHFD1* G1958A were determined as previously described [Frosst et al., 1995; Hol et al., 1998; Födinger et al., 2003; Pietrzyk and Bik-Multanowski, 2003; Dutta et al., 2005; Biselli et al., 2008]. The variants *MTRR* A66G, *TC2* A67G and *BHMT* G742A were genotyped by real time PCR allelic discrimination (*Taqman SNP Genotyping Assays* C__3068176_10; C__25967461_10 and C__11646606_20 respectively, *Applied Biosystems, Foster City,*

CA. USA). Automated sequencing was used to investigate *MTHFR* A1298C and T1317C polymorphisms as previously described [Alvarenga et al., 2008], except for the purification procedure, performed using the enzymes *Exonuclease I* and *Shrimp Alkaline Phosphatase* (*Fermentas Life Sciences*, Brazil), according to manufacturer's instructions.

Statistical analysis

Continuous variables are reported as median and variation. The maternal age was calculated considering the age of the mother at the birth of the DS child for the case group, and the age at birth of the last child for the control group. The Mood's median test and logistic regression analyses were used for comparison of maternal age and age at presentation between case and control groups.

Hardy-Weinberg (HW) equilibrium was tested by the chi-square test, using the BioEstat program, and genotype frequencies in DS, and control mothers were compared by the likelihood ratio test and logistic regression. The genotype comparisons were also performed applying the recessive model (wild-type homozygous + heterozygous versus mutant homozygous) and the dominant model (heterozygous + mutant homozygous versus wild-type homozygous) for the effect of the mutant allele. The contribution of combined genotypes of two polymorphisms for the maternal risk for DS was investigated by logistic regression analysis, considering only the dominant model due to the sample size and using the homozygous wild-type genotype of both combined polymorphisms as reference for the analysis.

The relationship between the number of uncommon alleles per woman considering all tested *loci* was analyzed using the Mann-Whitney test. The haplotype

frequencies of *MTHFR, TC2* e *CβS* genes were inferred by the *Haploview* program (version 4.0).

The comparison between groups regarding the median of Hcy, folate and MMA was performed using the Mood's median test followed by logistic regression analysis using quartiles. Spearman's correlation was used to investigate the correlation between the concentrations of Hcy, folate and MMA, the number of uncommon alleles and age at presentation. The correlation analysis between Hcy, folate and MMA concentrations were also performed in a subset of DS and control mothers paired by age at presentation.

The computer-assisted statistical analyses were carried out using the Minitab for Windows program (Release 12.22), and values of $P \leq 0.05$ were considered significant.

RESULTS

Age

The median maternal age was significantly higher in the case group (30.4 years; 12.9 – 46.3) compared to the control group (26.4 years; 15.4 – 40.7) ($P < 0.001$); when considering only women with maternal ages below 35 years, the same result was observed ($P < 0.001$). Stratifying the maternal ages into quartiles, there was no association between age and the maternal risk for DS. Regarding the age at presentation, the median age was significantly higher in the control group compared to the case group ($P < 0.001$).

Allele, genotype and haplotype distributions

Allele and genotype frequencies showed no difference between DS mothers and control groups. The genotype frequencies were in HW equilibrium in both groups, except for the polymorphisms *CβS* T833C and 844ins68, which presented higher

frequency of the mutant homozygous genotype in relation to the expected (P = 0.03 for both polymorphisms) in the case group.

The results of the logistic regression analysis are presented in the Table 1. The genotype *MTHFR* 677 CT was associated with increased maternal risk for DS compared to the CC genotype with OR = 1.77 (CI 95% = 1.06 – 2.95; P = 0.03) (Table 1). This association was also observed when considering only women with maternal ages below 35 years (OR = 1.89; CI 95% = 1.07 – 3.33; P = 0.03). In the latter group, the genotype *MTR* 2756 AG was also associated with increased maternal risk for DS with OR = 1.77 compared to the genotype AA (CI 95% = 1.00 – 3.13; P = 0.05).

When considering the dominant effect of the mutant alleles, higher frequency was observed for the genotypes *MTHFR* 677 CT or TT in the case group compared to the control group (P = 0.04), and the presence of these genotypes was associated with the maternal risk for DS with OR = 1.68 (CI 95% = 1.03 – 2.73; P = 0.04). Regarding the recessive effect of the mutant alleles, the genotype *TC2* 776 GG was more frequently observed in the case group compared to the control in relation to the AA or AG genotypes (P = 0.05) and presented a borderline statistical significance for an association with maternal risk for DS (OR = 2.04; CI 95% = 0.98 – 4.23; P = 0.06).

The higher frequencies of the genotypes *MTHFR* 677 CT or TT and *TC2* 776 GG in the case group, observed in the dominant and recessive models, respectively, were also observed in the group of women with maternal age less than 35 years old and associated with the increased maternal risk for DS (*MTHFR* 677: OR = 1.82; CI 95% = 1.06 – 3.15; P = 0.03; *TC2* C776G: OR = 2.30; CI 95% = 1.05 – 5.04; P = 0.04).

The median of the number of uncommon alleles per individual (case or control) for the 12 loci tested did not differ between groups (P = 0.30). The combined genotypes associated with the modulation of the maternal risk for DS are presented in the Table 2.

The haplotype frequencies of *MTHFR*, *TC2* and *CβS* genes are presented in the Table 3. Regarding the *MTHFR* gene, there was evidence of linkage disequilibrium between the polymorphisms C677T and A1298C (LOD = 11.05; D' = 1.0), C677T and T1317C (LOD = 3.23; D' = 1.0) and A1298C and T1317C (LOD = 3.83; D' = 1.0). A significantly higher frequency was observed for the C-A-T haplotype (wild-type alleles) in the control group compared to the case group (P = 0.01). According to the analysis of the *TC2* gene, the polymorphisms A67G and C776G are weakly linked (LOD = 2.46; D' = 0.63) and there was no difference in the haplotype frequencies between the groups. The variants at positions 833 and 844 of the *CβS* gene are strongly linked (LOD = 74.17; D' = 1.0) and the haplotype 833 T / 844 I and 833 C / 844 W were not observed in either group.

Lower folate concentration was observed in the case group compared to the control group (12.20 *vs* 14.60 ng/mL. P = 0.03). Similarly, the case group presented higher MMA concentration in relation to the controls (0.17 *vs* 0.15 umol/L, P = 0.003). Regarding Hcy, a lower concentration was observed in DS mothers compared to control mothers (6.27 *vs* 8.32 umol/L, P<0.001).

The distribution of Hcy, folate and MMA concentrations did not differ in relation to the genotypes of the polymorphisms studied in the case group (data not shown). However, higher Hcy concentration was associated with the presence of the genotypes *RFC1* 80 AA or AG compared to GG (P = 0.03) and of the genotype *BHMT* 742 GG compared to AA or GA (P = 0.009) in the control group. In relation to the

folate, a lower concentration was observed in women carrying the *MTHFR* 677 CT or TT genotypes compared to CC (P = 0.009). In addition, lower MMA concentration was associated with the presence of the genotypes *BHMT* 742 AA or GA compared to GG (P = 0.05).

A negative correlation between Hcy and folate concentrations was observed in case (P = 0.05) and control groups (P = 0.03), as well a positive correlation between Hcy and MMA concentrations (P = 0.006 and P = 0.002, respectively). In addition, a positive correlation between age at presentation and Hcy (P = 0.002) was observed in the control group. In the correlation analysis between Hcy, folate and MMA concentrations performed in a subset of DS and control mothers paired by the age at presentation, no differences were observed compared to the results obtained on the analysis of the whole group.

DISCUSSION

Advanced maternal age at conception represents an important risk factor for DS. The risk of bearing a child with DS is 1:1476 live births at 20 years old, and this risk increases at age 35 for 1:352 live births [Morris et al., 2002]. The higher maternal age median observed in our study in case mothers in relation to controls is consistent with the literature data.

Of all polymorphisms tested, only *CβS* T833C and 844ins68 were out of HW equilibrium only in the case group. Departure from HW equilibrium in this study may have resulted from selection or small sample size. The control group was in HW equilibrium for these polymorphisms, potentially indicating an association between the mutant alleles and maternal risk for DS, but no subsequent association was detected. No other polymorphisms deviated from HW equilibrium, so there does not appear to be a

widespread problem with the ascertainment of this sample set [Xu et al., 2002; Wittke-Thompson et al., 2005].

A higher frequency of the *MTHFR* 677 CT or TT genotypes was observed in the case group in relation to control group, and the mutant allele *MTHFR* 677 T was associated with increased maternal risk for DS. This risk was even higher when considering only women with maternal age less than 35 years old. These results are consistent with the literature that shows significantly higher frequency of the allelic variant *MTHFR* 677 T in homozigosity and heterozigosity in mothers of individuals with DS compared to control mothers, including in a Brazilian population [da Silva et al., 2005; Meguid et al., 2008; Wang et al., 2008]. These studies are based on the evidence that abnormal folate and methyl metabolism can lead to DNA hypomethylation and abnormal chromosomal segregation, given that the polymorphism *MTHFR* C677T was associated with decreased enzyme activity [Weisberg et al., 2001; James et al., 2003; Beetstra et al., 2005].

Recently, Coppedè et al. (2007) observed an association between the *MTHFR* 677T allele and the increased baseline frequency of binucleated micronucleated lymphocytes (BML), and previously these authors observed a statistically significant increased rate of aneuploidy of chromosome 21 in BML in mothers who gave birth to individuals with DS compared to control mothers [Migliore et al., 2006]. These data indicate that the *MTHFR* 677 T allele plays a role in the etiology of the chromosome 21 nondisjunction.

The *MTR* 2756 AG and *TC2* 776 GG genotypes were also associated with the modulation of the maternal risk factor for DS in the group of women aged less than 35 years. Few studies have analyzed the influence of the *MTR* A2756G polymorphism in

the maternal risk factor for DS [Bosco et al., 2003; Chango et al., 2005; da Silva et al., 2005]. Higher risk of DS offspring was related to the presence of the polymorphic allele *MTR* 2756 G, associated with higher plasmatic Hcy concentrations [Bosco et al., 2003], and also when combined with polymorphic alleles of other genes involved in folate metabolism [da Silva et al., 2005]. These findings suggest the influence of this polymorphism in the susceptibility of nondisjunction in young women. It is possible that the polymorphism *MTR* A2756G affects the folate pathway by decreasing the transmethylation rate of Hcy, even if the polymorphism has no apparent effect on Hcy concentrations in this study.

In regards to the *TC2* gene, which codifies a vitamin B_{12} transporting protein, there are no studies on the contribution of polymophisms in this gene to maternal risk for DS. Recently, the polymorphism *TC2* C776G was associated with variations in SAM concentrations in childbearing-age women, because women with *TC2* 776 CG or GG genotype tended to have lower concentrations of SAM than did women with the genotype 776CC [Barbosa et al., 2008]. Considering that SAM is the major methyl donor for DNA methylation reactions of DNA, it is possible that the variant *TC2* 776G exerts an influence on the maternal risk for DS, as observed in the present study. In addition, there is evidence of an association between the variant *TC2* 776 G and the maternal risk for offspring with neural tube defects [Gueánt-Rodriguez et al., 2003], whose occurrence is also influenced by genetic determinants of the folate metabolism.

Considering that some risk factors may interact to produce a synergic effect, the contribution of the combined genotypes to the maternal risk for DS was tested. The interaction between allelic variants involved in the folate metabolism and the

modulation of the maternal risk for DS observed in the present study were previously shown in other studies [Bosco et al., 2003; Scala et al., 2006; Wang et al., 2008].

Regarding haplotypes in *MTHFR* gene, the present study showed linkage disequilibrium between the polymorphisms C677T, A1298C and T1317C. The literature shows linkage disequilibrium (LD) between the *MTHFR* C677T and A1298C [Shi et al., 2003]. Next to the *MTHFR* A1298C variant, the silent polymorphism at position 1317 was also in linkage disequilibrium with C677T and A1298C. The higher frequency of the *MTHFR* 677C-1298A-1317T haplotype in the control group shows the protective maternal effect of these alleles against DS, because the mutant alleles 677T and 1298C were associated with the increase of the maternal risk for DS in several studies [da Silva et al., 2005; Meguid et al., 2008; Wang et al., 2008].

The linkage disequilibrium between the polymorphism *CβS* 833 and 844 were expected, since the variant at position 844 is found always in cis at position 833, and the polymorphisms T833C and 844ins68 are cited as a double mutation in cis [Pepe et al., 1999]. Regarding the *TC2* gene haplotypes, the linkage disequilibrium between the variants A67G and C776 are consistent with a previous study [Lievers et al., 2002].

The observation of lower Hcy concentration in the case group compared to control group is a surprising finding, considering that studies have reported an association between higher Hcy concentrations and the increased maternal risk for DS [James et al., 1999; Takamura et al., 2004; da Silva et al., 2005]. In addition, in a previous study by our group carried out in 58 DS mothers and 49 control mothers [Biselli et al., 2008], higher mean Hcy concentration was observed in the case group, and concentrations higher than 4.99 umol/L were associated with maternal risk for DS with a 4.62-fold increased risk. However, with the increasing of the sample size of both

groups (present study) an opposite association was observed. Recently, the study of Kohli et al. (2008) also showed significantly higher plasma Hcy concentrations in controls compared to mothers of babies with DS, as observed in our study. However, for the quantification of Hcy, Kohli et al. (2008) used random plasma samples, while in our study, fasting plasma samples were used. This unexpected result may be due to the effect of other genetic or environmental factors in the study population that affects Hcy concentrations.

In the present study, a negative correlation between Hcy and folate concentrations was observed, an expected finding considering the literature data [Barbosa et al., 2008]. In relation to genotypes, significantly higher Hcy concentrations were associated with the presence of *RFC1* 80 AA or AG compared to GG and *BHMT* 742 GG compared to GA or AA in the control group. The *RFC1* gene codifies a protein that participates in the intestinal folic acid absorption [Nguyen et al., 1997]. Recently, the *RFC1* 80GG genotype was associated with 7% lower plasma Hcy concentrations compared to the AA and AG genotypes [Devos et al., 2008]. It is possible that the A allele significantly impairs the ability of cells to take up folate, which subsequently reduces their capacity to remethylate Hcy, thereby causing it to accumulate and raise plasma concentrations. Although this polymorphism was not associated with variations in serum folate concentrations in the present study, it is possible that it exerts an influence on red blood cells folate concentrations.

Studies investigating the influence of *BHMT* G742A polymorphism in Hcy concentration are limited. Similarly to our findings, Ananth et al. (2007), in a study with placental abruption, observed that women carrying the GG and GA had higher Hcy concentrations compared to the AA genotype. In addition, Morin et al. (2003), observed

that mothers of children with spina bifida with the *BHMT* 742 AA genotype had lower Hcy concentration and higher serum folate concentration, although the difference was not significant. Considering that the BHMT enzyme catalyzes an alternative route of Hcy remethylation (Figure 1), it is possible that polymorphisms in the *BHMT* gene could result in modifications in the enzyme produced. Although obvious differences in catalytic activity of BHMT enzymes were not identified between the wild-type and mutant enzyme, it is possible that the two enzymes have different stability [Weisberg et al., 2003].

This is the first study that analyzes the influence of *BHMT* G742A on the modulation of the maternal risk for DS. Although this polymorphism was not associated with the risk for DS alone, it showed a protective effect in the presence of GA or AA genotypes when combined with the *TC2* 67 AA genotype. The protective effect of the *BHMT* 742A allele against DS is expected, because the AA genotype was associated with the protection against neural tube defects in offspring [Morin et al., 2003]. In addition, the polymorphism A67G in the *TC2* gene was never studied in DS mothers and its effect on the synthesis of transcobalamin is unclear [Afman et al. 2002].

The observation of significantly lower serum folate concentration in DS mothers compared to the control group is consistent with a study by Takamura et al., (2004), performed in a Japanese population. Other studies have shown that the folate deficiency is associated with chromosomal instability and chromosome 21 aneuploidy [James et al., 2003; Beetstra et al., 2005]. Although no DS mothers presented folate deficiency in the present study, it is possible that the lower folate concentration in this group could favor the occurrence of aneuploidy. Regarding the influence of the genotypes on the folate concentrations, the *MTHFR* 677 CT and TT genotypes have been associated with

lower folate concentrations in relation to CC [Kölling et al., 2004; Yang et al., 2008], as observed in the present study. The MTHFR enzyme catalyzes the conversion of 5-MTHF to tetrahydrofolate (Figure 1), the main circulating folate form, and the reduced enzyme activity due to the presence of the T allele could decrease the circulating folate concentration.

The higher MMA concentration observed in the case group could reflect lower concentrations of vitamin B_{12} in this group, as shown by the literature data [Barbosa et al., 2008], although the majority of the women in this study did not present concentrations that characterize deficiency of this vitamin. Vitamin B_{12} acts as co-factor of the l-methylmalonyl-co-enzyme-A mutase enzyme, which plays a role in a second pathway of Hcy degradation. The decreased availability of vitamin B_{12} prevents the conversion of methylmalonyl-coA to succinyl-coA, diverting the substrate to the MMA synthesis and leading to an elevation of its concentrations in the blood and urine [Klee, 2000]. It is possible that decreased vitamin B_{12} concentration leads to alterations in the folate metabolic pathway because the activity of the MTR enzyme, which catalyzes the remethylation of Hcy to methionine, is vitamin B_{12}-dependent. Studies have shown that decreased vitamin B_{12} concentrations results in reduced activity of MTR enzyme and DNA hypomethylation in animal models [Brunaud et al., 2003] and are negatively associated with the frequency of endogenous micronucleus formation, an indicator of chromosomal damage [Fenech et al., 1997]. Differing from our results, Takamura et al., (2004) found no significant differences in vitamin B12 concentrations between DS and controls.

There is evidence that the vitamin B_{12} deficiency induces the increase of Hcy concentration. Savage et al. (1994) showed that of 434 individuals with vitamin B_{12}

deficiency, 98.4% and 95.9% presented increased MMA and Hcy concentrations, respectively. The relation between vitamin B_{12} deficiency and increased MMA and Hcy concentration was also observed in a recent study of Selhub et al. (2007). These findings support the correlation between MMA and Hcy concentrations observed in the present study. Regarding the polymorphisms, lower MMA concentration was associated with the presence of *BHMT* 742 AA or AG genotypes. Studies have shown that the *BHMT* 742 A allele seems to improve the Hcy remethylation [Morin et al., 2003; Ananth et al., 2007]. Thus, it may be hypothesized that this improvement diverts the Hcy available to the remethylation pathway, reducing the transulfuration pathway and consequently the MMA synthesis.

In conclusion, the results of the present study indicate that the polymorphisms *MTHFR* C677T, *MTR* A2756G and *TC2* C776G, and some genotype combinations, modulate the maternal risk for DS. The polymorphisms *RFC1* A80G and *BHMT* G742A modulate the Hcy concentrations in control mothers, while the *MTHFR* C677T and *BHMT* G742A polymorphisms affect the folate and MMA concentrations, respectively. These findings are consistent with the influence of genetic polymorphisms in modulating folate metabolism and maternal risk for DS.

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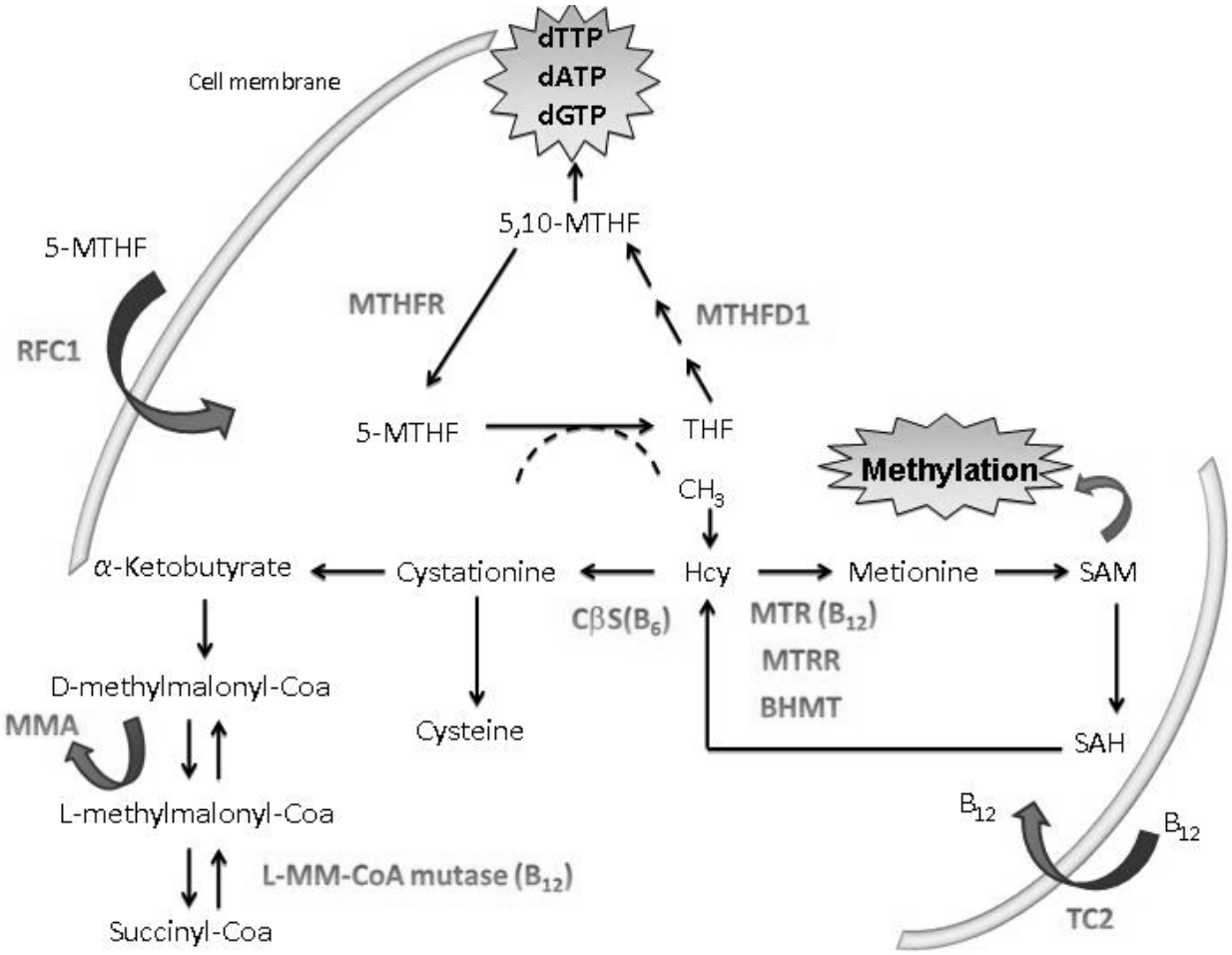

Figure 1. Folate metabolism. BHMT = Betaine-homocysteine methyltransferase; CßS = Cystathionine ß-synthase; CH_3 = Methyl, CH_2THF = Methylenetetrahydrofolate, CH_3THF = Methyltetrahydrofolate; dATP = Deoxyadenosine 5'-triphosphate; dGTP = Deoxyguanosine 5'-triphosphate; dTTP = Deoxythymidine 5'-triphosphate; Hcy = Homocysteine; L-MM-Coa mutase = L-methylmalonyl coenzyme A mutase; MMA = Methylmalonic acid; MTHFD1 = Methylenetetrahydrofolate dehydrogenase 1; MTHFR = Methylenetrahydrofolate reductase; MTR = Methionine synthase; MTRR = Methionine synthase reductase; RFC1 = Reduced folate carrier 1; SAH = S-adenosyl-homocysteine; SAM = S-adenosyl-methionine; TC2 = Transcobalamin 2; THF = Tetrahydrofolate.

Table 1. Genotype frequencies of 12 polymorphisms involved in the folate pathway in DS and control mothers.

Genotype	DS mothers n (%)	Control mothers n (%)	OR (CI 95%)	P
***MTHFR* C677T**				
CC	40 (38.1)	94 (50.81)	reference	
CT	55 (52.4)	73 39.46	1.77 (1.06 - 2.95)	0.028
TT	10 (9.5)	18 9.73	1.31 (0.55 - 3.08)	0.542
***MTHFR* A1298C**				
AA	51 (48.6)	101 (55.2)	reference	
AC	48 (45.7)	73 (39.9)	1.30 (0.79 - 2.14)	0.297
CC	6 (5.7)	9 (4.9)	1.32 (0.45 - 3.91)	0.616
***MTHFR* T1317C**				
TT	89 (84.8)	158 (86.3)	reference	
TC	16 (15.2)	23 (12.6)	1.23 (0.62 - 2.46)	0.548
CC	0 (0)	2 (1.1)	0.00	0.999
***MTR* A2756G**				
AA	62 (59.1)	127 (68.7)	reference	
AG	38 (36.2)	49 (26.5)	1.59 (0.94 - 2.68)	0.082
GG	5 (4.8)	9 (4.9)	1.14 (0.37 - 3.54)	0.823
***MTRR* A66G**				
AA	36 (34.3)	65 (35.1)	reference	
AG	53 (50.5)	89 (48.1)	1.08 (0.63 - 1.83)	0.789
GG	16 (15.2)	31 (16.8)	0.93 (0.45 - 1.93)	0.849
***RFC1* A80G**				
AA	29 (27.6)	53 (28.7)	reference	
AG	48 (45.7)	88 (47.6)	1.00 (0.56 - 1.77)	0.991
GG	28 (26.7)	44 (23.8)	1.16 (0.60 - 2.24)	0.652
***CβS* T833C**				
TT	83 (79.1)	145 (78.4)	reference	
TC	18 (17.1)	38 (20.5)	3.49 (0.63 - 19.49)	0.551
CC	4 (3.8)	2 (1.1)	0.83 (0.44 - 1.54)	0.154
CβS* 844ins68				
WW	83 (79.1)	145 (78.4)	reference	
WI	18 (17.1)	38 (20.5)	0.83 (0.44 - 1.54)	0.551
II	4 (3.8)	2 (1.1)	3.49 (0.63 - 19.49)	0.154
***TC2* A67G**				
AA	77 (73.3)	129 (69.7)	reference	
AG	26 (24.8)	49 (26.5)	0.89 (0.51 - 1.55)	0.677
GG	2 (1.9)	7 (3.8)	0.48 (0.10 - 2.36)	0.366
***TC2* C776G**				
CC	42 (40.0)	75 (40.5)	reference	
CG	46 (43.8)	93 (50.3)	0.88 (0.53 - 1.48)	0.638
GG	17 (16.2)	17 (9.2)	1.79 (0.83 - 3.86)	0.141
***BHMT* G742A**				
GG	56 (53.3)	77 (41.6)	reference	

GA	43 (41.0)	88 (47.6)	0.67 (0.41 - 1.11)	0.120
AA	6 (5.7)	20 (10.8)	0.41 (0.16 - 1.09)	0.075
***MTHFD1* G1958A**				
GG	34 (32.4)	72 (38.9)	reference	
GA	58 (55.2)	81 (43.8)	1.52 (0.89 - 2.57)	0.123
AA	13 (12.4)	32 (17.3)	0.86 (0.40 - 1.84)	0.699

*The results of the *CβS* 844ins68 genotypes were defined as W for the wild allele and I or the allele with the 68bp insertion.

Table 2. Combined genotypes associated with the modulation of the maternal risk for DS.

Combined genotypes	OR*	CI 95%	P
MTHFR 677 TC or TT / *MTHFR* 1298 AA	2.26	1.09 – 4.68	0.03
MTHFR 677 TC or TT / *MTHFR* 1298 AC or CC	2.69	1.24 – 5.84	0.01
MTHFR 677 TC or TT / *MTR* 2756 AA	1.89	1.02 – 3.53	0.04
MTHFR 677 TC or TT / *MTR* 2756 AG or GG	2.44	1.21 – 4.94	0.01
MTHFR 677 TC or TT / *TC2* 776 CC	3.18	1.42 – 7.15	0.005
MTHFR 677 TC or TT / *MTHFD1* 1958 GA or AA	2.41	1.13 – 5.14	0.03
MTHFR 1298 AC or CC / *RFC1* 80 AA	2.93	1.15 – 7.47	0.03
MTHFR 1317 TT / *MTR* 2756 AG or GG	1.76	1.02 – 3.02	0.04
MTR 2756 AG or GG / *MTHFD1* 1958 GA or AA	2.07	1.00 – 4.30	0.05
TC2 67 AA / *BHMT* 742 GA or AA	0.53	0.30 – 0.93	0.03

* The homozygous wild-type genotypes for both polymorphisms were used as reference for this analysis.

Tabela 3. Haplotype frequencies of the *MTHFR, TC2* and *CβS* genes in case and control groups.

Haplotypes	Case	Control	X^2	P
***MTHFR* 677 / 1298 / 1317**				
C / A / T	0.281	0.385	6.40	0.01
T / A / T	0.357	0.292	2.59	0.11
C / C / T	0.286	0.249	0.95	0.33
C / A / C	0.076	0.074	0.01	0.92
***TC2* 67 / 776**				
A / C	0.496	0.508	0.08	0.78
A / G	0.361	0.322	0.92	0.34
G / C	0.123	0.149	0.75	0.39
G / G	0.020	0.022	0.02	0.90
***CβS* 833 / 844**				
T / W	0.876	0.886	0.14	0.71
C / I	0.124	0.114	0.14	0.71
T / I	0	0	----	----
C / W	0	0	----	----

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Title: Folate metabolism in Brazilian individuals with Down syndrome

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Abstract

The presence of the *CβS* gene in triplicate in individuals with Down syndrome (DS) results in an altered profile of metabolites involved in the methionine / homocysteine (Hcy) pathway, including reduced plasma concentrations of Hcy, methionine, S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM). In addition to the presence of three copies of the *CβS* gene, recent evidence has suggested that genetic variants involved in folate metabolism can also alter the concentrations of the products of this metabolism in individuals with DS. Thus, the purpose of the present study was to analyze Hcy, serum folate and plasma methylmalonic acid (MMA) concentrations in 90 individuals with DS and to investigate twelve polymorphisms of the folate metabolism. Genotyping for the polymorphisms was carried out by polymerase chain reaction (PCR), PCR followed by enzyme digestion, allele-specific PCR, Allelic Discrimination and direct sequencing. Total plasma Hcy and MMA concentrations were measured by mass spectrometry and folate concentrations were measured by competitive immunoassay. The polymorphism *MTHFR* C677T, *MTR* A2756G, *MTRR* A66G, *TC2* C776G and *BHMT* G742A modulate the plasma Hcy in Brazilian individuals with DS, while the polymorphism *TC2* A67G modulate folate concentrations and C*β*S T833C and 844ins68 the MMA concentrations. These data may contribute to understanding the role of polymorphisms in genes related to the folate metabolism and its metabolic consequences in trisomy 21.

Key words: Down's syndrome: Folate: Polymorphism: Homocysteine

Introduction

Down syndrome (DS) is a chromosomal disorder resulting from the presence of three copies of chromosome 21[1]. The overexpression of genes coding for specific enzymes translates directly into biochemical aberrations, affecting the multiple interacting metabolic pathways culminating in cellular dysfunction and contributing to the unique pathogenesis of DS [2].

Individuals with DS present disturbed folate metabolism attributed to the presence of three copies of the *Cystathionine ß-synthase* (*CßS*) gene, located on chromosome 21 [2,3]. This gene codifies an enzyme involved in the homocysteine (Hcy) transsulfuration pathway, catalyzing the condensation of Hcy and serine to form cystathionine (Figure 1). An increase in the transsulfuration pathway via *CßS* overexpression indirectly reduces the concentration of Hcy available for the remethylation reaction, catalyzed by vitamin B_{12}–dependent enzyme methionine synthase (MTR), while, simultaneously, it promotes the accumulation of 5-methyltetrahydrofolate (5-MTHF). In addition, the increase in the transsulfuration pathway reduces the conversion of 5-MTHF to tetrahydrofolate (THF), the metabolically active form of folate, required for *de novo* synthesis of nucleotides for RNA and DNA synthesis. Consequently, a functional folate deficiency can be observed even in the presence of normal or elevated folate concentration [2]. Thus, the presence of the *CßS* gene in triplicate in DS individuals results in an altered profile of metabolites involved in the methionine / Hcy pathway, including reduced plasma concentrations of Hcy, methionine, S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM) [2,3].

In addition to the presence of three copies of the *CβS* gene, studies have shown that genetic variants involved in the folate metabolism can also alter the concentration of the products of this metabolism in individuals with DS [4,5]. In a previous study, we evaluated the influence of the polymorphisms *Methylenetrahydrofolate reductase* (*MTHFR*) C677T and A1298C, *Methionine synthase* (*MTR*) A2756G and *Reduced folate carrier 1* (*RFC1*) A80G on Hcy concentration in 56 individuals with DS and observed that the polymorphism A2756G of the *MTR* gene, which codifies a vitamin B_{12}-dependet enzyme, plays a role in modulation of Hcy concentrations in these individuals [5]. In the present study, we extended the analysis of the Hcy concentrations to 90 individuals with DS, and analyzed another eight polymorphisms of the folate metabolism. In addition, serum folate and plasma methylmalonic acid (MMA), an indicator of the vitamin B_{12} status, were quantified.

Subjects and methods

This study protocol was approved by both the Research Ethics Committee of Sao Jose do Rio Preto Medical School (CEP-FAMERP), in Sao Paulo state, and the National Research Commission (CONEP), Brazil. Ninety DS individuals with full trisomy 21 (49 male and 41 female) were recruited at the General Genetics Outpatient Service of Hospital de Base, Sao Jose do Rio Preto, SP, Brazil, after signing family-informed consent forms.

Fasting blood samples were collected for plasma and serum separation and DNA extraction. Total plasma Hcy and MMA concentrations were measured by mass spectrometry [6-8]. Hcy concentrations higher than 15 μmol/L were considered to characterize hyperhomocysteinemia [9] and MMA concentrations higher than 0.5

μmol/L defined vitamin B_{12} deficiency [8]. Folate concentrations were measured by competitive immunoassay (*Immulite kit*, *DPC Medlab*, Brazil) and concentrations below 3 ng/mL were considered to characterize folate deficiency, according to the manufacturer's instructions.

Genomic DNA was extracted from blood leukocytes as described by Miller et al. (1988) [10] or using a GFX^{TM} Genomic Blood DNA Purification Kit (*GE Healthcare*, EUA). The polymorphisms *MTHFR* C677T, *MTR* A2756G, *RFC1* A80G, *CβS* 844 ins68 and T833C, *Transcobalamin 2* (*TC2*) C776G and *Methylenetetrahydrofolate dehydrogenase 1* (*MTHFD1*) G1958A were determined as previously described [5,11-15].The polymorphisms *Methionine synthase reductase* (*MTRR*) A66G, *TC2* A67G and *Betaine-homocysteine methyltransferase* (*BHMT*) G742A were investigated by Allelic Discrimination using *Taqman* probes (*Applied Biosystems,* USA, *TaqMan* SNP Genotyping Assays C__3068176_10; C__25967461_10 and C__11646606_20). *MTHFR* A1298C and T1317C variants were determined by direct sequencing as described elsewhere [16], except for the purification process performed using the enzymes Exonuclease I and Shrimp Alkaline Phosphatase (*Fermentas Life Sciences*, Brazil), according to the manufacturer's instruction.

Statistical analyses

Concordance of genotype frequencies with Hardy-Weinberg equilibrium was tested by Chi-square test, using the BioEstat program (version 5.0). The Haploview program (version 4.0) was used to infer the haplotype frequencies. The Hardy-Weinberg equilibrium was not tested for the polymorphisms *RFC1* A80G, *CβS* T833C and *CβS* 844ins68, since we used a restriction fragment length polymorphism method, that does not enable the distinction of heteroallelic individuals containing one or two copies of

each allele. For the same reason, the haplotype frequencies of the polymorphisms T833C and 844ins68 on the *CβS* gene were not estimated.

Distributions of Hcy, folate and MMA were all skewed, thus a logarithmic transformation was performed. However, even after Log-transformation, the resulting MMA distribution was not normal. For this reason, the Hcy and folate data were analyzed and are presented as mean values in the logarithmic scale and the MMA data as median values.

To evaluate the effect of the polymorphisms on the variation of the biochemical parameters, three analyses were performed: a comparison of the mean (Hcy and folate) or median (MMA) concentrations considering the three possible genotypes of each polymorphism; a dominant model of the mutant allele, considering that its effect is evident in both heterozygous and homozygous; and a recessive model, considering that the effect of the mutant allele is evident only in homozygous. The Mood Median test was used for statistical analysis of MMA data and the T test and variance analysis (ANOVA) were employed for Hcy and folate data.

Statistical analyses were performed using Minitab for Windows (Release 12.22) program. For statistical significance, values of $P \leq 0.05$ were considered significant.

Results

The genotype frequencies of the polymorphisms (Table I) are all in Hardy-Weinberg equilibrium. Regarding the MTHFR 677-1298-1317 haplotypes, the frequency of haplotype T-A-T was 0.339, haplotype C-A-T was 0.328, haplotype C-C-T was 0.283, and haplotype C-A-C was 0.050. There was evidence of linkage disequilibrium among the polymorphisms C677T and A1298C (LOD = 5.88; D = 1.0),

C677T and T1317C (LOD = 1.07; D = 1.0), and A1298C and T1317C (LOD = 0.74; D = 1.0). The TC2 67-776 haplotypes presented frequencies of 0.593 for A-C, 0.291 for A-G, 0.071 for G-C and 0.044 for G-G. There was no evidence of linkage disequilibrium of the TC2 polymorphisms in this group of DS individuals (LOD = 0.02; D' = 0.07).

Eighty-seven plasma samples were Hcy-quantified (1.63 ± 0.51), and only two individuals presented hyper-hyperhomocysteinemia. Of 83 individuals evaluated for folate concentration (2.83 ± 0.51), none showed deficiency of this vitamin. In relation to the MMA concentration (median: 0.25 umol/L; range: 0.09 – 4.77 umol/L), 19 out of 85 individuals presented values that demonstrated a vitamin B_{12} deficiency.

Mean Hcy concentrations presented a significant variation according to the genotypes of some polymorphisms. Higher Hcy concentration was associated with the presence of the *MTHFR* 677 CC genotype in relation to TT genotype (P = 0.02); MTR 2756 AG in relation to AA genotype (P = 0.03); *TC2* 776 CC and CG compared to GG genotype (P = 0.04); and *BHMT* 742 GG in relation to AA genotype (P = 0.04). The mean folate concentrations did not present differences according to the genotypes. Regarding MMA, higher median concentration was associated with the presence of the heterozygous genotype for *CβS* T833C (P = 0.04) and *CβS* 844ins68 (P = 0.04) in relation to the wild-type homozygous genotypes.

When considering the dominant effect of the mutant allele, higher Hcy concentration was associated with the *MTR* 2756 AG or GG genotypes compared to AA (P = 0.02), and lower concentration was associated with the presence of *BHMT* 742 AA or GA compared to GG genotype (P = 0.03). There were no differences in the folate and MMA distributions in this model.

Considering the recessive effect of the mutant allele, Hcy concentration was significantly higher in the *MTRR* 66 GG genotype compared to AA or AG (P = 0.05) and significantly lower in the presence *MTHFR* 677 TT compared to CC or CT (P = 0.02), *TC2* 776 GG compared to CC or CG (P = 0.04) and *BHMT* 742 AA compared to GG or GA (P = 0.04). In addition, higher mean folate concentration was observed in individuals with the genotype *TC2* 67 GG compared to AA or AG (P = 0.03). There was no association between MMA and the polymorphisms.

Discussion

Studies have shown that the presence of three copies of the *CβS* gene and subsequent decreased MTR enzyme reaction in individuals with DS can result in disturbances in folate metabolism. The result is a functional folate deficiency that may contribute to the metabolic pathology of this complex genetic disorder [2]. Hcy, vitamin B_{12}, and folate are metabolic and nutritional factors directly related to the folate pathway, and alterations in their concentrations may indicate or lead to disturbances in this metabolism [2,17].

Several contributions have shown that genetic polymorphisms could influence plasma Hcy concentrations, either directly or by affecting plasma folate concentrations [4,18-22]. In a previous study [5], we showed that the *MTR* A2756G polymorphism exerts influence on Hcy concentrations of DS individuals because the heterozygous AG genotype was associated with higher Hcy concentration compared to the homozygous wild-type AA genotype. However, no association between Hcy concentrations and the polymorphisms *MTHFR* C677T and A1298C and *RFC1* A80G was found. In the present study, the *MTHFR* A1298C and *RFC1* A80G variants also showed no

association with Hcy concentrations, as well as with folate and MMA concentrations. On the other hand, the results of the analysis of the *MTR* 2756 genotypes with Hcy concentrations are consistent with our previous observation of an association between the mutant G allele and increased Hcy concentrations in DS individuals, as well as with the results of other studies of non-DS individuals [20,21].

Regarding the polymorphism *MTHFR* C677T, lower Hcy concentration was observed in the presence of the mutant homozygous genotype *MTHFR* 677 TT, an unexpected result considering the literature data, which shows that the occurrence of the *MTHFR* 677 T allele is associated with reduced MTHFR enzyme activity [23] and increased Hcy concentration [22]. In DS individuals, Licastro et al. (2006) [4] observed higher Hcy concentrations in subject's homozygous *MTHFR* 677TT, but no association was found in other studies [17,24]. It is important to consider that the metabolic configuration of individuals with DS differs from the non-DS individuals due to the overexpression of chromosome 21 genes involved in the folate metabolism, such as *CβS* and *RFC1* genes, which could modify the effect of genetic polymorphisms of this pathway on Hcy concentration.

According to our knowledge, this is the first study to investigate the influence of the polymorphisms in genes codifying the TC2 protein, a vitamin B_{12} transporter [25], the BHMT protein, which remethylates Hcy to methionine using the methyl donor betaine [26] and the MTHFD1 protein, which catalyzes the conversion of tetrahydrofolate to the corresponding 10-formyl, 5,10-methenyl and 5,10-methylene derivates [27] (Figure 1), in individuals with DS. In the present study, no associations were found between Hcy, folate and MMA concentrations and the polymorphism *MTHFD1* G1958A.

Regarding the *TC2* A67G polymorphism, higher folate concentration was associated with the presence of the homozygous GG genotype compared to GA or AA genotypes. Considering the association of the *TC2* 67 G allele with reduced transcobalamin protein concentrations observed by Afman et al. (2002) [28], the higher folate concentrations associated with the presence of the *TC2* 67 GG genotype in the present study could be explained by a possible reduction in vitamin B_{12} availability and consequent reduced MTR enzyme activity. However, the same study [28] showed that, although the *TC2* 67 G allele was associated with reduced transcobalamin protein concentrations, this polymorphism was not associated with alteration in red blood cell vitamin B_{12} concentrations. Thus, further investigations on these aspects are warranted.

Another polymorphism of the *TC2* gene, C776G, was associated with lower Hcy concentration in individuals homozygous to the mutant allele (GG genotype). This genotype was previously associated with lower concentration of the transcobalamin-vitamin B_{12} complex (holotranscobalamin) compared to the wild-type genotype, and an inverse relation was observed between plasma Hcy concentrations and plasma vitamin B-12 concentrations regardless of genotype [29]. However, studies regarding the influence of the *TC2* C776G polymorphism on Hcy concentrations demonstrated different data [29-32]. It has been suggested that the discrepancy between studies regarding the influence of genetic polymorphisms on Hcy concentrations is related to differences in age, ethnicity and gender of the study subjects, and other factors such as folate, vitamin B_{12} and B_6 intake, genetic factors, and study design [5,30,33].

Concerning the *BHMT* G742A polymorphism, our results showed an effect of the mutant *BHMT* 742 A allele decreasing the plasma Hcy concentration. The evaluation of the impact of this polymorphism on the protein functional properties did

not show differences in catalytic activity between the mutant and wild-type protein, but, it is possible that the two enzymes have different stability [34]. Although the functional effect of the *BHMT* G742A polymorphism has not been proven until the present, studies have suggested a protective role of the mutant homozygous AA genotype against neural tube defects [35] and cardiovascular disease [34]. In addition, the AA genotype was associated with lower Hcy concentrations in a previous study [35], consistent with our findings.

The impact of the A66G mutation of the *MTRR* gene on Hcy concentrations has been studied in several populations [17,36-38]. This polymorphism has not been associated with hyperhomocysteinemia, but reports in adults revealed that homozygosity for the G allele could contribute to a moderate increase in Hcy concentrations, independent of the serum levels of folate, vitamin B_6 and B_{12} [36]. In healthy children, the isolated analysis of this polymorphism showed no effect on plasma Hcy concentrations [37]. In individuals with DS taking folic acid supplements, the homozygous for the G allele had significantly higher tHcy concentrations than did those homozygous for the A allele [17]. Similarly, in our group of individuals with DS, those with the GG genotype presented higher Hcy concentrations compared to those AA or AG genotype.

Regarding the polymorphisms C*β*S 844ins68, it has been suggested that the allele with the insertion results in improvement of CBS enzyme activity [39,40], although other studies do not support a functional role for this polymorphism [17,41,42]. Since the C*β*S 844ins68 polymorphism occurs always *in cis* at position 833 (T→C) [43], the same effect for the presence of both mutant alleles could be expected. In view of the hypothesis that the mutant alleles at positions 833 and 844 of the C*β*S

gene improve the CBS enzyme activity, the association of the heterozygous C*β*S 844ins68 genotype with higher MMA concentrations compared to the wild-type genotype could be explained by the increased production of the substrate for MMA synthesis, resulting in MMA accumulation (Figure 1). However, further studies are warranted to determine the role of the polymorphisms C*β*S T833C and 844ins68 on concentration of metabolites involved in the folate pathway.

In conclusion, our results indicate that the polymorphisms *MTHFR* C677T, *MTR* A2756G, *MTRR* A66G, *TC2* C776G and *BHMT* G742A modulate the plasma Hcy in Brazilian individuals with DS, while the polymorphism *TC2* A67G modulates folate concentrations and C*β*S T833C and 844ins68 the MMA concentrations. These data could contribute to the understanding of the role of polymorphisms in genes related to the folate metabolism and its metabolic consequences in trisomy 21.

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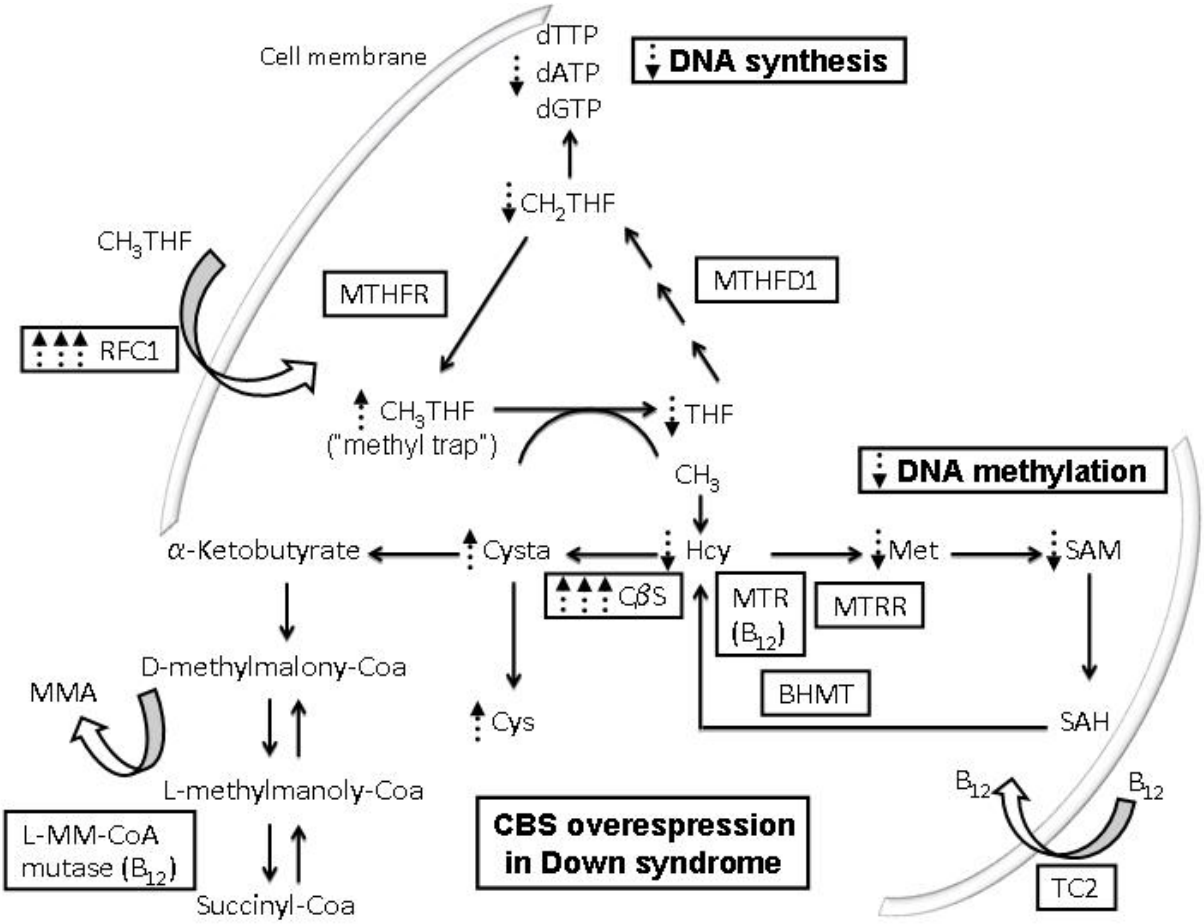

Figure 1. Folate metabolism in Down syndrome (DS) individuals. Arrows indicate direct and indirect alterations in metabolites induced by *cystathionine ß-synthase* (*CßS*) overexpression in DS individuals. BHMT = Betaine-homocysteine methyltransferase; CßS = Cystathionine ß-synthase; CH_3 = Methyl, CH_2THF = Methylenetetrahydrofolate, CH_3THF = Methyltetrahydrofolate; Cysta = cystathionine; Cys = cysteine; Hcy = Homocysteine; MMA = Methylmalonic acid; MTHFD1 = Methylenetetrahydrofolate dehydrogenase 1; MTHFR = Methylenetrahydrofolate reductase; MTR = Methionine synthase; MTRR = Methionine synthase reductase; RFC1 = Reduced folate carrier 1; SAH = S-adenosyl-homocysteine; SAM = S-adenosyl-methionine; TC2 = Transcobalamin 2; THF = Tetrahydrofolate.

Table I. Genotype frequencies of the polymorphisms in individuals with Down syndrome.

	Wild-type homozygous n (%)	**Heterozygous n (%)**	**Mutant homozygous n (%)**
***MTHFR* C677T**	41 (45.6)	37 (41.1)	12 (13.3)
***MTHFR* A1298C**	48 (53.3)	33 (36.7)	9 (10.0)
***MTHFR* T1317C**	81 (90.0)	9 (10.0)	0
***MTR* A2756G**	55 (61.1)	27 (30.0)	8 (8.9)
***MTRR* A66G**	32 (35.6)	46 (51.1)	12 (13.3)
***RFC1* A80G**[a]	15 (16.7)	62 (68.9)	13 (14.4)
***TC2* A67G**	72 (80.0)	15 (16.7)	3 (3.3)
***TC2* C776G**	37 (41.1)	45 (50.0)	8 (8.9)
***CβS* 844ins68**[a]	69 (76.7)	21 (23.3)	0
***CβS* T883C**[a]	69 (76.7)	21 (23.3)	0
***BHMT* G742A**[b]	46 (51.7)	31 (34.8)	12 (13.5)
***MTHFD1* G1958A**	33 (36.7)	37 (41.1)	20 (22.2)

[a]The genotyping methods do not differentiate the presence of one or two copies of each allele in heterozygous individual because the gene is located on 21 chromosome and is in triplicate in individuals with Down syndrome.

[b]Genotyping one individual was not possible.

3. CONCLUSÕES

3. Conclusões

1. O genótipo *TC2* 776 GG, os genótipos combinados *MTHFR* 677 TC ou TT / *TC2* 776 CC, *MTHFR* 677 TC ou TT / *MTHFD1* 1958 GA ou AA e *MTR* 2756 AG ou GG / *MTHFD1* 1958 GA ou AA, concentrações aumentadas de MMA plasmático e reduzidas de folato sérico são fatores de risco materno para a SD, enquanto os genótipos combinados *TC2* 67 AA / *BHMT* 742 GA ou AA apresentam um efeito protetor.
2. O genótipo *BHMT* 742 GG está associado ao aumento de Hcy e a presença dos genótipos *BHMT* 742 AA ou GA está associada às concentrações reduzidas de MMA no grupo de mães controles.
3. Os polimorfismos *TC2* C776G e *BHMT* G742A modulam as concentrações de Hcy plasmática, enquanto o polimorfismo *TC2* A67G afeta as concentrações de folato e os polimorfismos C*β*S T833C e 844ins68 as concentrações de MMA em indivíduos com SD.

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5-ANEXOS

FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO
AUTARQUIA ESTADUAL - LEI Nº 8899 ,de 27/09/94
(Reconhecida pelo Decreto Federal nº 74.179, de 14/06/74)

Parecer n.º 165/2004

COMITÊ DE ÉTICA EM PESQUISA

O Protocolo n.º 3340/2004 sob a responsabilidade de Érika Cristina Pavarino Bertelli com o título "Avaliação Genético-Clínica e Molecular em Síndrome d Down" está de acordo com a Resolução CNS 196/96 e foi **aprovado** por esse CEP.

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

São José do Rio Preto, 12 de julho de 2004.

Prof.ª Dr.ª Patrícia Maluf Cury
Coordenadora do CEP/FAMERP

Av. Brigadeiro Faria Lima, 5416 - Cep 15.090-000 Fone: (017) 210 - 5700
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MINISTÉRIO DA SAÚDE
Conselho Nacional de Saúde
Comissão Nacional de Ética em Pesquisa - CONEP

PARECER Nº2400/2004

Registro CONEP: 10618 (Este nº deve ser citado nas correspondências referentes a este projeto)

Registro CEP: 3340/04 **Processo nº 25000.106488/2004-41**
Projeto de Pesquisa: *"Avaliação genético clínica e molecular em Síndrome de Down."*
Pesquisador Responsável: Dra. Érika Cristina Pavarino Bertelli
Instituição: Faculdade de Medicina de São José do Rio Preto - FAMERP
Área Temática Especial: Genética Humana

Ao se proceder à análise das respostas ao parecer CONEP nº 2001/2004, relativo ao projeto em questão, considerou-se que:

1) tendo em vista a afirmação da pesquisadora responsável que será estabelecido um banco de material biológico, solicita-se que seja feito um banco de dados junto ao CEP da instituição, informando: quem será o responsável pelo banco, condições de armazenamento, segurança do banco, como será o acesso pelos pesquisadores a esse banco, de que forma será garantida a confidencialidade dos indivíduos que doarem o material para a formação desse banco;
2) as informações enviadas atendem aos aspectos fundamentais da Res. CNS 196/96 sobre diretrizes e normas regulamentadoras de pesquisas envolvendo seres humanos;
3) o projeto foi aprovado pelo Comitê de Ética em Pesquisa – CEP da instituição supracitada.

Diante do exposto, a Comissão Nacional de Ética em Pesquisa - CONEP, de acordo com as atribuições definidas na Resolução CNS 196/96, manifesta-se pela aprovação do projeto de pesquisa proposto com a recomendação 1. acima citada, devendo esta ser acompanhada pelo CEP, para posterior início da pesquisa.

Situação: Projeto aprovado com recomendação

Brasília, 29 de Novembro de 2004

WILLIAM SAAD HOSSNE
Coordenador da CONEP/CNS/MS