Joice Matos Biselli

METABOLISMO DO FOLATO E SÍNDROME DE DOWN: ANÁLISE DE POLIMORFISMOS GENÉTICOS E HOMOCISTEÍNA PLASMÁTICA

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

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Orientadora: Profª. Drª. Érika Cristina Pavarino Bertelli

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São José do Rio Preto, ___/___/___.

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DEDICATÓRIA

Aos meus pais Sandra e João Hélio

Pelo amor e confiança que depositaram em mim e pelo investimento em meus sonhos. Sem vocês minha realização não seria possível. Essa conquista é de vocês! Amo muito vocês.

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"Aceite com sabedoria o fato de que o caminho está cheio de contradições. Há momentos de alegria e desespero, confiança e falta de fé, mas vale a pena seguir adiante."

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"Grande coisa é haver recebido do céu uma partícula, uma sabedoria, o dom de achar a relação das coisas, a faculdade de as comparar e o talento de concluir".

Machado de Assis

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LISTA DE ABREVIATURAS E SÍMBOLOS

10-CHO-THF	*10-formyl tetrahydrofolate*
5-CH_3-THF	*5-methyltetrahydrofolate*
5-MTHFR	5-metiltetrahidrofolato
5,10-CH_2-THF	*5,10-methylenetetrahydrofolate*
ANOVA	*Variance analysis*
BP	*Base pair*
CβS	Cistationina β-sintase (*Cystathionine* β-*synthase*)
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (*Coordination for the Improvement of Higher Education Personnel*)
CEP	*Research Ethics Committee*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico (*National Council for Scientific and Technological Development*)
CONEP	National Research Commission
DF	*Degrees of freedom*
DHF	*Dihydrofolate*
DNA	Ácido desoxirribonucléico (*Desoxirribonucleic acid*)
DS	*Down syndrome*
dATP	*Adenine*
dGTP	*Guanine*
dTTP	*Thymine*
dUTP	*Uracyl*
EDTA	*Ethylenediaminetetracetic acid*
FAMERP	Faculdade de Medicina de São José do Rio Preto (*São José do Rio*

	Preto Medical School)
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo (*São Paulo State Research Foundation*)
FUNFARME	Fundação Faculdade Regional de Medicina de São José do Rio Preto
HB	Hospital de Base
Hcy	Homocisteína (*Homocysteine*)
IQ	*Intelligence quotient*
LC-MS/MS	*Liquid chromatography – tandem mass spectrometry*
μmol/L	Micromol / litro
MTHFR	Metilenotetrahidrofolato redutase (*Methylenetetrahydrofolate reductase*)
MTR	Metionina sintase (*Methionine synthase*)
MTRR	Metionina sintase redutase (*Methionine synthase reductase*)
PCR	Reação em Cadeia da Polimerase (*Polymerase chain reaction*)
QI	Coeficiente de inteligência
RFC1	Carregador de folato reduzido 1 (*Reduced folate carrier 1*)
SAH	S-adenosilhomocisteína (*S-adenosylhomocysteine*)
SAM	S-adenosilmetionina (*S-adenosylmethionine*)
SD	Standad deviation
SD	Síndrome de Down
TCN	*Transcobalamin*
THF	*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*)

RESUMO

Introdução A Síndrome de Down (SD) é, na maioria dos casos, decorrente de não-disjunção cromossômica durante a meiose materna. Acredita-se que o metabolismo anormal do folato como resultado de polimorfismos genéticos pode levar à hipometilação do DNA e conseqüente não-disjunção cromossômica. **Objetivos** Estabelecer as freqüências de anomalias cromossômicas dos casos de SD atendidos no Serviço Ambulatorial de Genética do Hospital de Base (HB) de São José do Rio Preto para posterior seleção de pacientes com cariótipo compatível com trissomia livre do cromossomo 21; avaliar a influência dos polimorfismos *Metilenotetrahidrofolato redutase* (*MTHFR*) C677T e A1298C, *Metionina sintase* (*MTR*) A2756G e *Carregador de folato reduzido 1* (*RFC1*) A80G e das concentrações de homocisteína (Hcy) plasmática no risco materno para a SD; investigar o impacto dos polimorfismos *MTHFR* C677T e A1298C, *MTR* A2756G e *RFC1* A80G nas concentrações de Hcy em indivíduos com SD. **Casuística e Método** Para investigação molecular e dosagem de Hcy foram incluídos no estudo 56 indivíduos com SD com resultado cariotípico 47,X_,+21, 72 mães de indivíduos com trissomia livre do 21 (mães SD) e 194 mães de indivíduos sem a síndrome (mães controle). A quantificação de Hcy plasmática foi realizada pela técnica de cromatografia líquida/espectrometria de massas seqüencial. O DNA foi extraído a partir de leucócitos do sangue periférico para investigação dos polimorfismos *MTHFR* C677T, *MTR* A2756G e *RFC1* A80G pela reação em cadeia da polimerase (PCR) e digestão enzimática, e do polimorfismo *MTHFR* A1298C pela técnica de PCR alelo-específica. **Resultados** As freqüências de alterações cromossômicas nos pacientes com SD foram de 92,2% (n = 357) para trissomia livre do 21, 6,2% (n = 24) para translocação e 1,5% (n = 6) para mosaicismo. A análise

molecular nos grupos de mães SD e controle mostrou que a mediana do número de alelos polimórficos para os quatro *loci* testados foi maior no grupo de mães SD em relação ao grupo controle (P = 0,02), e a presença de três ou mais alelos polimórficos aumenta o risco de prole com SD em 1,74 vezes (P = 0,048). Risco materno aumentado para a SD foi observado também na presença de concentração de Hcy plasmática maior que 4,99 μmol/L (P = 0,003). As freqüências alélicas para os polimorfismos no grupo de indivíduos com SD foram 0,37 para *MTHFR* 677T, 0,21 para *MTHFR* 1298C, 0,18 para *MTR* 2756G e 0,47 para *RFC1* 80G. A concentração média de Hcy neste grupo foi de 5,2 ± 3,3 μmol/L. Concentrações de Hcy foram significantemente elevadas na presença do genótipo heterozigoto *MTR* 2756AG em relação ao genótipo tipo selvagem *MTR* 2756AA (P = 0,025). **Conclusões** A presença de três ou mais alelos polimórficos para *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G e *RFC1* A80G, e concentração de Hcy plasmática acima de 4,99 μmol/L são fatores de risco maternos para a SD. O genótipo heterozigoto *MTR* 2756AG está associado ao aumento das concentrações de Hcy de indivíduos com SD. Confirma-se, ainda, que a não-disjunção cromossômica, representada pela trissomia livre do 21, é a principal causa da SD.

ABSTRACT

Introduction Down syndrome (DS) is, in the most cases, resulting from chromosomal nondisjunction during maternal meiosis. It is believed that the abnormal folate metabolism as result of genetic polymorphisms may lead to DNA hypomethylation and consequent chromosomal nondisjunction. **Objective** To establish the chromosomal anomalies frequencies of DS cases consulted by Genetics Outpatient Service of Hospital de Base (HB) in São José do Rio Preto to subsequent selection of patients with free trisomy 21; to evaluate the influence of the *Methylenetetrahydrofolate reductase* (*MTHFR*) C677T and A1298C, *Methionine sinthase* (*MTR*) A2756G and *Reduced folate carrier 1* (*RFC1*) A80G polymorphisms and of plasma homocysteine (Hcy) concentrations as maternal risk factors for DS; to investigate the impact of the *MTHFR* C677T and A1298C, *MTR* A2756G and *RFC1* A80G polymorphisms on Hcy concentrations in DS individuals. **Subjects and Methods** To molecular investigation and plasma Hcy quantification were included in the study 56 DS individuals with karyotypic result 47,X_,+21, 72 mothers of DS individuals with free trisomy 21 (DS mothers) and 194 mothers who had no children with DS (control mothers). The Hcy quantification was performed by liquid chromatography – tandem mass spectrometry. DNA was extracted from leukocytes of peripheral blood to the investigation of the *MTHFR* C677T, *MTR* A2756G and *RFC1* A80G polymorphisms by polymerase chain reaction (PCR) and enzyme digestion, and the *MTHFR* A1298C polymorphism by allele-specific PCR. **Results** The frequencies of chromosomal alterations in DS patients were 92.2% (n = 357) for free trisomy 21, 6.2% (n = 24) for translocation and 1.5% (n=6) for mosaicism. The molecular analysis in DS mothers and control group showed that the median of the number of polymorphic alleles for the four *loci* tested was higher

in DS mothers as compared to the control group (P = 0.02), and the presence of three or more polymorphic alleles increase the risk for having a child with DS in 1.74 times (P = 0.048). Elevated maternal risk for DS was also observed in the presence of Hcy concentration higher than 4.99 μmol/L (P = 0.003). The allele frequencies for the polymorphisms in DS group were 0.37 for *MTHFR* 677T, 0.21 for *MTHFR* 1298C, 0.18 for *MTR* 2756G and 0.47 for *RFC1* 80G. The Hcy mean concentration in this group was 5.2 ± 3.3 μmol/L. The Hcy concentrations were significantly increased in the presence of *MTR* 2756AG heterozygous genotype as compared to the *MTR* 2756AA wild-type genotype (P = 0.025). **Conclusions** The presence of three or more polymorphic alleles for *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *RFC1* A80G and plasma Hcy concentrations higher than 4.99 μmol/L are maternal risk factors for DS. The *MTR* 2756AG heterozygous genotype is associated with increased Hcy concentrations in DS individuals. This study confirms yet that chromosomal nondisjunction, represented by free trisomy 21, is the most frequent cause of DS.

INTRODUÇÃO

I. INTRODUÇÃO

A síndrome de Down (SD) é a cromossomopatia mais comum na espécie humana, resultante da presença de três cópias do cromossomo 21. Sua incidência aproximada é de 1 em 600 nativivos,[1,2] constituindo a principal causa genética de retardo mental.[3,4] O fenótipo da SD é complexo e variável entre os indivíduos, que podem apresentar uma combinação de características dismórficas,[5-7] cardiopatias congênitas,[8] deficiência imunológica,[9] entre outras.

Considerando que a SD ocorre em 95% dos casos como um modelo de não-disjunção cromossômica caracterizado pela trissomia livre do cromossomo 21,[7] vários estudos são realizados na tentativa de elucidar os fatores que influenciam a não-disjunção meiótica na espécie humana. Sabe-se que a origem parental do cromossomo 21 extra é materna em cerca de 80% dos casos, e a maioria dos erros ocorre durante a primeira divisão meiótica.[10]

A idade materna avançada é considerada um importante fator de risco para ocorrência da SD.[10-12] Este risco pode estar relacionado ao processamento anormal de configurações cromossômicas suscetíveis à não-disjunção, ocorridas durante a meiose I, como resultado da degradação do processo meiótico (falha em componentes das fibras do fuso, em proteínas de coesão das cromátides irmãs ou em proteínas de controle *checkpoints*)[13] e/ou a falhas no mecanismo de seleção uterina contra embriões geneticamente comprometidos.[14]

Estudo de James et al.[15] propôs que a ocorrência da SD independente da idade materna está relacionada à hipometilação do DNA como conseqüência do metabolismo anormal do folato. A metilação do DNA ocorre quase exclusivamente nas repetições de dinucleotídeos CpG no genoma humano. Esta modificação epigenética do DNA possui

vários papéis funcionais, incluindo controle da expressão gênica[16] e a manutenção da estabilidade genômica.[17] De fato, estudos mostraram que a hipometilação do DNA genômico está associada à instabilidade cromossômica e segregação anormal dos cromossomos.[17,18]

O folato desempenha um papel importante na manutenção da metilação do DNA e, assim, da estabilidade genômica.[19] O metabolismo do folato é responsável, em uma de suas vias, pela síntese de S-adenosilmetionina (SAM), o maior doador intracelular de grupos metil para reações de metilação do DNA, proteínas e lipídios.[20] Algumas enzimas desempenham funções importantes na manutenção das concentrações de SAM e, conseqüentemente, nas reações de metilação celulares.

A enzima Metilenotetrahidrofolato redutase (MTHFR) catalisa a conversão do 5,10 metilenotetrahidrofolato para 5-metiltetrahidrofolato (5-MTHFR), a principal forma circulante de folato, que atua como doador de grupos metil para a remetilação da homocisteína (Hcy) para metionina. Esta reação de remetilação é catalisada pela enzima Metionina sintase (MTR), que requer a vitamina B_{12} (metilcobalamina) como co-fator, e resulta na formação de SAM. Participando também deste metabolismo, a enzima Cistationina β-sintase (CβS), dependente de vitamina B_6, desenvolve papel crucial no metabolismo da Hcy, convertendo-a em cistationina na chamada via de transsulfuração.[20,21]

Proteínas que transportam folato também são importantes na manutenção das reações de metilação celulares, uma vez que são responsáveis pela quantidade de folato disponível nas células. A proteína carregadora de folato reduzido, codificada pelo gene *RFC1* (*Reduced folate carrier 1*) (cromossomo 21q22.2-q22.3)[22] localiza-se na membrana das células da mucosa intestinal e participa do processo de absorção do ácido

fólico, realizando o transporte do 5-MTHFR para o interior de uma variedade de células.[23,24]

Polimorfismos em genes que codificam enzimas envolvidas no metabolismo do folato podem interferir nas concentrações de Hcy e SAM. Alguns polimorfismos em genes que participam desta via metabólica têm sido investigados como fatores de risco maternos para a SD [15,25-37]. Dois polimorfismos no gene *MTHFR* (cromossomo 1p36.3),[38] C667T e A1298C, estão associados à redução da atividade da enzima produzida.[39,40] A contribuição do polimorfismo *MTHFR* C677T para o aumento das concentrações de Hcy plasmática foi evidenciada por diversos estudos,[29,35,41-44] assim como sua influência no risco materno para a SD.[15,28-30,34-36] Para o polimorfismo *MTHFR* A1298C o aumento das concentrações de Hcy é observado por alguns autores,[44-46] embora outros não confirmem esta associação;[29,42,47] seu papel como fator de risco para a SD é também contraditório.[27-31, 33,34]

O polimorfismo no gene *MTR* (cromossomo 1q43), uma substituição de adenina por guanina na posição 2756 (A2756G),[48] também está relacionado com alterações na via metabólica do folato, embora seu efeito na atividade enzimática não tenha sido avaliado *in vitro*.[49] A influência deste polimorfismo nas concentrações de Hcy é contraditório, uma vez que alguns estudos associaram concentrações elevadas deste aminoácido à presença do alelo selvagem (A),[49-51] enquanto outros observaram relação com o alelo polimórfico (G).[52,53] Entretanto, muitos estudos não evidenciaram relação entre este polimorfismo e alterações nas concentrações de Hcy. [29,33,54-59] Até o presente, poucos estudos avaliaram a influência do polimorfismo *MTR* A2756G no risco materno para a SD.[29,31,33] O risco aumentado para prole com SD foi relacionado à presença do alelo polimórfico em associação a concentrações elevadas de Hcy,[33] e quando

combinado a alelos polimórficos de outros genes envolvidos no metabolismo do folato,[29] embora esses achados não tenham sido confirmados por Chango et al.[31]

O gene *RFC1* apresenta-se polimórfico no nucleotídeo 80 (A→G)[60] e a avaliação do impacto deste polimorfismo nas propriedades funcionais da proteína RFC1 resultante da variante polimórfica demonstrou que existem somente mínimas diferenças na sua afinidade por substratos e/ou eficiência de transporte em relação à enzima do tipo selvagem.[61] Até o presente, somente dois estudos avaliaram a influência do polimorfismo *RFC1* A80G no risco materno para a SD. Chango et al.[31] não observaram contribuição desta variante no risco materno para a SD em população francesa, enquanto Coppede et al.[25] sugerem um papel para este polimorfismo, juntamente com o polimorfismo *MTHFR* C677T, no risco para a SD em população italiana. A influência da interação entre os polimorfismos *RFC1* A80G e *MTHFR* C677T também foi observada nas concentrações de Hcy plasmática de indivíduos saudáveis.[60]

Os estudos que avaliam a contribuição de um único gene envolvido no metabolismo do folato para o risco materno para a SD são conflitantes. As divergências podem ser resultantes de variações geográficas nas freqüências dos alelos de um determinado gene entre diferentes populações, das diferenças no número amostral dos grupos estudados e dos fatores ambientais de cada região, como a condição nutricional. Todavia, a participação de polimorfismos em genes envolvidos no metabolismo do folato no risco para SD é reforçada pelo efeito combinado de alelos ou genótipos dos diferentes genes em várias populações. Estudos em populações brasileira,[29,30,34] canadense,[15] indiana,[28] americana,[36] irlandesa,[35] francesa[33] e italiana[25]

evidenciaram relação entre polimorfismos nesses genes e o risco materno aumentado para a SD.

Metabolismo do folato em indivíduos com SD

Indivíduos com SD apresentam alterações no metabolismo do folato devido à presença de três cópias do gene *CβS*, presente no cromossomo 21.[62,63] Um aumento na via de transulfuração da Hcy (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 (Figura 1).[21] O decréscimo da atividade da enzima MTR promove o acúmulo de 5-MTHFR, que resulta na redução de sua conversão para tetrahidrofolato, a forma metabolicamente ativa de folato, requerida para a síntese de RNA e DNA. Como conseqüência 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.[62]

O efeito funcional da variante A80G do gene *RFC1*, presente no cromossomo 21, e polimorfismos em genes localizados em outros cromossomos podem também contribuir para alterações em produtos derivados da via metabólica do folato em indivíduos com SD.[50,64,65] Alguns estudos mostraram freqüências elevadas de genótipos polimórficos, tais como *MTHFR* 677TT, [64] *MTR* 2756AG e *MTR* 2756GG,[33] 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.[50,64] Estudo de Guéant et al.[65] revelou associação entre concentrações

elevadas de Hcy e declínio do coeficiente de inteligência (QI) em pacientes com SD, bem como entre os alelos variantes *MTHFR* 677T e *Transcobalamina* 776G e redução de QI, possivelmente relacionada a alterações na via de remetilação da Hcy.

A presença de variantes genéticas envolvidas no metabolismo do folato em indivíduos com SD pode conferir aos mesmos uma vantagem de sobrevivência. Hobbs e colaboradores[66] observaram uma transmissão preferencial do alelo *MTHFR* 677T de pais heterozigotos para crianças com SD, e sugeriram que este achado pode conferir uma vantagem metabólica, uma vez que a expressão elevada da enzima CβS nesses indivíduos está associada com concentrações reduzidas de Hcy, metionina e SAM. A Hcy é aminoácido essencial para a síntese de DNA dependente de folato e reações de metilação celulares, importantes para o desenvolvimento fetal. Segundo estes autores, uma interação materno-fetal do polimorfismo *MTHFR* C677T também poderia proporcionar um balanço favorável na distribuição de folato entre a síntese de DNA e a metilação celular nos indivíduos com SD. A transmissão preferencial do alelo 677T de pais para filhos com SD foi também observada por Rai *et al*. (28), confirmando a hipótese de Hobbs *et al*. (66).

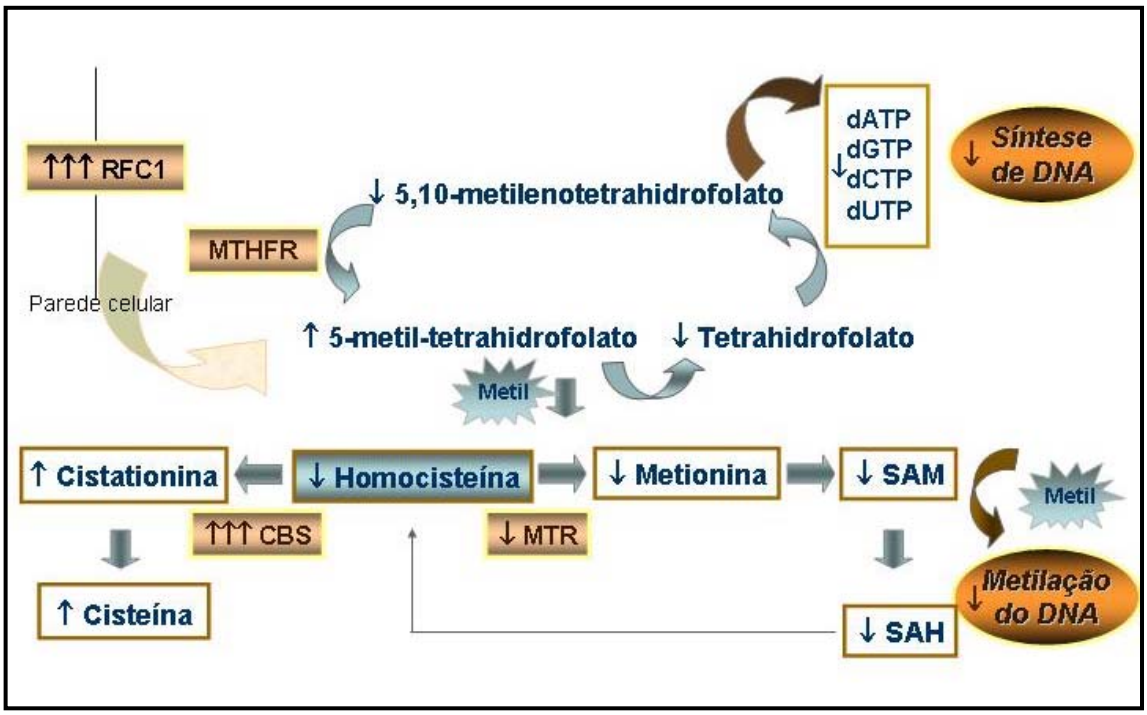

Figura 1. Metabolismo do folato em indivíduos com SD. As setas indicam alterações diretas e indiretas nos metabólitos, induzidas pela expressão elevada do gene *C*β*S*.

OBJETIVOS

Com base nas evidências apresentadas, este estudo teve como objetivo:

1. Estabelecer as freqüências de anomalias cromossômicas dos casos de SD atendidos no Serviço Ambulatorial de Genética do Hospital de Base de São José do Rio Preto, para posterior seleção de pacientes com resultado cariotípico compatível com trissomia livre do 21;

2. Avaliar a influência dos polimorfismos *MTHFR* C677T e A1298C, *MTR* A2756G, *RFC1* A80G e das concentrações de Hcy plasmática no risco materno para a SD;

3. Investigar o impacto dos polimorfismos *MTHFR* C677T e A1298C, *MTR* A2756G e *RFC1* A80G nas concentrações circulantes de Hcy em indivíduos com SD portadores de trissomia livre do 21.

ARTIGOS CIENTÍFICOS

II. ARTIGOS CIENTÍFICOS

***ARTIGO I*:**

Título: Cytogenetic profile of Down syndrome cases consulted by a general genetics outpatient service in Brazil.

Autores: Joice Matos Biselli, Eny Maria Goloni-Bertollo, Mariângela Torreglosa Ruiz, Érika Cristina Pavarino-Bertelli.

Periódico: *Down syndrome Research and Practice*, aceito para publicação em fevereiro de 2007 (aprovação no Anexo III).

ARTIGO II:

Título: Genetic polymorphisms involved in folate metabolism and elevated concentrations of plasma homocysteine: maternal risk factors for Down syndrome in Brazil.

Autores: Joice Matos Biselli; Eny Maria Goloni-Bertollo; Bruna Lancia Zampieri; Renato Haddad; Marcos Nogueira Eberlin; Érika Cristina Pavarino-Bertelli.

Periódico: *Prenatal Diagnosis*, a ser submetido para publicação.

ARTIGO III:

Título: Polymorphism *MTR* A2756G (but not *MTHFR* C677T, A1298C and *RFC1* A80G) is a determinant of homocysteine concentrations in Brazilian individuals with Down syndrome.

Autores: Joice Matos Biselli, Eny Maria Goloni-Bertollo, Renato Haddad, Marcos Nogueira Eberlin, Érika Cristina Pavarino-Bertelli.

Periódico: *Brazilian Journal of Medical and Biological Research*, a ser submetido para publicação.

Title: Cytogenetic profile of Down syndrome cases consulted by a general genetics outpatient service in Brazil.

Authors: Joice Matos Biselli, Eny Maria Goloni-Bertollo, Mariângela Torreglosa Ruiz, Érika Cristina Pavarino-Bertelli.

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Abstract

Down syndrome (DS), or trisomy 21, can be caused by three types of chromosomal abnormalities: free trisomy 21, translocation or mosaicism. The cytogenetic diagnosis, made through karyotypic examination, is important mainly to determine recurrence risks to assist genetic counseling. The objective of this work was to carry out a cytogenetic profile of confirmed cases of DS consulted in the General Genetics Outpatient Service in a teaching hospital - Hospital de Base in São José do Rio Preto – from the implantation of the service in 1973 to November 2005 with the purpose of establishing the frequencies of patients' cytogenetic abnormalities. A retrospective study was performed, in which karyotypic results of patients with DS consulted in the General Genetics Outpatient Service of HB-FAMERP from 1973 to November 2005 were investigated. The results of cytogenetic analysis were obtained from the consultation register and patients' hospital records. The results show 387 DS cases confirmed by karyotypic examinations. Of these, 357 (92.2%) patients have free trisomy of chromosome 21; 24 (6.2%) have translocation involving chromosome 21; and 6 (1.5%) have mosaicism. Nondisjunction was the main cause of DS, as the majority of the patients suffer from free trisomy of chromosome 21. The cytogenetic pattern of DS is variable among different studies.

Keywords: Cytogenetics, Down syndrome, genetic counseling, mosaicism, translocation, trisomy 21.

Introduction

Down Syndrome (DS) is the commonest chromosomal disorder in humans with a prevalence of 1:660 newborns (Devlin and Morrison, 2004). It can be caused by three types of chromosomal abnormalities: free trisomy 21, translocation or mosaicism (Newberger, 2000).

Free trisomy 21 is characterized by the presence of three complete copies of chromosome 21, generally resulting from nondisjunction during maternal meiosis (Lamb et al., 1996) and is seen in about 95% of the cases. Translocations are attributed to 3-4% of the cases, with the Robertsonian translocation involving chromosomes 14 and 21 being the most common type. Mosaicism, characterized by some cells containing 46 chromosomes and others with 47 chromosomes, is reported in 1% of DS cases[2]. These rates of occurrence of cytogenetic abnormalities are described in the basic literature but papers report variations in the cytogenetic pattern of the syndrome (Verma et al., 1991; Mutton et al., 1996; Jyothy et al., 2000; Mokhtar et al., 2003; Kava et al., 2004; Ahmed et al., 2005).

Although there is considerable variation in the physical features of individuals with DS, they present with a range of characteristics that enable clinical diagnosis of the syndrome (Prasher, 1994; Kava et al., 2004; Ahmed et al., 2005). Even so, cytogenetic diagnosis is important to confirm the clinical diagnosis and, principally, to determine the risk of recurrence thereby helping genetic counseling. This risk differs greatly between the cases as free trisomy and mosaicism generally do not repeat in siblings of DS carriers (an approximate risk of 1% for under 30-year-old

women), whilst translocation may be recurrent. For translocations, if both genitors present with normal karyotypes, the risk of recurrence is 2% to 3%. However, if one of the parents is the carrier of a balanced translocation, the risk of recurrence depends on this parent's gender and on the type of translocation (Newberger, 2000). In the case of Robertsonian translocations, the recurrence risk is around 10 to 15% when the mother is the carrier and from 2 to 15% when the carrier of this balanced translocation is the father (Jorde et al., 2006). On the other hand, if one of the genitors is the carrier of a balanced translocation involving two chromosomes 21 the recurrence risk for DS is 100%. Thus, once diagnosed as a case of DS due to a translocation, a karyotypic analysis of both parents is recommended (Newberger, 2000).

The objective of this work was to establish the cytogenetic abnormality frequencies of patients with DS in the Genetics Outpatient Service of a teaching hospital, to contribute to the knowledge of these data in a South American such as Brazil.

Material and methods

A retrospective study was performed, in which karyotypic results of patients with DS consulted in a public healthcare service that routinely performs karyotypic examinations only by conventional cytogenetic technique (chromosome GTG-banding) (Gustashaw, 1997). The results of cytogenetic analysis were obtained from the consultation register and patients' hospital records. All patients with available karyotypic results were included in the study.

Statistical analysis of the data was achieved utilizing the Likelihood Ratio Test with a level of significance ≤ 5%.

Results

Over the 32 years of the existence of the general genetics outpatient service of HB-FAMERP in São José do Rio Preto, 387 cases of DS confirmed by cytogenetic analysis were registered. Of these, 357 (92.2%) patients had free trisomy, 24 (6.2%) presented with translocation involving the chromosome 21, and 6 (1.5%) patients had mosaicism (Figure 1).

Discussion

According to the literature, DS is a consequence of free trisomy of chromosome 21 in about 95% of cases, translocation in 3-4% and mosaicism in 1% (Newberger, 2000).

Recent publications on cytogenetic studies of patients with DS show differences in the frequencies of these chromosomal abnormalities. Ahmed et al. (2005) observed, in a sample of 295 patients, frequencies of 95.6%, 3.7% and 0.7% respectively for free trisomy, translocation and mosaicism. These frequencies do not significantly differ from those observed by Mutton et al. (1996) with 95% for free trisomy, 4% for translocations and 1% for mosaicism in a total of 5737 patients (P-value = 0.98). The study of Verma et al. (1991), which included the karyotyping results of 645 patients, identified 93% of free trisomy, 4.1% of translocations and 2.6% of mosaicism. Kava et al. (2004) observed frequencies of 95%, 3.2% and 1.8% for free trisomy, translocation and mosaicism, respectively in a series of 221 patients. The study of

Jyothy et al. (2000) of 101 patients differs by the high frequency of mosaicism (7.69%), even higher than the frequency of translocations (4.39%). Mohktar et al. (2003) observed a lower frequency of translocations (2.7%) with values of 96.6% for free trisomy and 0.7% for mosaicism for the 673 in their cohort. On the contrary, our study differs from the studies mentioned above because of the high frequency of translocations with a frequency of 92.2% for free trisomy, 6.2% for translocations and 1.5% for mosaicism. An analysis of all these results, including our own data, revealed that the observed frequencies are significantly different among the different studies (P-value < 0.05).

No specific reason can explain this discrepancy in the frequencies of cytogenetic abnormalities among the different investigations. Ahmed et al. (2005) attributed this divergence to differences in the study periods, maternal ages and the populations studied. The majority of the cited studies, including ours, considered the karyotyping results of only live-born infants born to mothers of any age (Verma, 1991; Jyothy et al., 2000; Mokhtar et al., 2003; Kava et al., 2004). Exceptions were Mutton et al. (1996), who also considered data coming from miscarriages and pre-natal diagnoses, and Ahmed et al. (2005), who included only over 18-year-old patients in their study.

Even so, all the investigations pointed to chromosomal nondisjunction as the main cause of DS, as the majority of the patients suffer from free trisomy of chromosome 21, with nondisjunction generally due to maternal meiosis (Lamb et al., 1996).

It is well known that advanced maternal age increases the risk of having a child with DS (Newberger, 2000; Zheng and Byers, 1992; Lamb et al., 2005). The risk of a woman of up to 25 years old having a child with DS is 1:1300 and 1:365 at 35 years old. At 45 years old, this risk increases to 1:30 (Newberger, 2000). However, a recently published metanalysis showed that this risk does not continue to rise with age for women older than 45 years old. Possible explanations for this fact are early miscarriages in older women and fertility treatment including egg donation, pre-implantation diagnosis (Morris et al., 2005). Apart from advanced maternal age, several hypotheses have already been raised to try to explain the etiology of chromosomal non-disjuncture in humans (Sherman et al., 1991; Gaulden, 1992; Avramopoulos et al., 1996; James et al., 1999; Hassold and Sherman 2000; Sheth and Sheth, 2003; da Silva et al., 2005; Pavarino-Bertelli et al., 2005). More recently, hypomethylation of the centromeric DNA as a result of abnormal folate metabolism has been implicated in abnormal chromosomal segregation and studies point to the role of polymorphisms in some genes involved in homocysteine metabolism as risk factors for DS (James et al., 1999; Sheth and Sheth, 2003; da Silva et al., 2005).

Cytogenetic investigations of individuals who present clinical characteristics of the syndrome are fundamental to establish a precise diagnosis, which has a direct implication in the genetic counseling process. Additionally, the karyotype of affected individuals identify cases that may have been inherited making an investigation of the parents' karyotypes necessary as they may be carriers of a balanced translocation involving chromosome 21. In this case, the cytogenetic investigation of the genitors is essential to establish the risk of recurrence of the syndrome in future generations.

Thus, all individuals with a diagnosis suggestive of DS should be referred to a genetic counseling service.

The growth of the General Genetics Outpatient Service of HB-FAMERP over the last few years, together with the great publicity related to genetics and its applications in medicine, has led to an increase in the number of cytogenetic diagnoses of DS individuals. Currently, around two new cases of DS are identified every month, many more than at the start of the service. Our investigation did not include cases with clinical diagnosis of DS but without karyotyping confirmation at the time of the study to identify the chromosomal abnormality involved, thus a greater number of cases with a diagnosis of possible DS have been screened by the service.

It is important to stress that in a broad review of publications, reports on this theme in the Brazilian population were not found. Although our outpatients' service is located in the city of São José do Rio Preto, these data do not reflect the prevalence of DS in this city, as the hospital to which the outpatients' service is attached covers a region of 99 municipals (Hospital de Base, 2005), reflecting the cytogenetic pattern of DS in the southwest region of Brazil.

In conclusion, the cytogenetic pattern of DS is variable among different studies. Free trisomy of chromosome 21, resulting from a chromosomal nondisjunction, is the most frequent cause. All cases with a diagnostic hypothesis of DS should be referred to a genetic counseling service.

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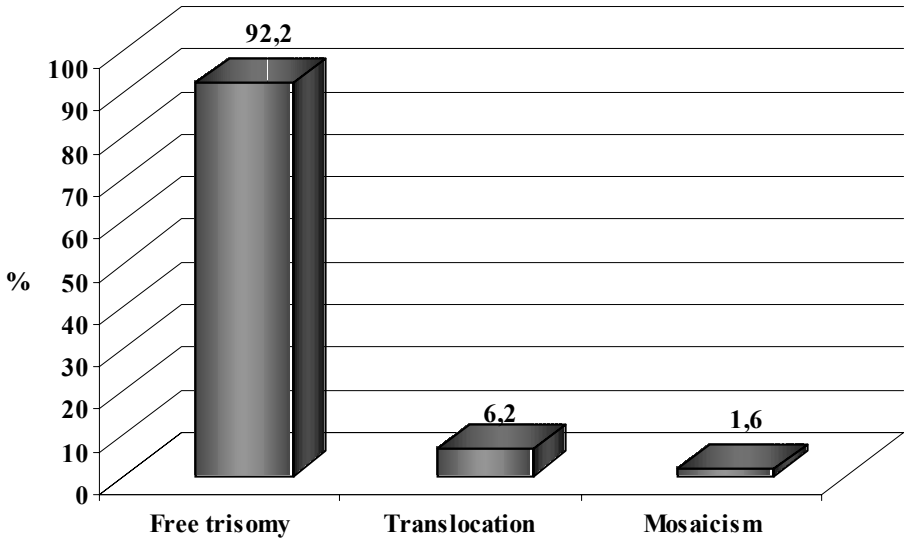

Figure 1. Frequencies of cytogenetic abnormalities in Brazilian patients with Down syndrome.

Title: Genetic polymorphisms involved in folate metabolism and elevated concentrations of plasma homocysteine: maternal risk factors for Down syndrome in Brazil.

Key-words: Trisomy 21; Down syndrome; Nondisjunction, Genetic; Polymorphism, Genetic.

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Abstract

Objective: To investigate the polymorphisms C677T and A1298C in *Methylenetetrahydrofolate reductase* gene (*MTHFR*), A2756G in *Methionine synthase reductase* (*MTR*) gene and A80G in *Reduced folate carrier I* (*RFC1*) gene, and plasma homocysteine (Hcy), on the maternal risk for Down syndrome (DS). **Methods:** Seventy-two DS mothers and 194 mothers who had no children with DS were evaluated. The investigation of the *MTHFR* C677T, *MTR* A2756G and *RFC1* A80G polymorphisms was performed by Polymerase Chain Reaction (PCR) and enzyme digestion and the *MTHFR* A1298C polymorphism by allele-specific PCR. Hcy quantification was carried out by liquid chromatography – tandem mass spectrometry (LS-MS/MS). **Results:** The median of the number of polymorphic alleles for the four *loci* tested was greater in DS mothers as compared to the control group, and the presence of three or more polymorphic alleles increase the risk for having a child with DS in 1.74 times. Elevated maternal risk for DS was also observed when plasmatic Hcy concentration was higher than 4.99 μmol/L. **Conclusions:** The presence of three or more polymorphic alleles for *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *RFC1* A80G and plasma Hcy concentrations greater than 4,99 μmol/L are maternal risk factors for DS.

Introduction

Down syndrome (DS) is caused by the presence of three copies of chromosome 21, in most cases due to the failure in chromosomal segregation during maternal meiosis (meiotic nondisjunction) (Jyothy *et al*., 2001). Some studies suggest that genomic DNA hypomethylation may be associated with chromosomal instability and abnormal segregation (James *et al*., 2003; Sciandrello *et al*., 2004).

Folate plays an important role in cellular methylation reactions (Fenech *et al*., 2002). Its metabolism is responsible, on one pathway, for the synthesis of S-adenosylmethionine (SAM), the main methyl group donor for methylation reactions of DNA, proteins and lipids. Some enzymes play important roles in the maintenance of SAM concentrations. The enzyme methionine synthase (MTR) catalyzes the remethylation of homocysteine (Hcy) to methionine, and this reaction results in SAM formation. The enzyme MTR requires 5-methyltetrahydrofolate as a methyl group donor for the remethylation of Hcy to methionine, and the formation of this radical depends on the action of the enzyme methylenetetrahydrofolate reductase (MTHFR) (Finkelstein, 2000).

Folate-transporting proteins are also important to the maintenance of DNA methylation, since they are responsible for the amount of folate available in the cells. The reduced folate carrier 1 (RFC1) protein is located in the intestinal mucosa membrane and plays a role in folic acid absorption, transporting 5-methyltetrahydrofolate into the cells (Nguyen *et al*., 1997).

Several studies have associated polymorphisms in genes involved in folate metabolism, such as *MTHFR*, *MTR* and *RFC1*, to an elevated maternal risk for SD (James *et al*., 1999; Grillo *et al*., 2002; Acácio *et al*., 2005; da Silva *et al*., 2005; Scala

et al., 2006; Coppede *et al*., 2006). The observation of significantly higher Hcy concentrations in DS mothers as compared to control mothers is another evidence that folate metabolism may be abnormal in these women (Bosco *et al*., 2003; Takamura *et al*., 2004; da Silva *et al*., 2005).

The aim of this study was to analyze the effect of four genetic polymorphisms, *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *RFC1* A80G, and plasma Hcy concentrations, on the maternal risk for DS.

Methods

The 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. In this study, 72 women who had given birth to children with full trisomy 21 were included (DS mothers), recruited from the Genetics Outpatient Service of Hospital de Base (HB) of the São José do Rio Preto Medical School (FAMERP), São Paulo, Brazil. Control mothers (n = 194) who had no children with DS were enrolled at the FAMERP Campus and at the HB Clinical Analysis Laboratory. Maternal age was calculated considering the age of the mother at the birth of the DS child for DS mothers, and the age at the birth of the last child for the control group. Mothers of DS individuals with translocation or mosaicism were excluded from the study. The exclusion criterion for the control group was the presence of a child with a genetic syndrome.

Genomic DNA was extracted from blood leukocytes (Abdel-Rahman *et al*., 1994). Polymorphisms at four *loci* were evaluated, two of them in the *MTHFR* gene (C677T and A1298C), one in the *MTR* gene (A2756G), and one in the *RFC1* gene

(A80G). The *MTHFR* A1298C polymorphism was determined by allele-specific PCR, using primers to separately amplify wild-type and mutated alleles (allele A: forward 5′-GGA GCT GAC CAG TGA AGA -3′ and reverse 5′- TGT GAC CAT TCC GGT TTG -3′ to amplify a 77 base-pair fragment; allele C: forward 5′-CTT TGG GGA GCT GAA GGA -3′ and reverse 5′- AAG ACT TCA AAG ACA CTT G - 3′ to amplify a 120 base-pair fragment). As positive controls of gene amplification, were used primers flanking the 677 polymorphic region of the *MTHFR* gene, described by Frosst *et al.* (1995), resulting in a 198 base-pair fragment. This strategy makes it possible to determine the *MTHFR* C677T genotype, after digestion of this fragment by enzyme *Hinf I*, which recognizes the allele T. Genotyping for the *MTR* A2756G polymorphism was carried out by PCR, using specific primers (forward 5′- CCA GGG TGC CAG GTA TAC AG -3′; reverse 5′ GCC TTT TAC ACT CCT CAA AAC C -3') to amplify a 498 base-pair fragment, followed by digestion with enzyme *Hae* III, that digests the fragment in the presence of the mutated G allele. The *RFC1* A80G polymorphism was determined using primer sequences described by Födinger *et al*. (2003) and digestion with enzyme *Cfo I* for recognizing the G allele.

Hcy quantification was possible only for 58 DS mothers and 49 control mothers. Plasma Hcy concentrations were determined by liquid chromatography-tandem mass spectrometry (LS-MS/MS) as previously described (Haddad *et al*., 2001; Vellasco *et al*., 2002), in overnight fasted mothers.

Statistical analysis

Data are presented as mean ± standard deviation (SD), number or frequency. To compare the maternal ages in the two groups, Student's *t* test and logistic regression with ages grouped into quartiles were used.

Hardy-Weinberg equilibrium was tested by the chi-square test, using the *BioEstat* program, and genotype frequencies in the SD and control mothers were compared by the likelihood ratio test and logistic regression. The relationship between the number of polymorphic alleles for the four *loci* tested per case or control mother and the maternal risk for DS was analyzed by Mann-Whitney's test followed by logistic regression analysis. For this analysis, the samples were divided into subsets (0-2 and 3-5 alleles). *MTHFR* haplotypes were inferred using the Phase program (version 2.1), which creates population frequency estimates of the haplotypes. The combined *MTHFR* genotypes (677 / 1298) distribution in the two groups was analyzed by the likelihood ratio test and logistic regression, excluding the only woman with the genotype 677CT / 1298CC. In the logistic regression analysis the 677CC / 1298AA genotype was considered as reference.

The comparison between groups regarding the Hcy concentrations was performed using Mood's median test followed by logistic regression analysis of Hcy grouped into quartiles. The distribution of Hcy concentrations according to the genotypes was carried out by the ANOVA test, using the logarithm scale of Hcy concentrations. For the combined *MTHFR* genotype analysis, we excluded the only woman with the genotype 677CT / 1298CC.

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

Results

Characterization of groups in relation to maternal age The mean maternal age of DS mothers was 31.7 ± 8.4 years (range: 13-46), and in the control group it was 27.1 ± 5.8 years (range: 15-41). As expected, there was a prevalence of older mothers in the case group ($P < 0.0005$), and an increased risk for DS was observed above the age of 32.4 years (OR 4.61; CI 95% 2.53 – 8.39; $P < 0.0005$). For women older than 35 years, the risk for DS was even higher (OR 10.18; CI 95% 4.90 – 21.15; $P < 0.0005$).

Genotype analysis and risk for Down syndrome The allele frequencies of *MTHFR* 677T, *MTHFR* 1298C, *MTR* 2756G and *RFC1* 80G are presented in Table 1. Genotype frequencies for the four polymorphisms were in Hardy-Weinberg equilibrium in both groups ($P > 0.05$) and showed no difference between DS mothers and control groups ($P = 0.85$ for *MTHFR* C677T; $P = 1.0$ for *MTHFR* A1298C; $P = 1.0$ for *MTR* A2756G; $P = 0.1$ for *RFC1* A80G). The combined *MTHFR* 677/1298 genotypes distribution showed no differences between DS and control mothers ($P = 2.4$), and no association was found with the risk for DS ($P = 0.88$ for CC/AC; $P = 0.20$ for CT/AA; $P = 0.38$ for CC/CC; $P = 0.17$ for CT/AC; $P = 0.27$ for TT/AA). In addition, the logistic regression analysis for all polymorphisms showed no association between the variant genotypes and the risk for DS either (Table 2). We also evaluated the genotype frequencies between the groups considering only women with maternal ages below 35 years and also below 32.4 years. However, no differences were found between case and control

mothers in these analyses. The distribution of the number of polymorphic alleles per individual (case or control) for the four *loci* tested is presented in Table 3. The median number of polymorphic alleles was higher in the group of DS mothers than in the control group (medians 3 and 2 for case and control groups, respectively; P = 0.02). The presence of three or more polymorphic alleles increases the risk of having a child with DS by 1.74 fold (OR 1.74; CI 95% 1 – 3.02; P = 0.048).

Haplotype analysis for *MTHFR* C677T and A1298C The haplotypes were inferred by the Phase program, and a higher frequency of the C-A haplotype was observed in both groups (0.390 and 0.468 in DS and control mothers, respectively). The frequency of haplotype T-A was 0.353 and 0.280, and of haplotype C-C 0.256 and 0.246, in the DS mothers and control group, respectively. The estimated frequency of haplotype T-C was 0.001 in DS mothers and 0.006 in control mothers. So, although haplotype T-C was present in our study population, its frequency was lower than the expected allele combination frequency calculated by the Phase program, confirming negative selection of this haplotype.

Homocysteine Concentrations The median Hcy concentrations were significantly higher in DS mothers (5.38 μmol/L) as compared to the control group (4.22 μmol/L) (P = 0.01), as shown in Figure 1, and a higher maternal risk for DS was observed when the Hcy concentrations were higher than 4.99 μmol/L (OR 4.62, CI 95% 1.69-12.59, P = 0.003).

Plasma Homocysteine Concentrations and Their Relation to Genotypes The analysis of Hcy distribution by genotypes showed that in women with *MTHFR* 1298CC genotype the Hcy concentrations varied significantly according to the group (case or control) (P = 0.036). In DS mothers, the Hcy concentrations were higher in the presence

of the 1298CC genotype, while in the control group these concentrations were lower (Figure 2).

Discussion

The association between advanced maternal age and the occurrence of trisomies has been well established. A Brazilian study conducted in the southeastern region of the country showed that the maternal age of women with DS children was significantly higher than that of women without DS children (Beiguelman *et al*., 1996), and our results confirm this finding.

The occurrence of DS independently of maternal age represents evidence of other risk factors for this syndrome. Several studies have correlated the maternal risk for DS to an abnormal folate metabolism (James *et al*., 1999; Grillo *et al*., 2002; da Silva *et al*., 2005; Acácio *et al*., 2005; Scala *et al*., 2006; Coppede *et al*., 2006). The observation of an increased occurrence of abnormal chromosome 13 and 21 segregation events in peripheral lymphocytes of young mothers with DS children suggests a generalized susceptibility to chromosomal malsegregation events, taking place in both the meiotic and the mitotic process (Migliore *et al*., 2006).

Folate deficiency has been linked to chromosomal instability and chromosome 21 aneuploidy (Wang *et al*., 2004; Beetstra *et al*., 2005), and the genome-damaging effect of folate deficiency in cultured lymphocytes may be modulated by the *MTHFR* genotype (Kimura *et al*., 2004). Certain studies have associated the presence of one or two *MTHFR* 677T alleles with an elevated maternal risk for DS (Hobbs *et al*., 2000; James *et al*., 1999), some of them carried out in Brazilian populations (Grillo *et al*., 2002; da Silva *et al*., 2005). The *MTHFR* A1298C variant was associated with the

maternal risk for DS when in heterozygosity and in combination with the 677CT genotype (Grillo *et al*., 2002; Acacio *et al*., 2005).

Linkage disequilibrium (LD) between *MTHFR* C677T and A1298C alleles has been reported (Stegmann *et al*., 1999; Shi *et al*., 2003). However, this LD is not complete, once the presence of some individuals with the haplotype T-C was observed in some studies (Shi *et al*., 2003; Parle-McDermott *et al*., 2003; Scala *et al*., 2006). In our study, in a total sample of 266 women, only one with this haplotype was observed, confirming its negative selection.

Regarding *MTR* A2756G, Bosco *et al*. (2003) observed an elevated risk for DS in the presence of the *MTR* 2756G allele, in combination with elevated Hcy concentrations. However, the association of this polymorphism with the risk for DS was not confirmed by Chango *et al*. (2005). Few studies have evaluated the *RFC1* A80G polymorphism in DS mothers. Coppede *et al*. (2006) showed a role for the 80GG genotype combined with the *MTHFR* 1298AA genotype in the maternal risk for DS in an Italian population. Another study (Chango *et al*., 2005) found no association between the variant *RFC1* A80G and the risk for DS.

In the present study, even though in the isolated analysis the polymorphisms were not associated with the maternal risk for DS, we observed a 1.74 fold increase in maternal risk for DS in the presence of three or more polymorphic alleles (P= 0.048). The higher median number of polymorphic alleles observed in our DS mothers as compared to the control mothers corroborates the findings of another Brazilian study (da Silva *et al.,* 2005). These authors investigated polymorphisms 844ins68 in the *Cystathionine* β-*synthase* gene (*CBS*) and A66G in the *Methionine synthase reductase* gene (*MTRR*), in addition to the polymorphisms in genes *MTHFR* and *MTR*, analyzed

in our study. They observed a 1.259 fold increase in the chance of having a DS child associated with the presence of each one of the polymorphic alleles studied.

Another factor that may modulate the maternal risk for DS is plasma Hcy concentration. Hcy is an amino acid formed during folate metabolism, and the elevation of its concentrations, indicative of alterations in this metabolic pathway, has been associated with an elevated risk for DS (James *et al*., 1999; Takamura *et al*., 2004; da Silva *et al*., 2005). Our results confirm this association, even though none of the women in both groups presented hyperhomocysteinemia, characterized by Hcy concentration above 15 μmol/L (American Society of Human Genetics, 1998).

Several studies have shown the contribution of genetic polymorphisms to the increase of plasma Hcy concentrations. The contribution of the *MTHFR* 677T allele is well established (Takamura *et al*., 2004; da Silva *et al*., 2005; Ulvik *et al*., 2006), while the participation of the A1298C polymorphism is still controversial (Weisberg *et al*., 2001; Castro *et al*., 2003; da Silva *et al*., 2005). Polymorphism *MTR* A2756G has also been associated with variations in Hcy concentrations. Some authors have associated elevated Hcy concentrations with the presence of the wild-type allele (A) (Harmon *et al*., 1999; Chen *et al*., 2001; Fillon-Emery *et al*., 2004), whereas others observed a correlation with the polymorphic allele (G) (Feix *et al*., 2001; Laraqui *et al*., 2006). An influence of the *RFC1* A80G polymorphism on Hcy concentrations of healthy individuals has been observed, but only when associated with the *MTHFR* C677T polymorphism (Chango *et al*., 2000).

Our results show that the Hcy concentrations are significantly different in women with an *MTHFR* 1298CC genotype according to the group (case or control), being higher in DS mothers and lower in control mothers. Martínez-Frías *et al*. (2006)

also observed that the Hcy concentrations varied significantly between DS and control mothers in the presence of some genotype combinations of polymorphisms *MTHFR* A1298C and *MTRR* A66G. Our observations may be attributed to the combinations of the *MTHFR* A1298C genotype with genotypes of other polymorphisms and/or to differences in environmental factors between the groups, such as nutritional status (Bailey *et al*., 2002). In addition, these results may be interpreted as a contribution of the elevated maternal Hcy concentration to the survival of a DS fetus, as proposed by Hobbs *et al.* (2002). According to these authors, a maternal-fetal genotype interaction of the *MTHFR* C677T polymorphism would provide a favorable balance of folate distribution between both DNA synthesis and cellular methylation in DS individuals, due to the increase in Hcy concentrations. It is possible that other polymorphic alleles involved in the folate metabolism produce a similar effect, which could explain our findings.

In the light of the results obtained, we concluded that the presence of three or more polymorphic *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *RFC1* A80G alleles, and of Hcy concentrations above 4.99 μmol/L, are maternal risk factors for DS. This study confirms the association between abnormal folate metabolism and DS in Brazil.

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Table 1. Allele frequencies of the *MTHFR* C677T, A1298C, *MTR* A2756G and *RFC1* A80G polymorphisms in DS and control mothers.

Polymorphism	**Allele**	**DS mothers**	**Control mothers**
MTHFR 677	T	0.3542	0.2861
MTHFR 1298	C	0.2569	0.2526
MTR 2756	G	0.2014	0.1881
RFC1 80	G	0.5417	0.5000

Table 2. Genotype frequencies of polymorphic alleles *MTHFR* C677T, A1298C, *MTR* A2756G and *RFC1* A80G in DS (n = 72) and control (n = 194) mothers.

Genotype	DS mothers n (%)	Control mothers n (%)	OR (95%CI)	P
***MTHFR* 677**				
CC	29 (40.3)	100 (51.5)	reference	
CT	35 (48.6)	77 (39.7)	1.69 (0.92-3.08)	0.089
TT	8 (11.1)	17 (8.8)	1.70 (0.62-4.66)	0.305
***MTHFR* 1298**				
AA	40 (55.6)	108 (55.7)	reference	
AC	27 (37.5)	74 (38.1)	1.07 (0.58-1.96)	0.832
CC	5 (6.9)	12 (6.2)	1.53 (0.48-4.95)	0.473
***MTR* 2756**				
AA	47 (65.3)	129 (66.5)	reference	
AG	21 (29.2)	57 (29.4)	1.01 (0.55-1.87)	0.965
GG	4 (5.6)	8 (4.1)	1.63 (0.46-5.83)	0.451
***RFC1* 80**				
AA	15 (20.8)	50 (25.8)	reference	
AG	36 (50.0)	94 (48.5)	1.25 (0.62-2.53)	0.533
GG	21 (29.2)	50 (25.8)	1.38 (0.63-3.03)	0.426

Table 3. Distribution of the *MTHFR* 677T, 1298C, *MTR* 2756G and *RFC1* 80G alleles per individual in the two groups.

	Number of polymorphic alleles					
	0	**1**	**2**	**3**	**4**	**5**
Case (n , %)	1 (1.4)	12 (16.7)	14 (19.4)	26 (36.1)	14 (19.4)	5 (6.9)
Control (n, %)	9 (4.6)	32 (16.5)	58 (29.9)	64 (33.0)	23 (11.9)	8 (4.1)

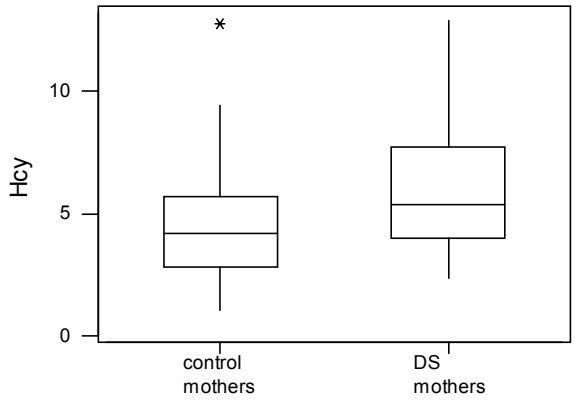

Figure 1. Distribution of plasma Hcy concentrations (μmol/L) in the two groups. Control group: median 4.22 μmol/L (range: 1.1 – 12.8); DS mothers group: median 5.38 μmol/L (1.4 – 12.9).

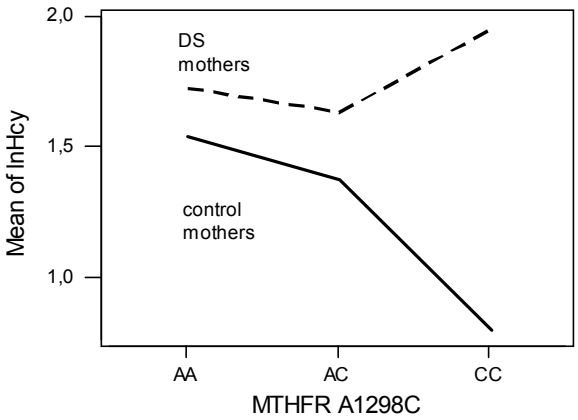

Figure 2. Hcy concentrations according to *MTHFR* A1298C genotypes.

Title: Polymorphism *MTR* A2756G (but not *MTHFR* C677T, A1298C and *RFC1* A80G) is a determinant of homocysteine concentrations in Brazilian individuals with Down syndrome.

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Short running: Polymorphisms and homocysteine in individuals with DS.

Key words: Down syndrome; Trisomy 21; Polymorphism, genetic; Homocysteine.

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Abstract

Individuals with Down syndrome present decreased homocysteine (Hcy) concentration, reflecting a functional folate deficiency secondary to overexpression of *Cystathionine* β-*synthase* (*CβS*) gene. Plasma Hcy may be influenced by genetic polymorphisms, thus we evaluated the influence of polymorphisms C677T and A1298C in the *Methylenetetrahydrofolate reductase* gene (*MTHFR*), A2756G in the *Methionine synthase* gene (*MTR*) and A80G in the *Reduce folate carrier 1* gene (*RFC1*) on Hcy concentrations in Brazilian DS patients. Fifty-six individuals with free trisomy 21 were included in the study. Plasma Hcy concentrations were measured, and genotyping for polymorphisms was carried out. The mean Hcy concentration was 5.2 ± 3.3 μmol/L. There was no correlation between Hcy concentrations and age, gender or the genotypes *MTHFR* C677T, A1298C and *RFC1* A80G. However, Hcy concentrations were significantly increased in the *MTR* 2756AG heterozygous genotype as compared to the *MTR* 2756AA wild-type genotype. Our results suggest that the heterozygote genotype *MTR* 2756AG is associated with the increase in plasma Hcy concentrations in Brazilian patients with DS.

Introduction

Down syndrome (DS), or trisomy 21, results from the gene expression of an extra chromosome 21, which occurs in most cases due to the failure of normal chromosomal segregation during maternal meiosis (1). Individuals with DS present decreased homocysteine (Hcy) concentrations (2). Hcy is an amino acid formed during the folate metabolism. It can be remethylated to methionine by the enzyme Methionine synthase (MTR), with transference of the methyl group of methyltetrahydrofolate, formed by the action of the enzyme MTHFR, or can be converted to cysteine in the transsulfuration pathway, which requires the enzyme Cystathionine b-synthase (CβS) (3). The decreased Hcy concentration observed in individuals with DS is consistent with the location of the *CβS* gene on chromosome 21 and its overexpression (2) (Figure 1) (4).

Plasma Hcy is influenced by modifiable and nonmodifiable factors, such as gender, vitamin status and genetic factors (5,6). The polymorphisms C677T and A1298C of the *Methylenetetrahydrofolate reductase* (*MTHFR*) gene are important genetic determinants of Hcy concentrations. The polymorphic alleles result in an enzyme with reduced specific activity (7,8), consequently leading to increased Hcy concentrations (9,10).

Another polymorphism in gene encoding protein involved in the homocysteine-folate metabolism is an adenine-to-guanine substitution in nucleotide 2756 of the *MTR* gene. Some studies (11,12) found that this polymorphism had an effect in increasing the Hcy concentration in the presence of the wild-type allele (A), whereas others (13,14) observed an association between elevated Hcy and this polymorphism in the presence of

the mutated allele (G). However, other studies did not observe any association between this polymorphism and alterations in Hcy concentrations (15,16).

Variant A80G of the *RFC1* (*Reduced folate carrier 1*) gene may also contribute to alterations in products derived from this metabolic pathway (11). The RFC1 protein is located in the intestinal mucosa membrane and plays a role in folic acid absorption, transporting 5-methyltetrahydrofolate into the cells (17).

The aim of the present study was to evaluate the influence of the *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *RFC1* A80G polymorphisms on Hcy concentrations in Brazilian patients with DS.

Subjects and Methods

Fifty-six DS individuals with full trisomy 21 were recruited after family informed consent from the General Genetics Outpatient Service of Hospital de Base in São José do Rio Preto, Brazil.

After overnight fasting, blood samples from all the 56 individuals were collected into EDTA-containing tubes. Genomic DNA was extracted from blood leukocytes (18). Polymorphisms were determined by polymerase chain reaction (PCR) with upstream and downstream primers flanking the target sequence. Mutation C677T in the *MTHFR* gene was detected using primer sequences described by Frosst *et al*. (19), followed by *Hinf* I digestion. The A1298C polymorphism was determined by allele-specific PCR, using primers to separately amplify wild-type and mutated alleles, as follow: forward: 5′- GGA GCT GAC CAG TGA AGA -3′, and reverse: 5′- TGT GAC CAT TCC GGT TTG -3′, to amplify a 77 base pair (bp) fragment corresponding to allele A (wild-type); forward: 5′-CTT TGG GGA GCT GAA GGA -3′, and reverse: 5′- AAG ACT TCA

AAG ACA CTT G -3′, to amplify a 120 bp fragment corresponding to allele C (mutated). A pair of primers was used to amplify a 198 bp segment of the *MTHFR* gene as positive control of gene amplification (19). Genotyping for the *MTR* A2756G polymorphism was carried out using specific primers (forward: 5′ CCA GGG TGC CAG GTA TAC AG 3′, and reverse: 5′ GCC TTT TAC ACT CCT CAA AAC C 3′) to amplify a 498 bp fragment spanning the polymorphism, followed by digestion with enzyme *Hae* III, that digests the fragment in presence of the mutated G allele. The *RFC1* A80G polymorphism was determined as described by Födinger *et al*. (2003). This method for *RFC1* A80G genotyping does not distinguish individuals who have one (AAG) or two (AGG) polymorphic alleles, once DS individuals present three alleles for this gene, which is located on chromosome 21.

Hcy was determined by liquid chromatography – tandem mass spectrometry (LS-MS/MS), as previously reported (21,22). Hcy concentrations >15μmol/L were considered to characterize hyperhomocysteinemia (23).

Data are presented as mean $\pm$ standard deviation (SD), number or frequency. The distribution of the Hcy values in relation to age was analyzed using the Spearman Correlation. To the comparisons of Hcy concentrations between genders and between the genotypes were employed the T Test and ANOVA, respectively, using the logarithm scale of Hcy concentrations. Hcy concentrations according to the combined *MTHFR* genotypes (677 and 1298) were evaluated by Mood's Median test. *MTHFR* haplotypes were inferred using the Phase program (version 2.1), which creates population frequency estimates of the haplotypes.

The computer-assisted statistical analyses were carried out with the *Minitab for Windows* (Release 12.22) program. Hardy-Weinberg equilibrium (HWE) was tested by

chi-square, using the *BioEstat* program, except for *RFC1* A80G, which presents three alleles in DS individuals. For statistical significance, values of $P \leq 0.05$ were considered.

Results

The study group consisted of 56 DS individuals (24 female and 32 male) with ages ranging between 1 month and 30 years (mean age 4.8 ± 6.7 years).

The allele frequencies observed were 0.37 for *MTHFR* 677T, 0.21 for *MTHFR* 1298C, 0.18 for *MTR* 2756G and 0.47 for *RFC1* 80G. The allele distribution of the polymorphisms is in HWE ($\chi^2 = 0.41$; df = 1; P = 0.521 for *MTHFR* C677T; $\chi^2 = 3.71$; df = 1; P = 0.054 for *MTHFR* A1298C; $\chi^2 = 1.22$; df = 1; P = 0.269 for *MTR* A2756G).

The *MTHFR* C677T and A1298C haplotypes frequencies inferred by Phase program were 0.4116 for C-A, 0.2134 for C-C, 0.3742 for T-A and 0.0008 for T-C. However, the T-C haplotype was not present in our sample of DS patients. Thus, the T-C frequency in our population was lower than the expected allele combination frequency calculated by the Phase program, confirming negative selection for this haplotype.

The mean Hcy concentration was 5.2 ± 3.3 μmol/L. There was no correlation between Hcy concentrations and age (Rs = 0.065; P = 0.633) and the Hcy concentrations were not different between genders (P = 0.627).

Mean Hcy concentrations according to genotypes for the polymorphisms studied are presented in Table I. There was no association between Hcy concentrations and genotypes *MTHFR* C677T (P = 0.110), *MTHFR* A1298C (P = 0.727), *RFC1* A80G (P = 0.769) and combined *MTHFR* 677/1298 genotypes (P = 0.135). However, the Hcy concentrations were significantly increased in the *MTR* 2756AG heterozygous genotype

as compared to the *MTR* 2756AA wild-type genotype (P = 0.025). Only one individual (female, 9 months of age) presented hyperhomocysteinemia (20.9 μmol/L); she was the carrier of a heterozygous genotype to polymorphism *MTR* A2756G (*MTR* 2756AG / *MTHFR* 677CC / *MTHFR* 1298AA / *RFC1* 80AAA).

Discussion

Previous studies have hypothesized that patients with DS have disturbed folate metabolism (2,24). Pogribna *et al*. (2001) reported that the plasma profile of the metabolites involved in the methionine / homocysteine pathway in DS children, including decreased Hcy concentrations, reflect a functional folate deficiency secondary to overexpression of *CBS* gene, present on chromosome 21. The increase in the activity of the transsulfuration pathway of Hcy, which results from the overexpression of *CBS,* may promote a ‘‘folate trap’’ by decreasing the cellular concentration of Hcy and its subsequent remethylation pathway .

Several studies failed to confirm reduced Hcy concentrations in DS patients (11, 25,26). Furthermore, in a study with elderly DS patients, Licastro *et al*. (27) observed that their plasma Hcy was higher and folate lower than in the controls; blood concentrations of vitamin B12 were also lower in the DS patients than in the controls. The authors attributed most of the cases of mild hyperhomocysteinemia in the elderly DS patients to a nutritional folate and B12 deficiency.

In our study, the mean Hcy concentration of DS patients (5.2 ± 3.3 μmol/L) was close to the one observed by Pogribna *et al*. (2) in DS children (5.1 ± 1.1 μmol/L), but lower than the observed by Fillon-Emery (11) (9.8 ± 0.7 μmol/L) and Licastro *et al*. (27) (12.51 ± 1.46 μmol/L) in adult and elderly DS patients, respectively, and by Bosco

et al. (26) (8.5 μmol/L) in a group of DS patients of several ages (median age: 13 years old). This may be due to the fact that in our study only five of the 56 DS individuals were adults.

MTHFR is one of the key enzymes in the folate-dependent remethylation of Hcy to methionine. The C677T polymorphism was associated with increased concentrations of Hcy in several studies (5,9,10,27). In DS patients, increased Hcy concentrations were observed in elderly DS subjects with a *MTHFR* 677TT genotype (27). However, Fillon-Emery *et al*. (11) found no difference in Hcy concentrations according to the *MTHFR* C677T genotype in adults with trisomy 21, but observed an increase in Hcy concentrations in the presence of genotypes *MTHFR* 1298 AA and AC as compared to the mutated homozygote (CC) in a group of patients taking folic acid supplements. Studies in other populations have not detected any significant differences in Hcy concentrations between A1298C genotypes (29,30). However, elevated Hcy concentrations have been reported for the double heterozygote 677CT / 1298AC (28). In our study on DS patients, the Hcy concentrations did not differ among the *MTHFR* genotypes, according to the results of a Brazilian study on healthy children (30).

The frequency of the *MTHFR* 677TT genotype observed by us (16.07%) was elevated compared to other studies on the Brazilian population (~10%) (30,31), but close to those observed by Hobbs *et al*. (2002) (15%) and Fillon-Emery *et al*. (2004) (12.8%) in DS patients. With regard to the *MTHFR* A1298C polymorphism, the prevalence of individuals which were homozygous 1298CC in this study (8.93%) was higher than that observed in a large group of healthy Brazilian children (5.5%) (30) but close to the one observed in French DS patients (7.7%) (11).

Linkage disequilibrium (LD) between *MTHFR* 677T and 1298C alleles has been reported (32,33). However, this LD is not complete, once there are studies showing the presence of some individuals with the rare haplotype T-C (34,35). In this study, no individual with a T-C haplotype was observed, confirming negative selection of this haplotype.

The frequency of the *MTR* 2756GG genotype (5.36%) in our DS patients was higher than the one observed in French DS patients (2.6%) (11). The contribution of the *MTR* A2756G polymorphism to Hcy concentrations is not yet well clarified. This polymorphism has been investigated as a risk factor for some diseases, such as vascular disease, neural tube defects, coronary artery disease and DS, and the evaluation of its influence on Hcy concentrations becomes of great interest. However, most of the studies did not find any relationship between this polymorphism and alterations in the Hcy concentrations (15,16), and the few studies that observed this relationship are contradictory with regard to the allele involved. Harmon *et al*. (12) analyzed the relationship between the A2756G genotype and Hcy concentration in a cohort of 625 working men aged 30-49 years and presented evidence that the 2756AA genotype was associated with a modest increase in plasma Hcy concentrations. Similar results were observed in a prospective study of myocardial infarction (36). In a group of DS patients taking folic acid supplements, higher Hcy concentrations were also observed in individuals with the *MTR* 2756AA genotype compared to the *MTR* 2756AG genotype (11). On the other hand, Laraqui *et al*. (13) recently observed a significant contribution of the 2756G allele to a moderate increase in Hcy concentrations in patients with coronary artery disease. In the present study, the Hcy concentrations were significantly

increased in the *MTR* 2756AG heterozygous genotype compared to the *MTR* 2756AA wild-type genotype, which is in accordance to the observations of Laraqui *et al*. (13).

Genotype *RFC1* 80AAA was associated with elevated Hcy concentrations as compared to *RFC1* 80AG in a group of DS patients using vitamin supplementation (11). The influence of this polymorphism on Hcy concentrations was also observed in healthy individuals, but only when combined with the *MTHFR* C677T polymorphism (37).

Elevated Hcy concentrations may be prejudicial, once they have been associated with elevated risk of cardiovascular disease, neurodegenerative disorders, psychiatric disorders and decline of cognitive performance (13,25,38). Guéant *et al*. (25) observed that lower intelligence quotient (IQ) was associated to higher Hcy concentrations in DS patients, and this association was also observed in the presence of the *MTHFR* T allele alone and in combination with the *Transcobalamin* (*TCN*) 776G polymorphic allele. However, they not observed relationship between IQ and *MTHFR* A1298C, *MTR* A2756G and methionine synthase reductase (*MTRR*) A66G.

According to Hobbs *et al*. (4), the presence of genetic variants involved in the folate metabolism in DS individuals may confer them a survival advantage. These authors observed a preferential transmission of the *MTHFR* 677T allele from heterozygote fathers to DS children, and they suggested that this may represent a metabolic advantage, once the overexpression of the CβS enzyme in these individuals is associated with decreased Hcy, methionine and SAM concentrations. Hcy is essential to the folate-dependent DNA synthesis and to cellular methylation reactions, which are important to the fetal development. According to these authors, a maternal-fetal genotype interaction of the *MTHFR* C677T polymorphism would also provide a favorable balance of folate distribution between both DNA synthesis and cellular

methylation in DS individuals. The preferential transmission of the 677T allele from fathers to SD children was also observed by Rai *et al*. (39), confirming the hypothesis of Hobbs *et al*. (4).

In conclusion, our results suggest that the heterozygote genotype *MTR* 2756AG is associated with the increase in plasma Hcy concentrations in Brazilian patients with DS. However, due to the contradictory results found in different studies, this relationship should be confirmed in a larger sample.

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Table I. Mean Hcy concentrations according to genotypes for polymorphisms *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *RFC1* A80G.

Genotypes	n (%)	Homocysteine (µmol/L)[a]
***MTHFR* C677T**		
CC	23 (41.07)	5.83 ± 4.25
CT	24 (42.86)	5.14 ± 2.17
TT	3 (16.07)	3.59 ± 2.17
***MTHFR* A1298C**		
AA	37 (66.07)	5.10 ± 3.39
AC	14 (25)	5.49 ± 3.17
CC	5 (8.93)	4.86 ± 3.46
***MTR* A2756G**		
AA	39 (69.64)	4.71 ± 2.61
AG	14 (25)	6.93 ± 4.51
GG	3 (5.36)	3.05 ± 1.13
***RFC1* A80G**		
AAA	10 (18.2)	5.92 ± 5.96
AG[b]	38 (69.1)	4.90 ± 2.74
GGG	7 (12.7)	5.22 ± 2.21

[a] Mean ± SD.

[b] The genotyping method used in this study does not distinguish AAG from AGG individuals.

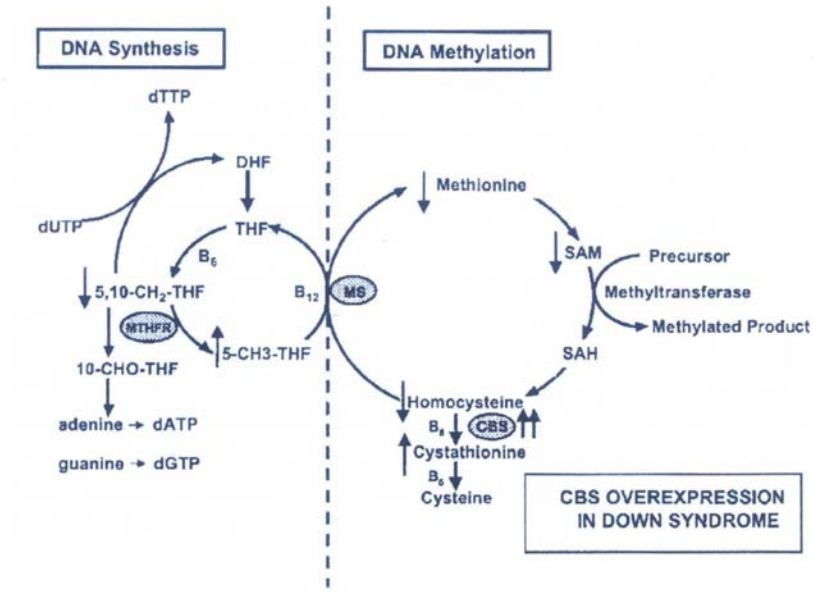

Figure 1. Folate metabolism in DS individuals. Arrows indicate direct and indirect alterations in metabolites, induced by *CβS* overexpression in DS individuals (4). Legend: dTTP = thymine; dUTP = uracyl; DHF = dihydrofolate; THF = tetrahydrofolate; 5,10-CH_2-THF = 5,10-methylenetetrahydrofolate; 5-CH_3-THF = 5-methyltetrahydrofolate; 10-CHO-THF = 10-formyl tetrahydrofolate; MTHFR = methylenetetrahydrofolate reductase; MS = methionine synthase; CβS = cystathionine β-synthase; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine.

CONCLUSÕES

III. CONCLUSÕES

1. A trissomia livre do cromossomo 21 é a causa mais freqüente da SD nos pacientes atendidos no Serviço Ambulatorial de Genética do Hospital de Base de São José do Rio Preto.
2. A presença de três ou mais alelos polimórficos para *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G e *RFC1* A80G, e concentração de Hcy plasmática acima de 4,99 μmol/L são fatores de risco maternos para a SD;
3. O genótipo heterozigoto *MTR* 2756AG está associado ao aumento da concentração de Hcy em indivíduos com SD.

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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

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----- Original Message -----
From: "down syndrome research and practice editor"
<dsrp-editor@downsed.org>
To: <erika@famerp.br>
Cc: "Joice Biselli" <joicebiselli@famerp.br>; "down syndrome research and practice editor" <dsrp-editor@downsed.org>
Sent: Wednesday, February 14, 2007 8:57 AM
Subject: Cytogenetic profile of Down syndrome cases consulted by a general genetics outpatient service in Brazil

Dear Prof. Bertelli,

Thank you for submitting your paper ("Cytogenetic profile of Down syndrome cases consulted by a general genetics outpatient service in Brazil") for publication in Down Syndrome Research and Practice. I am pleased to inform you that the paper has been reviewed and that we would like to accept it for publication.

To proceed with publication, we need you to complete, sign and return (post or fax) the attached agreement. When this is received we will proceed with the editorial process. This will first involve editing the paper to conform to the journal's style. We ask to agree to changes to the text made at this stage prior to laying out the final copy for print publication. Once agreed, the print layout will be prepared and at this point we will ask to approve a proof.

You paper will be published in an issue in volume 12 during 2007. If we can proceed with editing quickly enough we may include in issue 1 of volume that is due to be published within a few weeks.

We are making Down Syndrome Research and Practice fully Open Access with advance publication online. Therefore, as soon as the print layout proof is approved, we will publish online. The new web sites supporting these changes should become operational during March.

I hope this meets with your approval and look forward to hearing from you.

Regards,

Professor Sue Buckley OBE
The Down Syndrome Educational Trust
http://www.downsed.org/