2012

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MOLECULAR BIOLOGY REPORTS, DORDRECHT, v. 39, n. 10, pp. 9277-9284, OCT, 2012
http://www.producao.usp.br/handle/BDPI/42529

Mol Biol Rep (2012) 39:9277–9284
DOI 10.1007/s11033-012-1629-5

Genetic polymorphisms modulate the folate metabolism of Brazilian individuals with Down syndrome

J. M. Biselli · B. L. Zampieri · E. M. Goloni-Bertollo · R. Haddad · M. F. R. Fonseca · M. N. Eberlin · H. Vannucchi · V. M. Carvalho · E. C. Pavarino

Received: 23 August 2011 / Accepted: 16 April 2012 / Published online: 19 August 2012

Abstract Individuals with Down syndrome (DS) carry three copies of the *Cystathionine β-synthase* (*CβS*) gene. The increase in the dosage of this gene results in an altered profile of metabolites involved in the folate pathway, including reduced homocysteine (Hcy), methionine, S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM). Furthermore, previous studies in individuals with DS have shown that genetic variants in genes involved in the folate pathway influence the concentrations of this metabolism's products. The purpose of this study is to investigate whether polymorphisms in genes involved in folate metabolism affect the plasma concentrations of Hcy and methylmalonic acid (MMA) along with the concentration of serum folate in individuals with DS. Twelve genetic polymorphisms were investigated in 90 individuals with DS (median age 1.29 years, range 0.07–30.35 years; 49 male and 41 female). Genotyping for the polymorphisms was performed either by polymerase chain reaction (PCR) based techniques or by direct sequencing. Plasma concentrations of Hcy and MMA were measured by liquid chromatography-tandem mass spectrometry as previously described, and serum folate was quantified using a competitive immunoassay. Our results indicate that the *MTHFR* C677T, *MTR* A2756G, *TC2* C776G and *BHMT* G742A polymorphisms along with MMA concentration are predictors of Hcy concentration. They also show that age and Hcy concentration are predictors of MMA concentration. These findings could help to understand how genetic variation impacts folate metabolism and what metabolic consequences these variants have in individuals with trisomy 21.

Keywords Down syndrome · Folate · Genetic polymorphism · Homocysteine · Methylmalonic acid

J. M. Biselli · B. L. Zampieri · E. M. Goloni-Bertollo · E. C. Pavarino (✉)
Departamento de Biologia Molecular, Faculdade de Medicina de São José do Rio Preto (FAMERP), Unidade de Pesquisa em Genética e Biologia Molecular (UPGEM), Av. Brigadeiro Faria Lima, n.o 5416–Bloco U-6, São José do Rio Preto, SP CEP: 15.090-000, Brazil
e-mail: erika@famerp.br

R. Haddad · M. F. R. Fonseca · M. N. Eberlin
Departamento de Química, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil

H. Vannucchi
Departamento de Clínica Médica, Universidade de São Paulo (USP), Ribeirão Preto, SP, Brazil

V. M. Carvalho
Instituto de Pesquisa Fleury, São Paulo, São Paulo, Brazil

Introduction

Down syndrome (DS) is a chromosomal disorder caused by the presence of three copies of chromosome 21 [1]. The overexpression of genes involved in metabolic processes results in biochemical aberrations. The effect on the vast, integrated network of metabolic pathways leads to cellular dysfunction and contributes to the unique pathogenesis of DS [2].

Individuals with trisomy 21 present abnormalities in folate metabolism that are attributed to the additional copy of the *Cystathionineβ-synthase* (*CβS*) gene located on chromosome 21 [2, 3]. The *CβS* gene encodes an enzyme that catalyzes the condensation of homocysteine (Hcy) and serine to form cystathionine in the Hcy transsulfuration pathway (Fig. 1). Thus, overexpression of the *CβS* gene leads to an increase in the activity of this pathway. As a result of the enhanced condensation of Hcy and

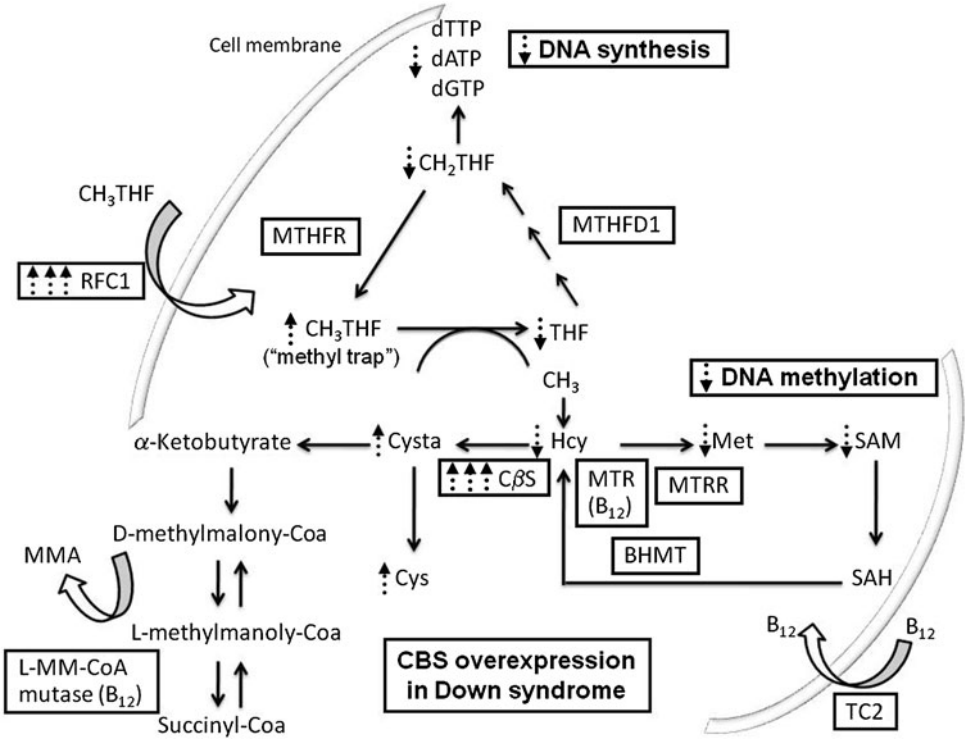

Fig. 1 Folate metabolism in Down syndrome individuals. *Arrows* indicate direct and indirect alterations in metabolites induced by cystathionine *β*-synthase (C*β*S) overexpression in DS individuals. *BHMT* Betaine-homocysteine methyltransferase, *CβS* Cystathionine *β*-synthase, *CH_3* Methyl, *CH_2THF* Methylenetetrahydrofolate, *CH_3THF* Methyltetrahydrofolate, *Cysta* cystathionine, *Cys* cysteine; *dATP* Deoxyadenosine 5′-triphosphate, *dGTP* Deoxyguanosine 5′-triphosphate, *dTTP* Deoxythymidine 5′-triphosphate, *Hcy* Homocysteine, *L-MM-Coamutase* L-methylmalonyl coenzyme A mutase, *Met* methionine, *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, *TCN2* Transcobalamin 2, *THF* Tetrahydrofolate

cystathionine, there is a reduction in the concentration of Hcy that is available for the remethylation reaction, which is catalyzed by the vitamin B_{12}-dependent enzyme methionine synthase (MTR). Simultaneously, the hyperactivity of the Hcy transsulfuration pathway leads to an accumulation of 5-methyltetrahydrofolate (5-MTHF) and a reduction in the conversion of 5-MTHF to tetrahydrofolate (THF). THF is the metabolically active form of folate and is required for de novo synthesis of nucleotides required for RNA and DNA synthesis. Consequently, a functional folate deficiency can be observed even in the presence of a normal or elevated concentration of folate [2]. In DS individuals, the extra copy of the *CβS* gene 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].

Furthermore, studies have shown that genetic variants involved in the folate metabolism can also affect the concentration of products derived from this metabolic process in individuals with DS [4, 5]. Previously, we evaluated the influence of the polymorphisms *Methylenetrahydrofolate reductase* (*MTHFR*) C677T and A1298C, *MTR*A2756G, and *Reduced folate carrier 1* (*RFC1*) A80G on Hcy concentration in 56 individuals with DS. We observed that the polymorphism A2756G of the *MTR* gene, which encodes a vitamin-B_{12}-dependent enzyme, influences Hcy concentrations in this population [4]. In the present study, we extend this analysis to 90 individuals with DS. Here, we investigate the association between twelve polymorphisms, *MTHFR* T1317C, *Methionine synthase reductase* (*MTRR*) A66G, *Transcobalamin 2* (*TCN2*) A67G and C776G, *Betaine homocysteine methyltransferase* (*BHMT*) G742A, *Methylenetetrahydrofolate dehydrogenase 1* (*MTHFD1*) G1958A, *CβS* T833C and 844ins68, *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *RFC1*A80G and the concentrations of serum folate, plasma Hcy, and plasma methylmalonic acid (MMA), which is an indicator of vitamin B_{12} status.

Materials and methods

This study's protocol was approved by both the Research Ethics Committee of São José do Rio Preto Medical School

(CEP-FAMERP, 165/2004) in São Paulo State, and the National Research Commission (CONEP) of Brazil. Ninety DS individuals with full trisomy 21 (median age 1.29, range 0.07–30.35 years-old; 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 family-informed consent forms were signed.

Fasting blood samples were collected for DNA extraction and separation of plasma and serum. Total plasma concentrations of Hcy and MMA were measured by liquid chromatography-tandem mass spectrometry [6–8]. Hcy concentrations greater than 15 μmol/L were considered to indicate hyperhomocysteinemia [9], and MMA concentrations greater than 0.5 μmol/L defined vitamin B_{12} deficiency (the conventional reference for vitamin B_{12} deficiency that was used was a concentration below 200 ng/L) [8, 10]. Folate concentrations were measured in serum using a competitive immunoassay (Immulite kit, DPC Medlab, Brazil), and concentrations less than 6.81 nmol/L were considered to indicate folate deficiency in accordance with the manufacturer's instructions.

Genomic DNA was extracted from peripheral blood mononuclear cells using either a protocol described by Miller et al. [11] or a GFX™ Genomic Blood DNA Purification Kit (GE Healthcare, USA). The genotypes of individuals at the polymorphisms *MTHFR*C677T, *MTR*A2756G, *RFC1*A80G, *CβS* 844ins68, *CβS* T833C, and *MTHFD 1*G1958A were determined using previously described methods [4, 12–15]. The variant C776G of the *TCN2* gene was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using the forward primer 5′-CATCAGAACAGTGCGAGAGG-3′ and the anti-sense primer described by Pietrzyk et al. [16]. The PCR products were digested with the *Scrf1* enzyme. The polymorphisms *MTRR*A66G, *TCN2*A67G and *BHMT*G742A were genotyped by allelic discrimination using *Taqman* probes (Applied Biosystems, Foster City, CA, USA, *TaqMan* SNP genotyping assays C__3068176_10; C__25967461_10 and C__11646606_20, respectively). *MTHFR*A1298C and T1317C variants were genotyped by direct sequencing as described elsewhere [17] with the exception that the purification process used in this study was performed using the enzymes exonuclease I and shrimp alkaline phosphatase (Fermentas Life Sciences, Brazil). This purification step was done in accordance with the manufacturer's instructions.

Statistical analyses

Concordance of genotype frequencies with Hardy–Weinberg equilibrium was evaluated by the Chi-squared test using the BioEstat program (version 5.0), except for the polymorphisms of *RFC1* and *CβS* genes located on chromosome 21. Because we used an RFLP-based method to analyze variants in *RFC1* and *CβS*, we were unable to distinguish between heteroallelic individuals carrying one or two copies of each allele.

Distributions of age and concentrations of plasma Hcy, serum folate and plasma MMA were all skewed; therefore, a logarithmic transformation was performed. To evaluate the effect of the polymorphisms on each biochemical parameter, linear regression analyses were performed. For Hcy analysis, the genotypes for each polymorphism (dominant and recessive models, separately), age, gender, folate concentration, and MMA concentration were used as predictors. For folate analysis, the predictors were the genotypes, age, gender, Hcy concentration, and MMA concentration. For MMA analysis, the genotypes, age, gender, folate concentration and Hcy concentration were used as predictors.

Statistical analyses were performed using the Minitab for Windows (Release 14) program. *P* values equal to or less than 0.05 were considered significant.

Results

The genotype frequencies of the polymorphisms (Table 1) are all in Hardy–Weinberg equilibrium. Hcy concentration was quantified in 87 plasma samples (mean 5.78 ± 3.20 μmol/L; median 4.75 μmol/L; range 1.26–20.90 μmol/L), and only two individuals presented hyperhomocysteinemia. Of the 83

Table 1 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 individuals (the gene is located on chromosome 21 and is in triplicate in individuals with Down syndrome)

[b] Genotyping one individual was not possible

individuals in which folate concentration was measured (mean: 19.42 ± 11.48 ng/mL; median 15.80 ng/mL; range 4.70–72.0 ng/mL), none were deficient for this vitamin. Based on MMA concentrations (mean 0.53 ± 0.80 μmol/L; median 0.25 μmol/L; range: 0.09–4.77 μmol/L), we concluded that 19 out of 85 individuals presented vitamin B_{12} deficiency.

Table 2 shows the mean values of Hcy, folate and MMA that are associated with the genotypes in the dominant and recessive models. Linear regression analyses considering the dominant effect of the variant alleles showed that the genotype *MTR* 2756 AG or GG is associated with increased Hcy concentration (coefficient 0.267; $P = 0.038$). Considering the recessive effect of the variant alleles, the genotypes *MTHFR* 677 TT (coefficient −0.420; $P = 0.022$), *TC2* 776 GG (coefficient −0.464; $P = 0.050$) and *BHMT* 742 AA (coefficient −0.394; $P = 0.036$) were associated with decreased Hcy concentration.

MMA concentration was inversely associated with age in both dominant (coefficient −0.063; $P = 0.001$) and recessive (coefficient −0.062; $P < 0.0001$) models. Both models also showed that MMA concentration and Hcy concentration are mutually predictive; MMA concentration is a predictor of Hcy concentration (Dominant model: coefficient 0.184; $P = 0.010$; Recessive model: coefficient 0.158; $P = 0.025$) and vice versa (Dominant model: coefficient 0.574; $P = 0.010$; Recessive model: coefficient 0.503; $P = 0.025$).

Discussion

Previous studies have shown that the presence of three copies of the *CβS* gene and the resulting decrease in the MTR-mediated reaction lead to disturbances in folate metabolism in individuals with DS. The result is a functional folate deficiency that may contribute to the metabolic pathology of this complex genetic disorder [2]. Although the role of abnormal folate metabolism in the DS phenotype is still unclear, Locke et al. [17] reported an association between the polymorphisms *RFC1* A80G and *MTHFR* A1298C and the occurrence of atrioventricular septal defect in individuals with DS. Moreover, it has been hypothesized that abnormal folate metabolism due to CBS overexpression could be related to the impaired DNA repair capability observed in DS [18].

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 folate metabolism [2, 19]. Previous studies have shown that genetic polymorphisms may influence plasma concentrations of Hcy either directly or by affecting plasma folate concentrations [5, 20–25]. Here, the results

Table 2 Mean values of Hcy, folate and MMA according to the genotypes in the dominant and recessive models

Polymorphism	Hcy (μmol/L)	Folate (ng/mL)	MMA (μmol/L)
Dominant model			
MTHFR C677T			
CC	6.43	21.47	0.54
CT and TT	5.27	17.86	0.53
MTHFR A1298C			
AA	5.48	19.23	0.59
AC and CC	6.20	19.90	0.48
MTHFR T1317C[a]			
TT	5.70	19.69	0.55
TC	6.97	18.31	0.45
MTR A2756G[b]			
AA	5.17	20.31	0.41
AG and GG	6.79	18.47	0.73
MTRR A66G			
AA	5.87	21.04	0.64
AG and GG	5.67	19.78	0.35
RFC1 A80G			
AA	6.57	23.29	0.50
AG and GG	5.37	19.79	0.19
TC2 A67G			
AA	5.98	19.53	0.54
AG and GG	5.15	19.67	0.50
TC2 C776G			
CC	5.85	17.53	0.47
CG and GG	5.24	21.54	0.55
MTHFD1 G1958A			
GG	6.10	19.47	0.45
GA and AA	5.29	19.76	0.59
BHMT G742A			
GG	6.60	20.10	0.65
AA and GA	4.96	19.21	0.43
CBS 844ins68[a]			
SS	5.95	20.09	0.52
SM	5.37	17.61	0.58
CBS T833C[a]			
TT	5.95	20.09	0.52
TC	5.37	17.61	0.58
Recessive model			
MTHFR C677T[b]			
CC and CT	6.08	20.16	0.58
TT	3.82	15.14	0.26
MTHFR A1298C			
AA and AC	5.62	19.35	0.54
CC	7.54	21.21	0.51
MTR A2756G			
AA and AG	5.84	19.60	0.49
GG	5.56	19.16	1.07

Table 2 continued

Polymorphism	Hcy (μmol/L)	Folate (ng/mL)	MMA (μmol/L)
MTRR A66G			
AA and AG	5.52	19.46	0.54
GG	7.87	20.20	0.48
RFC1 A80G			
AA and AG	5.99	19.88	0.57
GG	4.74	17.63	0.32
TC2 A67G			
AA and AG	5.84	19.49	0.54
GG	4.88	21.20	0.29
TC2 C776G[b]			
CC and CG	6.02	18.92	0.56
GG	3.57	26.47	0.22
MTHFD1 G1958A			
GG and GA	5.89	19.69	0.53
AA	5.57	19.10	0.54
BHMT G742A[b]			
GG and GA	6.04	19.81	0.57
AA	4.53	18.04	0.32

[a] The homozygous mutant genotype was not present in the sample

[b] Polymorphism associated with modulation of Hcy in the linear regression

indicate that the polymorphism *MTHFR* 677 TT is associated with a decrease in Hcy concentration. This was an unexpected finding as previous studies have shown that the *MTHFR* 677 T allele is associated with reduced MTHFR enzyme activity [26] and increased Hcy concentration [5, 20, 23–25]. Licastro et al. [5] observed elevated Hcy concentrations in elderly DS individuals with the *MTHFR* 677 TT genotype; however, in other studies that used both children and adults with DS, no association between the *MTHFR* C677T polymorphism and Hcy concentration was seen [19, 27]. Recently, Matteini et al. [28] observed an association between the *MTHFR* 677 TT genotype and decreased MMA concentration in patients with frailty syndrome. Furthermore, they showed that improved B_{12} status was associated with this genotype. While this finding is contrary to those of previous studies [23], it does corroborate with our results. One explanation for the discrepancies between the conclusions of these studies is that the examined populations were different from one another in age.

The results of the analysis of the association between *MTR* 2756 genotypes and Hcy concentrations are consistent with our previous observation of an association between the G allele and increased Hcy concentrations in DS individuals [4] and are also consistent with the results of other studies of non-DS individuals [21, 22]. MTR is an important vitamin B_{12}-dependent enzyme involved in folate metabolism. This enzyme catalyzes the transmethylation of Hcy to methionine in a reaction that utilizes methyl-tetrahydrofolate (*CH_3THF*) as a methyl group donor (Fig. 1). There are still no studies evaluating the difference between the enzymatic activity of the wild- and variant-types of MTR, but the association of the variant *MTR* 2756 G with increased Hcy concentration suggests that this polymorphism could result in an impaired enzyme [4, 21, 22].

To the best of our knowledge, this is the first study to investigate the influence of polymorphisms in genes encoding the BHMT and MTHFD1 proteins in individuals with DS. The former is a protein that remethylates Hcy to methionine using betaine as the methyl donor [29], and the latter is a protein known to catalyze the conversion of THF to the corresponding 10-formyl, 5,10-methenyl and 5,10-methylene derivatives [30] (Fig. 1). In the present study, no associations were found between Hcy, folate and MMA concentrations and the polymorphism *MTHFD1*G1958A. Our results, however, did show a connection between the variant *BHMT* 742 AA genotype and decreased plasma Hcy concentration. This polymorphism produces two distinct alloenzymes, which exhibit significant differences in Km values for Hcy and betaine [31]. The Km values of the variant alloenzyme are lower than those of the wild-type. The decreased Km of the alloenzyme may be responsible for the increased efficiency of Hcy remethylation, which requires betaine as a methyl group donor, [32], reducing Hcy concentration. Furthermore, studies have suggested a protective role of the homozygous AA genotype against neural tube defects [33] and cardiovascular disease [34], suggesting that the *BHMT* 742 G allele may have a deleterious effect on Hcy metabolism. Supporting this hypothesis and consistent with our findings, the *BHMT* 742 AA genotype was associated with lower Hcy concentrations in a previous study [33].

The TCN2 enzyme encodes a transport protein required for cellular uptake of B_{12}. This vitamin functions as co-factor of MTR, an important enzyme in folate metabolism that catalyzes the transmethylation of Hcy to methionine in a reaction that utilizes methyl-tetrahydrofolate (*CH_3THF*) as a methyl group donor (Fig. 1). In the present study, the *TCN2* 776 GG genotype was associated with reduced Hcy concentration. In agreement with our results, Brouns et al. [35] reported an association between the *TCN2* 776 G allele and a reduction in Hcy concentration observed in the amniotic fluid of pregnancies with a child having a complex birth defect an index for fetal metabolism. Previously published data show that the *TCN2* 776 G allele is associated with decreased transcription and decreased concentrations of cellular and plasma transcobalamin, the carrier protein that delivers vitamin B_{12} to cells [36]. This suggests

that the *TCN2* 776 G allele may encode a protein that is less efficient in delivering B_{12} to tissues. The negative impact of the *TCN2* 776 G allele on the intracellular availability of vitamin B_{12} is demonstrated by this variant's association with a lower mean concentration of the transcobalamin-vitamin B_{12} complex (holotranscobalamin) [36–38] in addition to increased MMA [39] and Hcy concentrations [37, 40]. Previous studies, however, did not find an association between this polymorphism and either holotranscolbalamin [41] or Hcy [38, 42]. Furthermore, an increase in Hcy concentration was shown to be associated with the heterozygous *TCN2* 776 CG genotype when compared to the homozygous genotypes [36]. The discrepancy between these studies may be the result of several variables, such as differences in age, ethnicity, gender of the study subjects, folate intake, vitamin B_{12} and B_6 intakes, genetic factors, and study design [4, 43, 44].

In this study, MMA concentration was inversely associated with age. Previous studies have shown that vitamin B_{12} concentration is inversely correlated with age [45, 46], corroborating with these findings, once MMA concentration reflects inversely the status of vitamin B_{12}. Vitamin B_{12} acts as a cofactor of the L-methylmalonyl-CoA mutase enzyme (Fig. 1), and its deficiency avoids the conversion of methylmalonyl-CoA to succinyl-CoA. This blockage diverts the substrate for MMA synthesis and leads to an increase in the concentrations of MMA and Hcy [47, 48]. Taken together, the results from previous studies that show a negative correlation between the concentration of vitamin B_{12} and Hcy [49] are in agreement with our observation of a positive correlation between the concentrations of MMA and Hcy.

Lastly, in July of 2004, the Brazilian government mandated that grain products must be fortified with folic acid [50]. The ensuing programs focused on increasing the dietary intake of folate to approximately 150 mg per person per day depending on the diet. This policy was implemented in order to reduce the incidence of neural tube defects. In the USA, fortification of grain products with folic acid led to a decrease in the mean Hcy concentration from 10.1 to 9.4 mmol/L [51]. Moreover, it is believed that the change in dietary intake of folate in the Brazilian population will significantly affect these values in the Brazilian population. We believe that such an observation would have an impact on the concentrations of folate and Hcy in our DS sample.

In conclusion, our results indicate that the *MTHFR* C677T, *MTR* A2756G, *TC2* C776G and *BHMT* G742A polymorphisms along with MMA concentration are predictors of Hcy concentration. They also show that age and Hcy concentration are predictors of MMA concentration. These findings could help to understand how genetic variation impacts folate metabolism and what metabolic consequences these variants have in individuals with trisomy 21.

Acknowledgments This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (04/15944-5 and 03/09931-5), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (302157/2008-5). J. M. Biselli and B. L. Zampieri are granted with a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (CGPP 046/2006).

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