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

Bruna Lancia Zampieri\textsuperscript{a}, Joice Matos Biselli\textsuperscript{a}, Eny Maria Goloni-Bertollo\textsuperscript{a}, Hélio Vannucchi\textsuperscript{b}, Valdemir Melechco Carvalho\textsuperscript{c}, José Antônio Cordeiro\textsuperscript{d} and Érika Cristina Pavarino\textsuperscript{a,*}

\textsuperscript{a}Unidade de Pesquisa em Genética e Biologia Molecular (UPGEM), Faculdade de Medicina de São José do Rio Preto (FAMERP), São José do Rio Preto, São Paulo, Brazil
\textsuperscript{b}Faculdade de Medicina de Ribeirão (USP), Ribeirão Preto, São Paulo, Brazil
\textsuperscript{c}Fleury, Centro de Medicina Diagnóstica, São Paulo, São Paulo, Brazil
\textsuperscript{d}Departamento de Epidemiologia e Saúde Coletiva da Faculdade de Medicina de São José do Rio Preto (FAMERP), São José do Rio Preto, São Paulo, Brazil

Abstract. Studies have shown that the maternal risk for Down syndrome (DS) may be modulated by alterations in folate metabolism. The aim of this study was to evaluate the influence of 12 genetic polymorphisms involved in folate metabolism on maternal risk for DS. In addition, we evaluated the impact of these polymorphisms on serum folate and plasma methylmalonic acid (MMA, an indicator of vitamin B\textsubscript{12} status) concentrations. The polymorphisms transcobalamin II (TCN2) c.776C>G, betaine-homocysteine S-methyltransferase (BHMT) c.742A>G, methylenetetrahydrofolate reductase (NAD(P)H) (MTHFR) c.677 C>T and the MTHFR 677C-1298A-1317T haplotype modulate DS risk. The polymorphisms MTHFR c.677C>T and solute carrier family 19 (folate transporter), member 1 (SLC19A1) c.80 A>G modulate folate concentrations, whereas the 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) c.66A>G polymorphism affects the MMA concentration. These results are consistent with the modulation of the maternal risk for DS by these polymorphisms.

Keywords: Down syndrome, genetic polymorphism, folate metabolism

1. Introduction

Down syndrome (DS), or trisomy 21 (MIM 190685), is the most common genetic disorder with a prevalence of 1 in 660 live births [27]. The only well-established risk factor for DS is advanced maternal age [7]. However, many DS children are born to mothers younger than 35 years, suggesting that other factors can also influence DS etiology. James et al. [48] hypothesized that pericentromeric hypomethylation, resulting from impaired folate metabolism secondary to a polymorphism on methylenetetrahydrofolate reductase (NAD(P)H) (MTHFR) gene, could impair chromosomal segregation and increase the risk for chromosome 21 nondisjunction in young mothers. Since then, several studies have revealed that polymorphisms in genes involved in the folate pathway modulate the maternal risk for DS [6,17,35,39,49] and the concentrations of metabolites involved in the folate pathway [14,30,43].

Folate metabolism vitally participates in the biosynthesis of nucleotides and S-adenosyl-methionine (SAM), the major methyl donor for DNA methylation reactions (Fig. 1). A folate deficiency has been associated with DNA hypomethylation, DNA damage, chromosomal instability, abnormal chromosome segregation and aneuploidy of chromosome 21 [45,47].
This study aimed to evaluate associations between 12 genetic polymorphisms, MTHFR c.677C>T, MTHFR c.1298A>C, MTHFR c.1317T>C, 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) c.2756 A>G, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) c.66A>G, cystathionine-beta-synthase (CBS) c.844ins68, CBS c.833T>C, solute carrier family 19 (SLC19A1), also known as reduced folate carrier – RFC1 c.80A>G, transcobalamin II (TCN2) c.776C>G, TCN2 c.67A>G, methenyltetrahydrofolate dehydrogenase (NADP+ dependent) 1, methylenetetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase (MTHFD1) c.1958G>A and betaine-homocysteine S-methyltransferase (BHMT) c.742G>A, and the maternal risk for DS. In addition, we evaluated the impact of the polymorphisms on serum folate and plasma methylmalonic acid (MMA, an indicator of the vitamin B_{12} status) concentrations.

2. Material and methods

The study protocol was approved by the Research Ethics Committee of São José do Rio Preto Medical School (CEP-FAMERP), State of São Paulo, and by the National Research Commission (CONEP), Brazil. Fasting peripheral blood samples were obtained from 105 women (case mothers) with karyotypically confirmed full trisomy 21 (translocation or mosaicism were not included) liveborn offspring and from 185 mothers with at least one healthy offspring and no history of miscarriage (control mothers). Case mothers were enrolled in the study during the following consultations: birth notification, maternal age assessment via a chi-square test using the BioEstat program, and the genotype frequencies were compared between case and control mothers by the likelihood ratio test. The Hardy-Weinberg (HW) equilibrium was assessed via a chi-square test using the BioEstat program, and the genotype frequencies were compared between case and control mothers by the likelihood ratio test. The relationship between the number of deleterious alleles for the 12 loci tested and the maternal risk for DS was assessed by logistic regression analysis. For this analysis, the sample was divided in two subsets (0–7 and 8–14 alleles for the total group and for the subgroup of women with maternal age \leq 35 years old) according to the median value of deleterious alleles (median = 7). Previously published data [2,9,16] and the results in
Table 1

| Polymorphism          | Allele deleterious |
|-----------------------|--------------------|
| MTHFR c.677C>T        | T                  |
| MTHFR c.1298A>C       | C                  |
| MTHFR c.1317T>C       | C                  |
| MTR c.2756A>G         | G                  |
| MTR c.66A>G           | G                  |
| SLC19A1 c.80A>G       | G                  |
| TCN2 c.67A>G          | G                  |
| TCN2 c.776C>G         | G                  |
| CBS c.844ins68        | I*                 |
| CBS c.833T>C          | C                  |
| MTHFD1 c.1958G>A      | A                  |
| BHMT c.742G>A         | A                  |

* The results of the CBS c.844ins68 genotypes were defined as I for the allele with the 68bp insertion and W for the wild-type allele.

Table 2

| Polymorphism          | DS mothers | Control mothers |
|-----------------------|------------|-----------------|
|                       | Genotype   | N  %            | N  %  P*          |
| MTHFR c.677C>T        | CC         | 40 38.1         | 94 50.8 0.09     |
|                       | CT         | 55 52.4         | 73 39.5          |
|                       | TT         | 10 9.5          | 18 9.7           |
| MTHFR c.1298A>C       | AA         | 51 48.6         | 101 55.2 0.56    |
|                       | AC         | 48 45.7         | 73 39.9          |
|                       | CC         | 6  5.7          | 9  4.9           |
| MTHFR c.1317T>C       | TT         | 89 84.8         | 158 86.3 0.55    |
|                       | TC         | 16 15.2         | 23 12.6          |
|                       | CG         | 9  4.9          | 4  2.2           |
|                       | GG         | 6  5.7          | 9  4.9           |
|                       | AG         | 38 36.2         | 49 26.5          |
|                       | GG         | 5  4.8          | 9  4.9           |
| MTR c.2756A>G         | AA         | 62 59.1         | 127 68.7 0.22    |
|                       | AG         | 36 34.3         | 65 35.1 0.91     |
|                       | AA         | 53 50.5         | 89 48.1          |
|                       | GC         | 16 15.2         | 31 16.8          |
| SLC19A1 c.80A>G       | AA         | 29 27.6         | 53 28.7 0.86     |
|                       | AG         | 48 45.7         | 88 47.6          |
|                       | GG         | 28 26.7         | 44 23.8          |
| CBS c.833T>C          | TT         | 83 79.1         | 145 78.4 0.26    |
|                       | TC         | 18 17.1         | 38 20.5          |
|                       | CC         | 4  4.8          | 2  1.1           |
| CBS c.844ins68*       | WW         | 83 79.1         | 145 78.4 0.26    |
|                       | WI         | 18 17.1         | 38 20.5          |
|                       | CC         | 4  4.8          | 2  1.1           |
|                       | AG         | 27 25.3         | 49 26.5          |
|                       | GC         | 16 15.2         | 31 16.8          |
|                       | GA         | 4  4.8          | 9  4.9           |
|                       | GG         | 6  5.7          | 20 10.8          |
|                       | AA         | 6  5.7          | 20 10.8          |
|                       | AG         | 53 50.5         | 89 48.1          |
|                       | GA         | 43 41.0         | 88 47.6          |
|                       | AA         | 57 54.3         | 77 41.6 0.10     |
|                       | GA         | 58 55.2         | 81 43.8          |
|                       | AA         | 13 12.4         | 32 17.3          |

* Likelihood Ratio Chi-Square test for genotypes.

The results of the CBS c.844ins68 genotypes were defined as W for the wild-type allele and I for the allele with the 68bp insertion.

3. Results

According to the likelihood ratio test, the genotype
Haplotype frequencies of the morphisms HW equilibrium in both groups, except for the poly-
omorphisms. The genotype frequencies were in frequencies were not different between DS and control groups (Table 2). The genotype frequencies were in HW equilibrium in both groups, except for the polymorphisms (Table 2). The genotype frequencies were in HW equilibrium in both groups, except for the polymorphisms (Table 2).

The median value of the folate concentration in DS mothers (12.2 ng/mL, 3.7–36.5) was significantly lower (P = 0.02) than in the case group (14.6 ng/mL; 5–74). Conversely, the median value of the MMA concentration in DS mothers (0.17 µmol/L, 0.05–0.81) was significantly (P < 0.0005) higher than in the case group (0.14 µmol/L, 0.05–0.81).

The haplotype frequencies of the MTHFR, TCN2 and CBS genes are presented in Table 3. The MTHFR gene exhibited linkage disequilibrium (LD) between the polymorphisms c.677C>T and c.1298A>C (LOD = 11.05; D' = 1.0), c.677C>T and c.1317T>C (LOD = 3.23; D' = 1.0) and c.1298A>C and c.1317T>C (LOD = 3.83; D' = 1.0). A significantly higher frequency of the C-A-T haplotype (wild-type alleles) was observed in the control group compared to the case group (P = 0.01). The TCN2 polymorphisms c.67A>G and c.776C>G are weakly linked (LOD = 2.46; D' = 0.63), whereas the CBS variants at positions 833 and 844 are strongly linked (LOD = 74.17; D' = 1.0). There was no difference in the haplotype frequencies for the TCN2 and CBS genes between the groups. The CBS haplotypes 833T/844I and 833C/844W were not present in either group.

When considering the dominant model using logistic stepwise regression analysis, maternal age (OR, 1.12; 95% CI, 1.075–1.174; P < 0.0005) and the MTHFR c.677 CT or TT genotypes (OR, 1.76; 95% CI, 1.011–3.073; P = 0.04) significantly contributed independently to DS risk. Maternal age (OR, 1.13; 95% CI, 1.080–1.183; P < 0.0005) and the TCN2 c.776 GG (OR, 2.45; 95% CI, 1.038–5.788; P = 0.04) and BHMT c.742 AA genotypes (OR, 0.26; 95% CI, 0.078–0.843; P = 0.02) were significant modifiers of DS risk under the recessive model (Table 4).

With respect to the factors that exert influence on biochemical parameters, folate concentrations below the 25th percentile were associated with the presence of MTHFR c.677 CT or TT (OR, 2.19; 95% CI, 1.223–3.920; P = 0.01), whereas MMA concentrations above the 75th percentile were associated with the MTRR c.66 AG or GG genotypes (OR, 1.98; 95% CI, 1.122–3.495; P = 0.02), both in the dominant model (Table 5).

When we analyzed only women ≤ 35 years old at conception, the most predictive independent risk factors for DS were maternal age (OR, 1.21; 95% CI, 1.098–1.321; P < 0.0005) and the MTHFR c.677 CT or TT genotypes (OR, 2.30; 95% CI, 1.135–4.661; P =
0.02) in the dominant model and the TCN2 c.776 GG genotype (OR, 3.47; 95% CI, 1.35–8.92; \( P = 0.01 \)) and BHMT c.742 AA genotypes (OR, 0.12; 95% CI, 0.015–0.974; \( P = 0.05 \)) in the recessive model (Table 4).

In women \( \leq 35 \) years old, folate concentrations below the 25th percentile were associated with the presence of MTHFR c.677 CT or TT (OR, 2.01; 95% CI, 1.05–3.83; \( P = 0.03 \)) in the dominant model and with the presence of SLC19A1 c.80 GG (OR, 2.20; 95% CI, 1.11–4.35; \( P = 0.02 \)) in the recessive model. The presence of MTRR c.66 AG or GG were associated with MMA concentrations above the 75th percentile (OR, 1.88; 95% CI, 1.02–3.44; \( P = 0.04 \)) in the dominant model (Table 5).

The median number of deleterious alleles did not differ between the groups, both in the total group and in the group of women \( \leq 35 \) years old.

4. Discussion

Although advanced maternal age at conception represents an important and well-established risk factor for DS [7], as confirmed in this study, the occurrence of DS births by young mothers suggests that other risk factors are also involved in the etiology of this syndrome. Abnormal folate metabolism has been identified as a maternal risk factor for DS in several populations [9]. This study reveals that polymorphisms in folate metabolism genes modulate the maternal risk for bearing a child with DS. This is the first study evaluating the role of MTHFR c.1317T>C, CBS c.833T>C, TCN2 c.67A>G, MTHFD1 c.1958G>A and BHMT c.742G>A polymorphisms in mothers of DS children in a Brazilian population. In addition, the influence of the polymorphisms BHMT c.742G>A and TCN2 c.67A>G on DS risk have never been studied until now.

The polymorphism TCN2 c.776 GG, which has been previously investigated for DS risk by our group in a smaller sample [21] and by Fintelmann-Rodrigues et al. [38], with negative results, was associated with increased DS risk in this study. Moreover, we observed that the TCN2 c.776 GG genotype influenced the risk for DS in both the total group and in the group with the conception age of women \( \leq 35 \) years. The presence of the TCN2 c.776 GG genotype has been shown to negatively affect the serum concentration of the TCN2 protein-vitamin B12 complex [28] and to be associated with low concentrations of SAM in childbearing-age women [43]. Considering that SAM is the major methyl donor for DNA methylation reactions, the variant TCN2 c.776C>G may influence maternal risk for DS by modifying the DNA methylation pattern. We are the first group to study the influence of the TCN2 c.67A>G polymorphism on maternal risk for DS, where no association was observed with DS risk. The LD between the variants TCN2 c.67A>G and c.776C>G observed in this study is consistent with a previous study [26].

Our group is the first to evaluate the role of the BHMT c.742G>A polymorphism on the risk of bearing a DS child, where an association between the BHMT c.742 AA genotype and decreased maternal risk for DS was observed. The BHMT protein catalyzes an alternative route of homocysteine (Hcy) remethylation (Fig. 1). The polymorphism produces two distinct alloenzymes, which exhibit significant differences in \( Km \) values for Hcy and betaine [10]. The \( Km \) values are lower for the variant alloenzyme compared to the wild-type. The low \( Km \) of the alloenzyme may be responsible for the increased efficiency of Hcy remethylation using betaine as a methyl group donor [42]. The decreased effect of the BHMT c.742A allele on DS risk could be expected when the maternal AA genotype...
was protective against neural tube defects (NTD) in the offspring [2,16]. Moreover, NTDs and DS are influenced by the same genetic factors involved in folate metabolism [13].

Polymorphisms in the *MTHFR* gene have been extensively analyzed for influences on folate and methyl metabolisms in maternal risk for DS. The *MTHFR* enzyme plays an important role in regulating DNA methylation reactions through the reduction of 5,10-methylentetrahydrofolate (5,10-MTHF) to 5-methylTHF (Fig. 1). A common polymorphism in the *MTHFR* gene, c.677 C>T, is known to decrease the affinity of the enzyme for its flavin adenine dinucleotide (FAD) cofactor, thereby decreasing the enzyme activity by approximately 35%, and the homozygous TT genotype reduces activity by 70% [41]. In this study, the presence of the *MTHFR* c.677 CT or TT genotypes was associated with increased maternal risk for DS, which corroborates previous associations between the *MTHFR* c.677 C>T polymorphism and the modulation of the maternal risk for DS [32,39,49]. Coppedè et al. [8] have observed an association between the *MTHFR* c.677 T allele and the occurrence of chromosome damage and missegregation events in mothers of DS individuals, supporting the role of this allele in the etiology of trisomy 21. Previously, these authors observed a significant increase in the rate of aneuploidy of chromosome 21 in these mothers [31].

The LD between the *MTHFR* polymorphisms c.677C>T, c.1298A>C and c.1317T>C observed in this study is consistent with the literature, which has illustrated LD between the *MTHFR* c.677 C>T and c.1298A>C [9,34]. Moreover, the silent polymorphism at position 1317 is near the one at 1298 position. The higher frequency of the *MTHFR* 677C-1298A-1317T haplotype in the control group confirms the protective maternal effect of these alleles against DS, which is indicated by the rare alleles 677T and 1298C that have been associated with increased maternal risk for DS in several studies [22,32,39,49]. In addition, this result corroborates the association between the 677T-1298C haplotype and the maternal risk for DS observed by Scala et al. [17].

*MTRR*, an enzyme codified by the *MTRR* gene, is responsible for the maintenance of the activated form of the MTR enzyme [29]. Several studies have observed an association between the *MTRR* c.66A>G polymorphism, alone and combined with other genetic variants, and DS risk and an elevated Hcy concentration [3, 5,6,36,40,44,49]. Additionally, a steady state kinetic analysis revealed a significant decrease in the affinity for MTRR accompanying a c.66A>G substitution, revealing a significant difference in the relative efficacies of the common *MTRR* polymorphism c.66A>G [15]. These findings further validate the association between the *MTRR* c.66 AG and GG genotypes and higher concentrations of MMA, likely a consequence of the variant enzyme activity that results in a higher Hcy concentration and consequently higher MMA concentrations.

The *SLC19A1* gene encodes an enzyme that participates in folic acid absorption, transporting for 5-methylTHF, an important determinant of folate concentration, to the interior of a variety of cells [50,52]. The *SLC19A1* gene is polymorphic at nucleotide 80 (A>G), and an assessment of the impact of this polymorphism on protein function has demonstrated a difference in its affinity for substrates and/or its efficiency in transport compared to the wild type enzyme [24]. Chang et al. [1] observed that *SLC19A1* c.80AA/ *MTHFR* c.677CT individuals exhibited higher plasma folate levels than *SLC19A1* c.80GG/ *MTHFR* c.677CT individuals, which corroborates our observations of lower folate concentrations in the presence of the *SLC19A1* c.80 GG genotype.

Because most polymorphisms, with the exception of *CBS* c.844ins68 and c.833 T>C, did not deviate from the HW equilibrium, our sample set was appropriately ascertained [18,19]. Departure from the HW equilibrium may have resulted from random selection or a small sample size.

A major strength of our study was the number of polymorphisms in folate metabolism genes investigated. Out of 12 polymorphisms, five have never been analyzed in a Brazilian population until now, including the *BHMT* c.742G>A and *TCN2* c.67A>G polymorphisms that influence the maternal risk for DS. A potential limitation of our study is that folate and MMA concentrations were not measured at the child’s delivery in both case and control mothers. Although the measurement of the concentrations at the time of conception would have been more relevant, the current quantification is a likely reflection of adult dietary patterns, once an adult’s dietary tends to have a similar pattern over the time. Also, the influence of the polymorphisms on MMA and folate concentrations was analyzed in the total group, including DS and control mothers, to investigate the polymorphism influence on the concentrations regardless of the presence of a DS child. The small size of the case group, which could reduce the power of the statistical analysis and complicate an investigation...
Fig. 1. Folate metabolism. BHMT = betaine–homocysteine S-methyltransferase; CBS = cystathionine-beta-synthase; CH₃ = methyl, 5,10-MTHF = 5,10-methylenetetrahydrofolate, 5-MTHF = 5-methyltetrahydrofolate; dATP = deoxyadenosine 5'-triphosphate; dGTP = deoxyguanosine 5'-triphosphate; dTTP = deoxythymidine 5'-triphosphate; Hcy = homocysteine; MMA = methylmalonic acid; MTHFD1 = methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase; MTHFR = methylenetetrahydrofolate reductase (NAD(P)H); MTR = 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR = 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; SLC19A1 = solute carrier family 19 (folate transporter), member 1; SAH = S-adenosyl-homocysteine; SAM = S-adenosylmethionine; TCN2 = transcobalamin II; THF = tetrahydrofolate.

of possible genotype combinations that may influence the maternal risk for DS, was an additional limitation of this study. However, studies that have investigated an association between folate gene polymorphisms and the risk of DS offspring have been conducted with relatively small sample sizes [9], primarily due to difficulties in the recruitment of these mothers, with significant results.

In conclusion, the results of this study indicate that the TCN2 c.776C>G, BHMT c.742A>G, and MTHFR c.677 C>T polymorphisms and the MTHFR c.677C-T haplotype modulate the risk for DS. The polymorphisms MTHFR c.677C>T and SLC19A1 c.80 A>G modulate folate concentrations, whereas the MTRR c.66A>G polymorphism affects MMA concentrations. These findings contribute to future research aimed at identifying metabolic interventions that will aid in preventing nondisjunction of the 21 chromosome. Future studies may benefit from sample sizes that are large enough to identify specific gene-gene interactions.

Acknowledgments

The authors are grateful to the mothers that participated in this study, to the Prof. Dr. Moacir F. Godoy for his help, to the Ding-Down workgroup (multidisciplinary group of health professionals - FAMERP) and to the FAMERP/FUNFARME for their collaboration in this work.

This study was supported by the FAPESP, CAPES and CNPq.

References

[1] A. Chango, N. Emery-Fillon, G.P. de Courcy, D. Lambert, M. Pfister, D.S. Rosenblatt and J.P. Nicolas, A polymorphism (80G>A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia, *Mol Genet Metab* 70 (2000), 310–315.

[2] A. Mostowska, K.K. Hozyasz, P. Wojcicki, M. Dziegielewksa and P.P. Jagodzinski, Associations of folate and choline metabolism gene polymorphisms with orofacial clefts, *J Med Genet* 47 (12) (2010), 809–815.

[3] A.P. Brandalize, E. Bandinelli, P.A. Dos Santos and L. Schuler-Faccini, Maternal gene polymorphisms involved in folate metabolism as risk factors for Down syndrome offspring in Southern Brazil, *Dis Markers* 29(2) (2010), 95–101.

[4] B.D.F. Guenther, C.A. Sheppard, P. Tran, R. Rozen, R.G. Matthews and M.L. Ludwig, The structure and properties of methylenetetrahydrofolate reductase from Escherichia coli suggest how folate ameliorates human hyperhomocysteinemia, *Nat Struct Biol* 6 (1999), 359–365.
in genes involved in folate metabolism as maternal risk factors for Down syndrome, *Am J Hum Genet* 67 (2000), 623–630.

[6] E. Pozzi, P. Vergani, L. Dalprà, R. Combi, D. Silvestri, F. Crosti, M. Dell’Orto and M.G. Valsecchi, Maternal polymorphisms for methylenetetrahydrofolate reductase and methionine synthetase reductase and risk of children with Down syndrome, *Am J Obstet Gynecol* 200(6) (2009), 636.e1–636.e6.

[7] E.G. Allen, S.B. Freeman, C. Druschel, C.A. Hobbs, L.A. O’Leary, P.A. Romitti, M.H. Royle, C.P. Torfs and S.L. Sherman, Maternal age and risk for trisomy 21 assessed by the origin of chromosome nondisjunction: a report from the Atlanta and National Down Syndrome Projects, *Hum Genet* 125 (2009), 41–52.

[8] F. Coppèdè, F. Miglieli, S. Bargagna, G. Siciliano, I. Antonucci, L. Stuppia, G. Palka and L. Migliore, Association of maternal polymorphisms in folate metabolizing genes with chromosome damage and risk of Down syndrome offspring, *Neurosci Lett* 449 (2009), 15–19.

[9] F. Coppèdè, The complex relationship between folate/homocysteine metabolism and risk of Down syndrome, *Mutat Res* 682(1) (2009), 54–70.

[10] F. Li, Q. Feng, C. Lee, S. Wang, L.L. Pelleymounter, I.J. van der Linden and M. den Heijer, Molecular genetic analysis of the human dihydrofolate reductase/homocysteine metabolism and risk of Down syndrome offspring, *Neurosci Lett* 449 (2009), 326–335.

[11] F.A. Hol, N.M.J. van der Put, M.P.A. Geurds and H.J. Blom, Molecular genetic analysis of the gene encoding the trifunctional enzyme MTHFD (methyltetrahydrofolate-dihydrogenase, methylenetetrahydrofolate-cyclohydrolase, formyltetrahydrofolate synthetase) in patients with neural tube defects, *Clin Genet* 53 (1998), 119–125.

[12] F.C. Parra, R.C. Amado, J.R. Lambertiucci, J. Rocha, C.M. Antunes and S.D. Penu, Color and genomic ancestry in Brazilians, *Proc Natl Acad Sci USA* 100 (2003), 177–182.

[13] G. Barkai, S. Arbusova, M. Berkenstadt, S. Heifetz and H. Gellekink, H.J. Blom, I.J. van der Linden and M. den Heijer, Frequency of Down’s syndrome and neural tube defects in the same family, *Lancet* 361(9366) (2003), 1331–1335.

[14] H. Gellekink, H.J. Blom, I.J. van der Linden and M. den Heijer, Molecular genetic analysis of the human dihydrofolate reductase gene: relation with plasma total homocysteine, serum and red blood cell folate levels, *Eur J Hum Genet* 15 (1) (2007), 103–109.

[15] H. Olteanu, T. Munson and R. Banerjee, Differences in the efficiency of reductive activation of methionine synthase and exogenous electron acceptors between the common polymorphic variants of human methionine synthase reductase, *Biochemistry* 41(45) (2002), 13378–13385.

[16] I. Morin, R. Platt, I. Weisberg, N. Sabbaghian, Q. Wu, T.A. Garrow and R. Rozen, Common variant in betaine-homocysteine methyltransferase (BHMT) and risk for spina bifida, *Am J Med Genet* 119A(2) (2003), 172–176.

[17] I. Scala, B. Granese, M. Sellitto, S. Salomé, A. Sammartino, A. Pepe, P. Mastroiacovo, G. Sebastiani and G. Andria, Analysis of seven maternal polymorphisms of genes involved in homocysteine/folate metabolism and risk of Down syndrome offspring, *Genet Med* 8(7) (2006), 409–416.

[18] J. Wiltke-Thompson, A. Pluzhnikov and N.J. Cox, Rational inferences about departures from Hardy-Weinberg equilibrium, *Am J Hum Genet* 76 (2005), 967–986.

[19] J. Xu, A. Turner, J. Little, E.R. Bleeker and D.A. Meyers, Positive results in association studies are associated with departure from Hardy-Weinberg equilibrium: hint for genotyping error? *Hum Genet* 111(6) (2002), 573–574.

[20] J.J. Pietrzyk and M. Bik-Multanowski, 776C>G polymorphism of the transcobalamin II gene as a risk factor for spina bifida, *Mol Genet Metab* 80 (2003), 364.

[21] J.M. Biselli, D. Bramati, V.F. Frigeri, B.L. Zampieri, E.M. Goloni-Bertollo and E.C. Pavarino-Bertelli, A80G polymorphism of reduced folate carrier 1 (RFC1) and C776G polymorphism of transcobalamin 2 (TCN2) genes in Down’s syndrome etiology, *São Paulo Med J* 126(6) (2008), 329–332.

[22] J.M. Biselli, E.M. Goloni-Bertollo, B.L. Zampieri, R. Haddad, M.N. Eberlin and E.C. Pavarino-Bertelli, The MTR A2756G polymorphism is associated with an increase of plasma homocysteine concentration in Brazilian individuals with Down syndrome, *Braz J Med Biol Res* 41(1) (2008), 34–40.

[23] J.R. Whetstone, A.J. Gifford, T. Witt, Y.X. Liu, R.M. Flaitley, M. Norris, M. Haber, J.W. Taub, Y. Ravindranath and L.H. Matherly, Single nucleotide polymorphisms in the human reduced folate carrier: characterization of a high-frequency GA variant at position 80 and transport properties of the His(27) and Arg(27) carriers, *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 7(11) (2001), 3416–3422.

[24] K. Yamada, Z. Chen, R. Rozen and R.G. Matthews, Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase, *Proc Natl Acad Sci U S A* 8(26) (2001), 14853–14858.

[25] K.J.A. Lievers, L.A. Afman and I.J. Klijtmans, Polymorphisms in the Transcobalamin Gene: Association with Plasma Homocysteine in Healthy Individuals and Vascular Disease Patients, *Clin Chem* 48(9) (2002), 1383–1389.

[26] K.L. Jones, *Smith’s Recognizable Patterns of Human Malformation*, (6th ed.), Philadelphia: Elsevier Saunders, 2006.

[27] K.M. von Castel-Dunwoody, G.P. Kauwell, K.P. Shelnutt, J.D. Wolthers and N.S. Scrutton, Protein interactions in the etiology, *Am J Obstet Gynecol* 200(6) (2009), 636.e1–636.e6.

[28] K.R. Wolthers and N.S. Scrutton, Protein interactions in the human methionine synthase-methionine synthase reductase complex and implications for the mechanism of enzyme reactivation, *Biochemistry* 46 (2007), 6696–6709.

[29] L. Laus, S. Liu, E.D. Ciappio, L.D. Parnell, J.B. Mason, K.L. Tucker and J.W. Crott, Associations between single nucleotide polymorphisms in folate uptake and metabolizing genes with blood folate, homocysteine, and DNA uracil concentrations, *Am J Clin Nutr* 88(4) (2008), 1149–1158.

[30] L. Migliore, G. Boni, R. Bernardini, F. Trippi, R. Colognato, I. Fontana, F. Coppédè and I. Sbrana, Susceptibility to chromosome malsegregation in lymphocytes of women who had a Down syndrome child in young age, *Neurobiol Aging* 27(5) (2006), 710–716.

[31] L.R. da Silva, N. Vergani, C. Galdieri Lde, M.P. Ribeiro Porto, S.B. Longhitano, D. Brunoni, V. D’Almeida and A.B. Alvarez Perez, Relationship Between Polymorphisms in Genes Involved in Homocysteine Metabolism and Maternal Risk for
Down Syndrome in Brazil, *Am J Med Genet* **135A**(3) (2005), 263–267.

[33] M. Födinger, J. Dierkes, S. Skoupy, C. Röhrer, W. Hagen, H. Putttinger, A.C. Hauser, A. Vychyiil and G. Sander-Plassmann, Effect of glutamate carboxypeptidase II and reduced folate carrier polymorphisms on folate and total homocysteine concentrations in dialysis patients, *J Am Soc Nephrol* **14**(S) (2003), 1314–1319.

[34] M. Shi, D. Caprau, P. Romitti, K. Christensen and J.C. Murray, Genotype frequencies and linkage disequilibrium in the CEPH human diversity panel for variants in folate pathway genes MTHFR, MTHFD, MTRR, RFC1, and GCP2, *Birth Defects Res A Clin Mol Teratol* **67** (2003), 545–549.

[35] M.F. Sadiq, E.A. Al-Refai, A. Al-Nasser, M. Khassawneh and M.K. El Awady, Methylenetetrahydrofolate reductase polymorphisms C677T and A1298C as maternal risk factors for Down syndrome in Jordan, *Genet Test Mol Biomarkers* **15**(1–2) (2011), 51–57.

[36] M.L. Martínez-Frian, B. Pérez, L.R. Desviat, M. Castro, F. Leal, L. Rodríguez, E. Mansilla, M.L. Martínez-Fernández, E. Bermejo, E. Rodríguez-Pinilla, D. Prieto and M. Ugarte, ECEMC Working Group, Maternal polymorphisms 677C-T and 1298A-C of MTHFR, and 66A-G MTRR genes: is there any relationship between polymorphisms of the folate pathway, maternal homocysteine levels, and the risk for having a child with Down syndrome? *Am J Med Genet A* **140** (2006), 987–997.

[37] M.P.S. Alvarenga, E.C. Pavarino-Bertelli and E.M. Goloni-Berto, Comparing the Identification for the of the MTHFR A1298C, *J Biomol Tech* **19**(2) (2008), 103–105.

[38] N. Fintelman-Rodrigues, J.C. Corrêa, J.M. Santos, M.M. Pimentel and C.B. Santos-Reboças, Investigation of CBS, MTR, RFC-1 and TC polymorphisms as maternal risk factors for Down syndrome, *Dis Markers* **26**(4) (2009), 155–161.

[39] N.A. Meguid, A.A. Darul, M. Khass, L.E. Hessieny, A. Ezzat and M.K. El Awady, Methylenetetrahydrofolate reductase polymorphism as a risk factor in Egyptian mothers with Down syndrome children, *Dis Markers* **24**(1) (2008), 19–26.

[40] P. Bosco, R.M. Guent-CRodriguez, G. Anello, C. Barone, F. Namour, F. Caraci, A. Roman, C. Romano and J.L. Guant, Methionine synthase (MTR) 2756 (A > G) polymorphism, double heterozygosity methionine synthase 2756 AG/methionine synthase reductase (MTRR) 66 AG, and elevated homocysteine are three risk factors for having a child with Down syndrome, *Am J Med Genet A* **121** (2003), 219–224.

[41] P. Frosst, H.J. Blom, R. Milos, P. Goyette, C.A. Sheppard, R.G. Matthews, G.H. Boers, M. den Heijer, L.A.J. Kluitmans, L.P. van den Heuve and R. Rozen, A candidate genetic risk factor for vascular disease: a common mutation in Methylene tetrahydrofolate reductase, *Nat Genet* **10**(1) (1995), 111–113.

[42] P.M. Ueland, HCholine and betaine in health and disease, *H Intérêt Metab Dis* **34**(1) (2011), 3–15.

[43] P.R. Barbosa, S.P. Stabler, R. Trentin, F.R. Carvalho, A.D. Luchessi, Hirata R.D., M.H. Hirata, R.H. Allen and E.M. Guerra-Shinohara, Evaluation of nutritional and genetic determinants of total homocysteine, methylmalonic acid and S-adenosylmethionine/S-adenosylhomocysteine values in Brazilian childbearing-age women, *Clinica Chimica Acta* **388**(1–2) (2008), 139–147.

[44] Q.H. Yang, L.D. Botto, M. Gallagher, J.M. Friedman, C.L. Sanders, D. Koontz, S. Nikolova, J.D. Erickson and K. Steinberg, Prevalence and effects of gene-gene and gene-nutrient interactions on serum folate and serum total homocysteine concentrations in the United States: findings from the third National Health and Nutrition Examination Survey DNA Bank, *Am J Clin Nutr* **88** (2008), 232–246.

[45] S. Beestra, P. Thomas, C. Salsbury, J. Turner and M. Fench, Folic acid deficiency increases chromosomal instability, chromosome 21 aneuploidy and sensitivity to radiation-induced micronuclei, *Mutat Res* **578** (2005), 317–326.

[46] S.A. Miller, D.D. Dykes and H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, *Nucleic Acids Res* **16**(3) (1988), 1215.

[47] S.J. James, I.P. Pogribny, B.J. Miller, S. Jernigan and S. Melnyk, Mechanisms of DNA damage, DNA hypomethylation, and tumor progression in the folate/methyl-deficient rat model of hepatocarcinogenesis, *J Nutr* **133** (2003), 3740S–37407S.

[48] S.J. James, M. Pogribna, I.P. Pogribny, S. Melnyk, R.J. Hine, J.B. Gibson, P.Y. Dafoya, D.H. Swenson, V.I. Wilson and D.W. Gaylor, Abnormal folate metabolism and mutation in the methylenetetrahydrofolate reductase gene may be maternal risk factors for Down syndrome, *Am J Clin Nutr* **70**(4) (1999), 495–501.

[49] S.S. Wang, F.Y. Qiao, L. Feng and J.J. Lv, Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome in China, *J Zhejiang Univ Sci B* **9**(2) (2008), 93–99.

[50] T.T. Nguyen, D.L. Dyer, D.D. Dunning, S.A. Rubin, K.E. Grant and H.M. Said, Human intestinal folate transport: cloning, expression, and distribution of complementary RNA, *Gastroenterology* **112**(3) (1997), 783–791.

[51] V.M. Carvalho and F. Kok, Abnormal folate metabolism and mutation in the methylenetetrahydrofolate reductase gene may be maternal risk factors for Down syndrome, *Am J Clin Nutr* **70**(4) (1999), 495–501.

[52] Z. Hou and L.H. Matherly, Oligomeric structure of the human chromosome 21 aneuploidy and sensitivity to radiation-induced micronuclei, *Mutat Res* **578** (2005), 317–326.