Disease Markers 28 (2010) 293–298
DOI 10.3233/DMA-2010-0704
IOS Press

Functional inference of methylenetetrahydrofolate reductase gene polymorphisms on enzyme stability as a potential risk factor for Down syndrome in Croatia

Jadranka Vranekovića, Ivana Babić Božovića, Nada Starčević Čizmarevića, Alena Buretić-Tomljanovića, Smiljana Ristića, Oleg Petrovićb, Miljenko Kapovića and Bojana Brajenović-Milića,*

[a]*Department of Biology and Medical genetics, School of Medicine, University of Rijeka, Rijeka, Croatia*
[b]*Department of Obstetrics and Gynecology, Rijeka University Hospital, Rijeka, Croatia*

Abstract. Understanding the biochemical structure and function of the methylenetetrahydrofolate reductase gene (*MTHFR*) provides new evidence in elucidating the risk of having a child with Down syndrome (DS) in association with two common *MTHFR* polymorphisms, C677T and A1298C. The aim of this study was to evaluate the risk for DS according to the presence of *MTHFR* C677T and A1298C polymorphisms as well as the stability of the enzyme configuration. This study included mothers from Croatia with a liveborn DS child ($n = 102$) or DS pregnancy ($n = 9$) and mothers with a healthy child ($n = 141$). *MTHFR* C677T and A1298C polymorphisms were assessed by PCR-RFLP. Allele/genotype frequencies differences were determined using χ^2 test. Odds ratio and the 95% confidence intervals were calculated to evaluate the effects of different alleles/genotypes. No statistically significant differences were found between the frequencies of allele/genotype or genotype combinations of the *MTHFR* C677T and A1298C polymorphisms in the case and the control groups. Additionally, the observed frequencies of the stable (677CC/1298AA, 677CC/1298AC, 677CC/1298CC) and unstable (677CT/1298AA, 677CT/1298AC, 677TT/1298AA) enzyme configurations were not significantly different. We found no evidence to support the possibility that *MTHFR* polymorphisms and the stability of the enzyme configurations were associated with risk of having a child with DS in Croatian population.

Keywords: Down syndrome, enzyme configuration, *MTHFR*, polymorphisms

1. Introduction

Down syndrome (DS) or trisomy 21 is the most prevalent autosomal trisomy detected at birth [2]. Approximately 95% of DS cases are of maternal origin, and the prevalence for DS increases with age of the mother [12]. A number of studies from distinct geographic regions have evaluated the role of an abnormal folate pathway as a possible risk for DS. The folate pathway plays an important function in DNA synthesis as well as in DNA methylation. Accurate methylation of genomic DNA, particularly in the centromeric region of the chromosome, is crucial for stabilization and proper separation of the chromosomes during cellular

*Corresponding author: Bojana Brajenović – Milić, Department of Biology and Medical genetics, School of Medicine, University of Rijeka, Braće Branchetta 20, 51000 Rijeka, Croatia. Tel.: +38 551 651128; Fax: +38 551 678896; E-mail: bojana@medri.hr.

ISSN 0278-0240/10/$27.50

division [14,17].

S-adenosylmethionine (SAM) is the major methyl donor for DNA, protein and lipid methylation, and its synthesis requires the activity of methylenetetrahydrofolate reductase (MTHFR), which catalyzes the conversion of 5,10 methylenetetrahydrofolate to 5-methyltetrahydrofolate, the major methyl donor for remethylation of homocysteine to methionine [15]. Polymorphisms in the human *MTHFR* gene (C677T and A1298C) have been identified, which result in a reduction of MTHFR enzymatic activity [21,35].

The association between the *MTHFR* C677T polymorphism and risk for having a child with DS was first reported ten years ago [20]. Since the initial study, many researchers have examined one or both *MTHFR* polymorphisms as a potential risk factor for DS, but these studies have yielded contradictory results [1,3–5, 7–11,18,20,24,26,27,29,30,33,36,39]. Interestingly, a recent study by Ulvik et al. demonstrated that the levels of total homocysteine (tHcy) associated with the various MTHFR combinations show significant differences; the levels of tHcy increase as the levels of folate decrease. Lowest serum folat and highest tHcy was found for the 677TT/1298AA genotype combinations [34]. These findings led Martinez-Frias to speculate that understanding the *MTHFR* gene structure and the mechanisms by which folate may reduce homocysteine levels in humans has provided a rationale for the conflicting epidemiological observations of the studies on the *MTHFR* C677T and A1298C polymorphisms and the risk of having a child with DS [23].

The aim of the present study was to assess the risk for DS in association with (i) maternal polymorphisms of *MTHFR* (C677T and A1298C) and (ii) stable or unstable MTHFR enzyme dimer configurations as defined by Ulvik et al. [34].

2. Subjects and methods

Mothers with DS children were recruited from the Downs syndrome associations in the larger cities of Croatia (Rijeka, Zagreb, Pula, Zadar, Split, Karlovac, Čakovec and Osijek). Blood samples were collected from a total of 111 women who had either a liveborn child with DS ($n = 102$) or a trisomy 21 pregnancy that was terminated ($n = 9$). All DS cases were full trisomy 21; cases with translocation or mosaicism were not included in the study. The karyotypes of the parents were confirmed as normal. The control group consisted of women who screened negative at the second trimester screening test for DS performed in the Department of Biology and Medical Genetics, School of Medicine, University of Rijeka. The control mothers gave birth to healthy children, and had no previous miscarriages or abnormal pregnancies; a total of 141 control DNA samples were collected from this group. The control and case groups were age-matched and of the same ethnicity (Caucasian). The mean age of the case and control groups was 31.3 ± 6.4 (range 16–43) and 31.4 ± 5.0 (range 18–41) years, respectively ($P = 0.867$). Maternal age was calculated as the age of the mother at the time of conception of the DS child for the case group or the child who was born after maternal serum screening for the control group.

For the purpose of this study, we marked *MTHFR* genotype combinations according to the stability of the enzyme configurations as follows [34]:

> Stable enzyme configurations = 1 for genotypes 677CC/1298AA, 677CC/1298AC, and 677CC/1298CC.
> Unstable enzyme configurations = 2 for genotypes 677CT/1298AA, 677CT/1298AC, and 677TT/1298AA.

The Ethical Committee of the Medical School, University of Rijeka, approved the study. Written informed consent was obtained from all participants in the study.

Genomic DNA was extracted from 3 ml of EDTA-treated blood using the QIAamp DNA blood FlexiGene DNA Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Genotyping analyses were performed using the PCR- RFLP technique. Genotyping for the *MTHFR* C677T and A1298C polymorphisms was performed using the method published by Coppede et al. [10]. Digested PCR fragments were separated by electrophoresis on a 3% agarose gel containing ethidium bromide.

Allele frequencies were calculated by gene counting, and deviations from those estimated by the Hardy-Weinberg equilibrium were examined using the χ^2 test. Group differences in genotype and allele were analyzed for statistical significance using the χ^2 test. Odds ratio (OR) and the 95% confidence intervals (CI) were calculated to for heterozygous and homozygous mutant genotypes was compared with the wild type. Results were considered statistically significant at $P < 0.05$.

3. Results

We first examined the distributions of the *MTHFR* C677T and A1298C genotypes in the case group of

Table 1
Genotype and allele frequencies of the *MTHFR* C677T and A1298C polymorphisms in women with a DS child (cases) and controls

Polymorphism	Genotype	Cases (%) N = 111	Controls (%) N = 141	OR (95%CI)	P
MTHFR C677T	CC	49 (44)	66 (47)	1.0 Reference	
	CT	49 (44)	64 (45)	1.03 (0.61–1.74)	0.908
	TT	13 (12)	11 (8)	1.60 (0.65–3.85)	0.303
	CT + TT	62 (56)	75 (53)	1.11 (0.67–1.83)	0.674
Allele	C	147 (66)	196 (70)	1.0 Reference	
	T	75 (34)	86 (30)	1.17 (0.80–1.71)	0.457
MTHFR A1298C	AA	48 (43)	63 (45)	1.0 Reference	
	AC	56 (51)	68 (48)	1.08 (0.64–1.81)	0.768
	CC	7 (6)	10 (7)	0.91 (0.32–2.59)	0.873
	AC+CC	63 (57)	78 (55)	1.06 (0.64–1.75)	0.819
Allele	A	152 (68)	194 (69)	1.0 Reference	
	C	70 (32)	88 (31)	1.01 (0.69–1.48)	0.985

Table 2
Combined genotypes frequencies of the *MTHFR* C677T and A1298C polymorphisms in women with a DS child (cases) and controls

MTHFR C677T	MTHFR A1298C	Cases (%) N = 111	Controls (%) N = 141	OR (95%CI)	P
CC	AA	12 (11)	17 (12)	1.0 Reference	
CT	AA	23 (21)	35 (25)	0.93 (0.37–2.30)	0.938
TT	AA	13 (12)	11 (8)	1.67 (0.56–4,98)	0.514
CC	AC	30 (27)	39 (27)	1.08 (0.45–2.62)	0.974
CT	AC	26 (23)	29 (20)	1.27 (0.51–3.15)	0.775
TT	AC	0	0		
CC	CC	7 (6)	10 (7)	0.99 (0.29–3.34)	0.766
CT	CC	0	0		
TT	CC	0	0		

women with a DS child and in the control group (Table 1). No significant difference in these distributions was observed when compared with those predicted from the Hardy-Weinberg equilibrium for either cases or controls. We analyzed the frequencies of the *MTHFR* C677T and A1298C alleles and genotypes in both groups, and no statistically significant differences in allele or genotype distributions were detected. Table 2 shows the frequencies of the nine genotype combinations derived from the C677T and A1298C polymorphisms in the case and control groups. We did not detect any statistically significant differences between the frequencies of genotype combinations in the two groups. We also assessed the frequencies of the enzyme configurations (stable or unstable) in the two groups, and no significant differences were found in frequencies between the case and the control group (Table 3).

4. Discussion

Here we present the first study on the genotype and allele frequencies of the C677T and A1298C *MTHFR* polymorphisms in a group of mothers in Croatia who had DS pregnancies or a live born DS child. Our findings confirm the general profile of genotype and allele distributions for *MTHFR* C677T and A1298C polymorphisms in the European population [16,22,28,32, 37]. We did not find any association between *MTHFR* C677T polymorphism with the risk for having a child with DS. The results are in agreement with studies performed in Mediterranean countries [4–11,24,33]. An association of the *MTHFR* 677T allele with DS has also been found in North and South America, Asia, and Egypt [13,18,20,24,27,36]. Much more contradictory reports have been presented for the *MTHFR* A1298C polymorphism [3,4,10,13,24,27,30]. When the combined maternal *MTHFR* C677T/A1298C genotypes were considered, we failed to find any significant association with the risk for offspring with DS. The present results are consistent with the findings obtained in Sicily, France, Turkey, and Brazil [3–5,8,29]. As expected, the combined genotypes 677CT/1298CC, 677TT/1298CC, and 677TT/1298AC were not observed in the case group or in the controls. The findings support linkage disequilibrium between the *MTH-*

Table 3
Frequencies of MTHFR enzyme dimer configuration according to stability in women with a DS child (cases) and controls

Configuration	Genotype MTHFR C677T/A1298C	Cases (%) N = 111	Controls (%) N = 141	OR (95%CI)	P
Stable	CCAA, CCAC, CCCC	46 (41)	65 (46)	1.20 (0.73–1.99)	0.540
Unstable	CTAA, CTAC, TTAA	65 (59)	76 (54)		

FR C677T and A1298C polymorphisms [1,4,10,13,31, 35]. Some authors have discussed the possibility that these genotype combinations decrease embryonic viability because they were found in fetuses but rarely (677TT/1298CC) or not at all in live births [6,19,23].

Our second aim was to evaluate the possibility of the stability of the enzyme configuration as an indicator for risk of having a child with DS. To the best of our knowledge, this is the first report of that kind. Ulvik et al demonstrated that six distinct enzyme dimer configurations are generated from the three common *MTHFR* alleles (C-A, C-C, and T-A) [34]. Thus, depending on the C677T/A1298C *MTHFR* genotype, one or three of these configurations is present for any given individual. Some of these enzyme configurations are unstable, and significant differences were detected in tHcy levels. The 677TT/1298AA genotype is associated with a strong increase of tHcy and decrease of folate in serum. Theoretically, 100% of the MTHFR enzyme dimers in 677TT/1298AA subjects are in the unstable configuration, and these patients will be at particular risk when their folate status is low, as the mutant enzyme requires much higher levels of folate for stabilizing enzyme activity [34,38]. Although not to the extent observed with the 677TT/1298AA genotype, the 677CT/1298AA and 677CT/1298AC genotypes exhibit enzymes in the unstable form as well. Other *MTHFR* genotype combinations, such as 677CC/1298AA, 677CC/1298AC, and 677CC/1298CC, have one or three stable enzyme dimer configurations [34]. We postulated that mothers with a DS child have more likely the *MTHFR* genotype combinations, which lead to producing MTHFR enzyme with more or less amount of unstable enzyme dimer configurations than mothers who have healthy child. The findings from this study, however, did not show any statistically significant differences in the frequencies of unstable form of MTHFR enzyme between cases and controls. We are aware that the relatively small number of cases in both groups was a limiting factor for completely addressing the question posed. However, some features of the present study should be stressed. First, we had an age-matched case-control study with absolute uniformity in ethnicity, and the control mothers had a negative personal and family history. The variation in allele or genotype frequencies among different populations due to ethnic variability or different stratification in the case-control study design is always emphasized [9,25,40].

In conclusion, a follow-up study with a larger group of patients is required, but this would not be enough to definitively address the problem. In addition to the genotype, the information regarding personal folate status is important because the postulated risk for DS is expected if the woman has an unstable form of the MTHFR enzyme and low folate status at the same time. To further resolve the problem, the impact of folate status on MTHFR enzyme configurations should be investigated, not in mothers who have a DS child, but in her mother [9,23,25]. Unfortunately, this information is essentially impossible to obtain. On the other hand, the effect of the maternal folate status on enzyme configuration could be investigated as a risk factor for trisomy 21 survival.

Acknowledgements

This study was, in part, supported by a grant (No.062-0000000-1349) from the Ministry of Science, Education and Sports, Zagreb, Croatia. The authors are thankful to all the mothers who participated in the study as well as Down's Syndrome Association presidents who helped us in collecting samples.

References

[1] G.L. Acácio, R Barini, C.S. Bertuzzo, E.C. Couto, J.M. Annichino-Bizzacchi and W.P. Júnior, Methylenetetrahydrofolate reductase gene polymorphisms and their association with trisomy 21, *Prenat Diagn* **25** (2005), 1196–1199.

[2] S.E. Antonarakis, R. Lyle, E.T. Dermitzakis, A. Reymond and S. Deutsch, Chromosome 21 and down syndrome: from genomics to pathophysiology, *Nat Rev Genet* **5** (2004), 725–738.

[3] J.M. Biselli, E.M. Goloni-Bertollo, B.L. Zampieri, R. Haddad, M.N. Eberlin and E.C. Pavarino-Bertelli, Genetic polymorphisms involved in folate metabolism and elevated plasma concentrations of homocysteine: maternal risk factors for Down syndrome in Brazil, *Genet Mol Res* **7** (2008), 33–42.

[4] K. Boduroğlu, Y. Alanay, B. Koldan and E. Tunçbilek, Methylenetetrahydrofolate reductase enzyme polymorphisms as maternal risk for Down syndrome among Turkish women, *Am J Med Genet A* **15** (2004), 5–10.

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

[6] G. Callejón, A. Mayor-Olea, A.J. Jiménez , M.J. Gaitán, A.R. Palomares, F. Martínez, M. Ruiz and A. Reyes-Engel, Genotypes of the C677T and A1298C polymorphisms of the MTHFR gene as a cause of human spontaneous embryo loss, *Hum Reprod* **22** (2007), 3249–3254.

[7] B. Chadefaux-Vekemans, M. Coudé, F. Muller, J.F. Oury, A. Chabli, J. Jaïs and P. Kamoun, Methylenetetrahydrofolate reductase polymorphism in the etiology of Down syndrome, *Pediatr Res* **51** (2002), 766–767.

[8] A. Chango, N. Fillon-Emery, C. Mircher, H. Bléhaut, D. Lambert, B. Herbeth, S.J. James, M.O. Réthoré and J.P. Nicolas, No association between common polymorphisms in genes of folate and homocysteine metabolism and the risk of Down's syndrome among French mothers, *Br J Nutr* **94** (2005), 166–169.

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

[10] F. Coppede, G. Marini, S. Bargagna, L. Stuppia, F. Minichilli, I. Fontana, R. Colognato, G. Astrea, G. Palka and L. Migliore, Folate gene polymorphisms and the risk of Down syndrome pregnancies in young Italian women, *Am J Med Genet A* **140** (2006), 1083–1091.

[11] F. Coppede, F. Migheli, 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.

[12] H.S. Cuckle, Primary prevention of Down's syndrome, *Int J Med Sci* **2** (2005), 93–99.

[13] 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 A* **135** (2005), 263–267.

[14] S. Gopalakrishnan, B.A. Sullivan, S. Trazzi, G. Della Valle and K.D. Robertson, DNMT3B interacts with constitutive centromere protein CENP-C to modulate DNA methylation and the histone code at centromeric regions, *Hum Mol Genet* **18** (2009), 3178–3193.

[15] P. Goyette, A. Pai, R. Milos, P. Frosst, P. Tran, Z. Chen, M. Chan and R. Rozen, Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR), *Mamm Genome* **9** (1998), 652–656.

[16] R.M. Gueant-Rodriguez, J.L. Gueant, R. Debard, S. Thirion, L.X. Hong, J.P. Bronowicki, F. Namour, N.W. Chabi, A. Sanni, G. Anello, P. Bosco, C. Romano, E. Amouzou, H.R. Arrieta, B.E. Sanchez, A. Romano, B. Herbeth, J.C. Guilland and O.M. Mutchinick, Prevalence of methylenetetrahydrofolate reductase 677T and 1298C alleles and folate status: a comparative study in Mexican, West African, and European populations, *Am J Clin Nutr* **83** (2006), 701–707.

[17] R. Heit, J.B. Rattner, G.K. Chan and M.J. Hendzel, G2 histone methylation is required for the proper segregation of chromosomes, *J Cell Sci* **122** (2009), 2957–2968.

[18] C.A. Hobbs, S.L. Sherman, P. Yi, S.E. Hopkins, C.P. Torfs, R.J. Hine, M. Pogribna, R. Rozen and S.J. James, Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome, *Am J Hum Genet* **67** (2000), 623–630.

[19] P.A. Isotalo, G.A. Wells and J.G. Donnelly, Neonatal and fetal methylenetetrahydrofolate reductase genetic polymorphisms: an examination of C677T and A1298C mutations, *Am J Hum Genet* **67** (2000), 986–990.

[20] S.J. James, M. Pogribna, I.P. Pogribny, S. Melnyk, R.J. Hine, J.B. Gibson, P. Yi, D.L. Tafoya, D.H. Swenson, V.L. 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** (1999), 495–501.

[21] S.S. Kang, E.L. Passen, N. Ruggie, P.W. Wong and H. Sora, Thermolabile defect of methylenetetrahydrofolate reductase in coronary artery disease, *Circulation* **88** (1993), 1463–1469.

[22] I. Lovricevic, B.D. Franjic, M. Tomicic, N. Vrkic, D. De Syo, N. Hudorovic, Z. Sonicki and R. Loncar, 5,10-Methylenetetrahydrofolate reductase (MTHFR) 677 C → T genetic polymorphism in 228 Croatian volunteers, *Coll Antropol* **28** (2004), 647–654.

[23] M.L. Martinez-Frias, The biochemical structure and function of methylenetetrahydrofolate reductase provide the rationale to interpret the epidemiological results on the risk for infants with Down syndrome, *Am J Med Genet A* **146A** (2008), 1477–1482.

[24] N.A. Meguid, A.A. Dardir, M. Khass, L.E. Hossieny, A. Ezzat and M.K. El Awady, MTHFR genetic polymorphism as a risk factor in Egyptian mothers with Down syndrome children, *Dis Markers* **24** (2008), 19–26.

[25] D. Patterson, Folate metabolism and the risk of Down syndrome, *Downs Syndr Res Pract* **12** (2008), 93–97.

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

[27] A.K. Rai, S. Singh, S. Mehta, A. Kumar, L.K. Pandey and R. Raman, MTHFR C677T and A1298C polymorphisms are risk factors for Down's syndrome in Indian mothers, *J Hum Genet* **51** (2006), 278–283.

[28] N. Rosenberg, M. Murata, Y. Ikeda, O. Opare-Sem, A. Zivelin, E. Geffen and U. Seligsohn, The frequent 5,10-methylenetetrahydrofolate reductase C677T polymorphism is associated with a common haplotype in whites, Japanese, and Africans, *Am J Hum Genet* **70** (2002), 758–762.

[29] C.B. Santos-Reboucas, J.C. Correa, A. Bonomo, N. Fintelman-Rodrigues, K.C. Moura, C.S. Rodrigues, J.M. Santos and M.M. Pimentel, The impact of folate pathway polymorphisms combined to nutritional deficiency as a maternal predisposition factor for Down syndrome, *Dis Markers* **25** (2008), 149–157.

[30] I. Scala, B. Granese, M. Sellitto, S. Salome, A. Sammartino, A. Pepe, P. Mastroiacovo, G. Sebastio and G. Andria, Analysis of seven maternal polymorphisms of genes involved in homocysteine/folate metabolism and risk of Down syndrome offspring, *Genet Med* **8** (2006), 409–4416.

[31] 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.

[32] I. Spiroski, S. Kedev, S. Antov, T. Arsov, M. Krstevska, S. Dzhekova-Stojkova, S. Kostovska, D. Trajkov, A. Petlichkovski, A. Strezova, O. Efinska-Mladenovska and M. Spiroski, Association of methylenetetrahydrofolate reductase (MTHFR-677 and MTHFR-1298) genetic polymorphisms with occlusive artery disease and deep venous thrombosis in Macedonians, *Croat Med J* **49** (2008), 39–49.

[33] L. Stuppia, V. Gatta, A.R. Gaspari, I. Antonucci, E. Morizio, G. Calabrese and G. Palka, C677T mutation in the 5,10-MTHFR gene and risk of Down syndrome in Italy, *Eur J Hum Genet* **10** (2002), 388–390.

[34] A. Ulvik, P.M. Ueland, A. Fredriksen, K. Meyer, S.E. Vollset, G. Hoff and J. Schneede, Functional inference of the methylenetetrahydrofolate reductase 677C > T and 1298A > C polymorphisms from a large-scale epidemiological study, *Hum Genet* **121** (2007), 57–64.

[35] N.M. van der Put, F. Gabreels, E.M. Stevens, J.A. Smeitink, F.J. Trijbels, T.K. Eskes, L.P. van den Heuvel and H.J. Blom, A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am J Hum Genet* **62** (1998), 1044–1051.

[36] 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** (2008), 93–99.

[37] B. Wilcken, F. Bamforth, Z. Li, H. Zhu, A. Ritvanen, M. Renlund, C. Stoll, Y. Alembik, B. Dott, A.E. Czeizel, Z. Gelman-Kohan, G. Scarano, S. Bianca, G. Ettore, R. Tenconi, S. Bellato, I. Scala, O.M. Mutchinick, M.A. López, H. de Walle, R. Hofstra, L. Joutchenko, L. Kavteladze, E. Bermejo, M.L. Martínez-Frías, M. Gallagher, J.D. Erickson, S.E. Vollset, P. Mastroiacovo, G. Andria and L.D. Botto, Geographical and ethnic variation of the 677C>T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide, *J Med Genet* **40** (2003), 619–625.

[38] 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* **98** (2001), 14853–14858.

[39] K. Yanamandra, J.A. Bocchini, Jr. and T.F. Thurmon, Absence of association of fetal MTHFR C677T polymorphism with prenatal Down syndrome pregnancies, *Eur J Hum Genet* **11** (2003), 1094–1095.

[40] E. Zintzaras, Maternal gene polymorphisms involved in folate metabolism and risk of Down syndrome offspring: a meta-analysis, *J Hum Genet* **52** (2007), 943–953.