METHYLENETETRAHYDROFOLATE REDUCTASE GENE POLYMORPHISMS AND THE PRENATAL RISK OF DOWN SYNDROME

RUXANDRA CRETU[1,*], DANIELA NEAGOS[1], ANDREEA TUTULAN-CUNITA[2], LAURENTIU CAMIL BOHILTEA[1], VERONICA STOIAN[3]

Keywords: Down syndrome, folate, methylenetetrahydrofolate reductase (*MTHFR*); *MTHFR* 677C>T; *MTHFR* 1298A>C

Abstract: The relationship between the metabolism of B9 vitamins (folates) and chromosomal nondisjunction leading to aneuploidy has drawn attention in the recent past. Here, we examine two polymorphisms in the gene encoding the folate metabolizing enzyme methylenetetrahydrofolate reductase and their impact upon the prenatal risk of Down syndrome (DS). Homozygous TT and heterozygous (CT) genotype frequencies of *MTHFR* at position 677 were higher among control mothers, showing no impact upon DS pregnancy risk. Similarly, the neither the homozygous CC genotype frequency at position 1298, nor the heterozygous AC genotypes associate with a higher risk of DS at a statistically significant level.

INTRODUCTION

Primary trisomy 21, the molecular event underlying Down syndrome (DS), is the consequence of the failure in normal chromosome 21 segregation during meiosis. A number of genetic and environmental factors have been suggested to play interactively a role in aneuploidization, among which dietary factors (Coppedè, 2009). In 1999, James *et al.* suggested that polymorphisms in folate metabolism genes leading to alterations of this pathway increase the risk of having infants with DS. In 95% of the syndrome cases, the nondisjunction event is of maternal origin, occurring primarily during meiosis I in the maturing oocyte. However, as the maternal meiose I occurs during the foetal development of the mother, the diet of the maternal grandmother might be significant in this case, while the diet of the mother may have impact upon the meiose II segregation failure. Only a few of DS cases (less than 5%) are due to errors occurring during paternal meiosis and little is known about the effect of paternal nutrition and aneuploidy in sperm. Still, Young *et al.* (2008) observed that men with high folate intake had lower frequencies of sperm with disomy 21 compared with men with lower intake, providing additional evidence for the importance of folates in human nondisjunction events.

Folic acid belongs to the family of B9 vitamins and is medically used for the primary prevention of severe congenital malformations and Down syndrome. The folate cycle is essential to two physiological processes: the synthesis of purines and pyrimidines required for DNA synthesis and repair, and cellular methylation associated with the methionine cycle. Alterations in the folate metabolism may be the result of some specific polymorphisms in the genes involved in its regulation and, due to the above-mentioned key roles of folate, these alterations can add to the risk of women having children with DS.

Methylenetetrahydrofolate reductase (*MTHFR*) is an important enzyme involved in folate metabolism; it catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) in 5-methyltetrahydrofolate (5-MeTHF), the latter representing the active form of folate that is involved in re-methylation of homocysteine to methyonine. *MTHFR* gene was mapped on human chromosome 1p36.3; it has 11 exons and exhibits multiple polymorphisms in general population, some of them with altered function in homozygous individuals. However, two polymorphisms of the *MTHFR* gene are found with higher frequency: 677C>T şi 1298A>C; these polymorphisms are thermolabile variants of the normal gene and determine the accumulation of homocysteine in circulatory system and the decrease of folic acid concentration.

The genetic polymorphism at position 677 of *MTHFR* is located in the exon 4, corresponding to the folate-binding site of the encoded protein. The cytosine to thymine transition mutation (677C>T) causes an alanine to valine substitution in the MTHFR protein and reduced enzyme activity (Frost *et al.*, 1995). The genotype *MTHFR* 677TT appears frequently in the general population and has major influences upon folate and homocysteine levels.

The second *MTHFR* polymorphism involves an adenosyne to cytosine substitution at base pair 1298 (1298A>C), causing a glutamate to alanine substitution in the MTHFR protein. The polymorphism is located in exon 7, within the presumptive regulatory domain (Goyette *et al.*, 1998). The 1298A>C mutation results in decreased MTHFR activity, with a stronger effect in the homozygous than in the heterozygous state, yet with a lesser impact than that of 677C>T (Van der Put *et al.*, 1998). Some previously published data show a frequency of 1298CC genotype of about 10% and an allele frequency of 1289C of about 36% among distinct populations (Botto *et al.*, 1999).

Beside *MTHFR*, polymorphisms in several other genes of the folate pathway affect the risk of DS pregnancy. For instance, folate receptors are responsible for 5-methyltetrahydrofolate binding and transport and inadequate receptor

function can lead to lower transport and a decrease in the intracellular concentration of active folate forms. Genes encoding folate receptors have been localized on chromosome 11 (11q13), as well as a soluble 5-MTHF receptor and an unexpressed folate receptor pseudogene (De Marco *et al.*, 2000); in addition, other genes for reduced folate carrier and cystathionine β-synthase are located on chromosome 21 and alteration in their dosage in trisomy 21 foetuses may further complicate the alterations occuring in this pathway (Coppedѐ, 2009).

The present study analyzes the association between *MTHFR* 677C>T and 1298A>C polymorphisms as maternal risk factors for meiotic nondisjunction of chromosomes 21, causing DS, in a cohort of Romanian mothers of DS children, in comparison with mothers.

MATERIALS AND METHODS

Our study include 72 women: 26 of them (ages 22-40), that gave birth to DS children, citogenetically confirmed as regular trisomy 21, including 7 women with a history of spontaneous miscarriages (26.92%); 46 control mothers that gave birth only to healthy children, without any history of miscarriages or abnormal pregnancies. All women in our study reside in the same geographic area and have a similar social background.

Genomic DNA was isolated from whole peripheral blood collected on EDTA, using peqGOLD blood DNA mini kit (*ATP Biotech*) following the manufacturer's instructions.

The *MTHFR* 677C>T and 1298A>C mutations were investigated by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. For 677C>T polymorphism, the primers were: forward 5'-TGA AGG AGA AGG TGT CTG CGG GA-3', reverse 5'-AGG ACG GTG CGG TGA GAG TG-3', amplifying a fragment of 198 bp. PCR conditions were 40 cycles of 30 sec at 94°C, 30 sec at 62°C, and 30 sec at 72°C, preceded by an initial denaturation of 2 min at 94°C and followed by a final extension of 7 min at 72°C. The presence of the T-allele generated a *Hinf*I site, producing a 175 bp fragment upon restriction in standard conditions, versus the 198 bp fragment of the C allele.

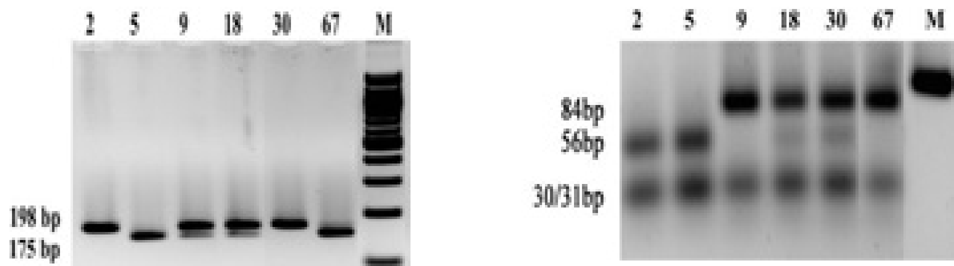
The primers for the PCR reaction to analyze the 1298A>C polymorphism were: forward: 5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3', reverse 5'-CAC TTT GTG ACC ATT CCG GTT TG-3'. PCR conditions were 38 cycles of 1 min at 92°C, 1 min at 60°C, and 30 sec at 72°C, preceded by an initial denaturation of 2 min at 92°C, and followed by a final extension of 7 min at 72°C. The amplified fragment is 163 bp; in the presence of cytosine, a *Mbo*II site is modified. Thus, while the wild type allele is restricted into five fragments of 56, 31, 30, 28, and 18 bp, while the mutated allele is digested only into four fragments of 84, 31, 30 and 18 bp (Van der Put *et al.*, 1998,).

Statistical Analysis

Allele frequencies were calculated for each genotype, and the differences in allele frequencies between mothers of children with DS and control mothers were determined using chi-square test. Expected genotype frequencies were calculated from the allele frequencies under the assumption of Hardy-Weinberg equilibrium. The interaction between the two *MTHFR* genotypes was evaluated by calculating the odds ratios (OR) for mutant genotypes, as compared to wild types for both of the *MTHFR* genotypes. Analyses were performed using the software SPSS.

RESULTS AND DISCUSSIONS

The investigation of the *MTHFR* 677 and 1298 polymorphisms was addressed by PCR amplification of genomic DNA using the primers described in *Materials and Methods* section, followed by restriction digestion with appropriate endonucleases. The results of the mutational analysis are shown for few representative cases in the figure 1.



a. b.

Fig 1a. PCR-RFLP (*Hinf*I) mutational analysis of *MTHFR* 677 polymorphism in exon 4. Lane 1 – case 2; 2 – case 5; 3 – case 9 ; 4 – case 18; 5–case 30; 6– case 67; M – pGEM 100 bp molecular weight marker (Promega). 1b. PCR-RFLP (*Mbo*II) Mutational analysis of *MTHFR* 1298 polymorphism in exon 7. Lane 1 – case 2; 2 – case 5; 3 – case 9 ; 4 – case 18; 5 – case 30 ; 6 – case 67; M – pGEM 100 bp molecular weight marker (Promega).

As observed in fig. 1a, cases 2 and 30 have the homozygous 677CC genotype, cases 5 and 67 – homozygous 677TT genotype and cases 9 and 18 – heterozygous 677CT genotype. For the polymorphic site at 1298 (fig. 1b), cases 2 and 5 have the AA genotype, case 9 and 67 – CC genotype and cases 18 and 30 – heterozygous AC genotype.

The allele frequencies of *MTHFR* 677C>T and 1298A>C in DS mothers and control mothers are listed in Table 1. *MTHFR* 677T allele frequency was 26.9% in DS mothers (χ^2: 1.83, P: 0.176), while the 1298C allele frequency was 44.2% (χ^2: 2.32, P: 0.127).

Table 1. Allele frequencies of *MTHFR* 677C>T and 1298A>C in mothers of DS children and control mothers.

Polymorphism	Allele	DS mothers (%)	Control mothers (%)	χ^2	P
MTHFR 677C>T	C	38 (73.1)	57 (62)	1.83	0.176
	T	14 (26.9)	35 (38)		
	Total	52	92		
MTHFR 1928A>C	A	29 (55.8)	63 (68.5)	2.32	0.127
	C	23 (44.2)	29 (31.5)		
	Total	52	92		

Genotype frequencies are listed in Table 2. All genotype frequencies among controls were consistent with Hardy–Weinberg equilibrium expectations.

Table 2. Genotype frequencies of *MTHFR* 677C>T and 1298A>C in mothers of DS children and in control mothers.

Polymorphism	Genotype	DS mothers (%)	Control mothers (%)	Odds ratio	95% CI	P
MTHFR 677C>T	CC	14 (53.8)	18 (39.1)	1	Reference	
	CT	10 (38.5)	21 (45.7)	0.612	0.22 - 1.71	0.35
	TT	2 (7.7)	7 (15.2)	0.367	0.07 - 2.05	0.24
	CT or TT	12 (46.2)	28 (60.9)	0.551	0.21 - 1.46	0.23
MTHFR 1298A>C	AA	8 (30.8)	20 (43.5)	1	Reference	
	AC	13 (50)	23 (50)	1.413	0.49 - 4.1	0.52
	CC	5 (19.2)	3 (6.5)	4.167	0.80 - 21.68	0.08
	AC or CC	18 (69.2)	26 (56.5)	1.73	0.626 - 4.78	0.29

CT heterozygous and TT homozygous genotype frequencies of *MTHFR* at position 677 were higher among controls mothers than among DS mothers (45.7% versus 38.5% and 15.2% versus 7.7% respectively, with an odds ratio of 0.612 (95% confidence interval (CI) 0.22–1.71 P value 0.35) and 0.367 (95% CI 0.07–2.05 P value 0.24), respectively).

CC homozygous genotype frequency at position 1298 was higher in DS mothers than in controls (19.2 % versus 6.5% respectively, with an odds ratio of 4.167 (95% CI 0,80 to 21.68, P value 0.08)) indicating that this polymorphism may have more genetic impact upon the risk of DS than the polymorphism at position 677, although, apparently not at a significant level. AC heterozygous genotype did not show any difference between the two groups.

We next compared the genotype frequencies between *MTHFR* 677CC, CT, TT and *MTHFR* 1298AA, AC, CC between DS mothers and control mothers. The results are presented in table 3 and show an association between 677CC/1289(A/C)C genotypes and DS infants (46.1% versus 26.1%, odds ratio 3, 95% CI 0.50-17.95).

Table 3. The association between *MTHFR* 677C>T and 1298A>C genotypes in DS mothers and control mothers

Genotype	DS mothers (%)	Control mothers (%)	Odds ratio	95% CI	P

	26 (total)	46 (total)			
677CC/1298AA	2 (7.7)	6 (13.0)	Reference		
677CT/1298AA	4 (15.4)	7 (15.21)	1.7143	0.23 - 12.89	0.598
677TT/1298AA	2 (7.7)	7 (15.21)	0.8571	0.09 - 8.07	0.893
677CT or TT/1298AA	6 (23.1)	14 (30.4)	1.2857	0.19 - 8.29	0.791
677CC/1298AC	7 (26.9)	9 (19.6)	2.33	0.35 - 15.30	0.371
677CC/1298CC	5 (19.2)	3 (6.52)	5	0.58 - 42.79	0.130
677CC/AC or CC	12 (46.1)	12 (26.1)	3	0.50 - 17.95	0.217
677CT/1298AC	6 (23.1)	14 (30.4)	1.28	0.19 - 8.29	0.791
1298CT or TT/1298AC or CC	6 (23.1)	14(30.4)	1.28	0.19 - 8.29	0.791

Down syndrome represents a major cause of mental retardation, affecting 1 in 700 live births (Cheffins *et al.*, 2000). It is caused by the failure of chromosome 21 to segregate normally during meiosis (Lejeune *et al.*, 1959). Despite substantial research, the cause of chromosome non-disjunction leading to trisomy 21 remains unclear. Abnormal folate metabolism due to common genetic polymorphisms has been described as a possible cause of DS (James *et al.*, 1999), as deficiences in cellular folate and methyl donors have been associated with abnormal DNA methylation, DNA strand breaks and abnormal chromosome segregation (James *et al.*, 2002).

Several association studies have been performed in the last decade (1999–2009), aimed at clarifying the role of folate and methyl metabolism in DS risk, almost all of them including the *MTHFR* 677C>T polymorphism. In our study, we investigated the prevalence of the *MTHFR* genotype variation in Romania, finding the frequencies of the CC, CT, and TT genotypes at 677 position among DS mothers of 53.8%, 38.5%, and 7.7%, respectively, and among control mothers - of 39.1%, 47.5%, and 15.2%, respectively (P values 0.23-0.35). These data do not point to any association between the polymorphisms as this locus and the risk of having DS infants.

James *et al* (1999) studied *MTHFR* 677C>T mutation in 57 mothers of DS children and 50 control mothers from United States and Canada and found a significant increase in the frequency of mutant allele (CT and TT), compared to controls. The frequencies of the CC, CT, and TT genotypes among the mothers of children with DS were 26.3%, 59.6 %, and 14% respectively. The corresponding frequencies among control mothers were 48%, 44%, and 8% respectively, therefore associating a higher risk of DS pregnancy to 677T allele.

In a similar approach, Hobbs *et al.* (2000) evaluated the frequencies of the *MTHFR* 677C>T mutations in DNA samples from 157 mothers of children with DS and 144 control mothers, in USA. They found that the *MTHFR* 677 C>T polymorphism is more prevalent among mothers of children with Down syndrome than among control mothers.

In unrelated studies, *MTHFR* 677C>T polymorphism resulted to be an independent risk factor for a DS offspring in the Egyptian and Chinese populations (Wang *et al.*, 2007; Meguid *et al.*, 2008; Wang *et al.*, 2008). However, none of the studies performed in Europe (O'Leary *et al.*, 2002; Chadefaux *et al.*, 2002; Stuppia *et al.*, 2002; Scala *et al.*, 2006; Martínez-Frías *et al.*, 2006; Coppedè *et al.*, 2009), Turkey or Japan (Takamura *et al.*, 2004; Boduroğlu *et al.*, 2004) found it to be an independent DS risk factor.

The contrast between the two categories of results could be due to variations in allele frequencies among different populations or to the different sizes of the case–control studies; however, it could also largely result from the contribution of environmental factors, such as dietary factors (Chadefaux *et al.*, 2002; Chango *et al.*, 2005; Coppedè *et al.*, 2006).

The *MTHFR* 1298A>C polymorphism has been studied less extensively than the 677C>T in relation with the risk of DS. The first evidence of an association between the 1298C allele and

DS risk was obtained in by Grillo *et al.* (2002), in a study on a Brazilian cohort; in addition, an interaction between MTHFR 1298A>C and 677C>T polymorphisms was shown to increase the DS risk (Grillo *et al.*, 2002; Acácio *et al.*, 2005). Similar results have been obtained in India (Rai *et al.*, 2006), Southern Italy (Scala *et al.*, 2006) and Egypt (Meguid *et al.*, 2008). Four out of five Brazilian studies indicate an increased risk of DS associated with the combinations of the *MTHFR* 1298C variant with other polymorphisms in genes of the folate metabolic pathway, including the *MTHFR* 677C>T allele. There are, however, four studies that failed to find any association between DS risk and the MTHFR 1298A>C polymorphism (either alone or combined with other variants). (Bosco *et al.*, 2003; Boduroğlu *et al.*, 2004; Chango *et al.*, 2005; Biselli et.al., 2008). Again, our data do not associate the presence of the mutant C allele at this locus with a higher risk of DS: the cumulated frequencies of AC and CC genotypes are 69.2% in DS mothers versus 56.5% in control mothers (P value 0.29). As expected, neither association analysis between different genotypes shows an increased risk for any particular combination.

CONCLUSIONS

We have, therefore, compared two polymorphisms in *MTHFR* gene in DS mothers under the age of 40 with controls. Allele frequencies at these positions do not point to any preferential association of a particular polymorphism and DS phenotype. The differences between *MTHFR* 677CT and *MTHFR* 677TT genotype frequencies in DS mothers and in controls were not significant. Therefore, the *MTHFR* 677C>T polymorphism is not a maternal risk factor for DS. Similarly, neither the *MTHFR* 1298A>C polymorphism does not associate with a higher risk, at a statistically significant level.

REFERENCES

Acácio G.L., R. Barini, C.S. Bertuzzo, E.C. Couto (2005) *Methylenetetrahydrofolate reductase gene polymorphisms and their association with trisomy 21*, Prenat. Diagn. 25: 1196–1199;

Antonarakis S.E., M.B. Petersen, M.G. McInnis, P.A. Adelsberger (1992) *The meiotic stage of nondisjunction in trisomy 21: determination by using DNA polymorphisms,* Am. J. Hum. Genet. 50: 544–550;

Biselli J.M., D. Brumati, V.F. Frigeri, B.L. Zampieri (2008) *A80G polymorphism of reduced folate carrier 1 (RFC1) and C776G polymorphism of transcobalamin 2 (TC2) genes in Down's syndrome etiology*, Sao Paulo Med. J. 126: 329–332.

Boduroğlu K., Y. Alanay, B. Koldan, E. Tunçbilek (2004) *Methylenetetrahydrofolate reductase enzyme polymorphisms as maternal risk for Down syndrome among Turkish women*, Am. J. Med. Genet. A 127: 5–10;

Bosco P., R.M. Gueant-Rodriguez, G. Anello, C. Barone (2003) *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: 219–224;

Botto L.D., Yang Q. (1999) *Methylenetetrahydrofolate reductase (MTHFR) and birth defects.* Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Birth Defects and Pediatric Genetics. Atlanta, GA;, www.cdc.gov/genetics/hugenet/rewievs/MTHFR.htm

Chadefaux-Vekemans B, M. Coudè, F. Muller, J.F. Oury (2002) *Methylenetetrahydrofolate reductase polymorphism in the etiology of Down syndrome*, Pediatr. Res. 51: 766–767;

Chango A., N. Fillon-Emery, C. Mircher, H. Bléhaut (2005) *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: 166–16;

Cheffins T., Chan A., Haan E., Ranieri E (2000) *The impact of maternal serum screening on the birth prevalence of Down's syndrome and the use of amniocentesis and chorionic villous sampling in South Australia*. BJOG, 107: 1453-1459;

Coppedè F. (2009) *The complex relationship between folate/homocysteine metabolism and risk of Down syndrome.I* Mut. Res. 682:54-70;

Coppedè F., F. Migheli, S. Bargagna, G. Siciliano (2009) *Association of maternal polymorphisms in folate metabolizing genes with chromosome damage and risk of Down syndrome offspring*, Neurosci. Lett. 449: 15–19;
Coppedè F., G. Marini, S. Bargagna, L. Stuppia (2006) *Folate gene polymorphisms and the risk of Down syndrome pregnancies in young Italian women, Am. J. Med. Genet*. A 140: 1083–1091;
De Marco P., Moronia., Merelloe., De Franchis R (2000) *Folate pathway gene alterations in patients with neural tube defects*. Am. J. Med. Genet. 95: 216-223;
Frost P, H.J. Blom, R. Milos, P. Goyette (1995) *A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase*, Nature Genet. 10: 111–113;
Goyette P., P. Aditya, M. Renate, P. Frosst (1998) *Chen Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR)*, Mammalian Genome 9: 652–656;
Grillo L.B., G.L. Acácio, R. Barini, W. Pinto Jr (2002) *Mutations in the methylenetetrahydrofolate reductase gene and Down syndrome*, Cad. Saude Publica 18: 1795–1797;
Hobbs C.A., Sherman S., Yi P., Hopkins S.E (2000) *Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome.* Am. J. Hum. Genet.67: 623-630;
James S., Pogribna M., Pogribny I., Melnyk S (1999) *Abnormal folate metabolism and mutation in the methylenetetrahydrofolate reductase gene may be maternal risk factors for Down syndrome*. Am. J. Clin. Nutr., 70: 495-501;
James S. J., Hobbs C. (2002) *Folate deficiency and molecular determinants of chromosome instability.* In: Folate and Human Development, Eds. E. Massaro, J.M. Rogers, Totowa, NJ, Humana Press pp. 43–69;
Lejeune J, Gautier M, Turpin R. (1959) Etude des chromosomes somatiqaues de neuf enfants mongoliens. Compte Rendu d'Acad Sci 248:1721–1722;
Martínez-Frías M.L., B. Pérez, L.R. Desviat, M. Castro (2006) *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: 987–997;
Meguid N.A., A.A. Dardir, M. Khass, L.E. Hossieny, (2008) *MTHFR genetic polymorphism as a risk factor in Egyptian mothers with Down syndrome children*, Dis. Markers 24: 19–26;
O'Leary V.B., A. Parle-McDermott, A.M. Molloy, P.N. Kirke (2002) *MTRR and MTHFR polymorphism: link to Down syndrome?* Am, J. Med. Genet. 107: 151–155;
Rai A.K., S. Singh, S. Mehta, A. Kumar (2006) *MTHFR C677T and A1298C polymorphisms are risk factors for Down's syndrome in Indian mothers*, J. Hum. Genet. 51: 278–283;
Scala I., B. Granese, M. Sellitto, S. Salomè (2006) *Analysis of seven maternal polymorphisms of genes involved in homocysteine/folate metabolism and risk of Down syndrome offspring*, Genet. Med. 8. 409–416;
Stuppia L., V. Gatta, A.R. Gaspari, I. Antonucci (2002) *C677T mutation in the 5,10-MTHFR gene and risk of Down syndrome in Italy*, Eur. J. Hum. Genet. 10: 388–390;
Takamura N., T. Kondoh, S. Ohgi, K. Arisawa (2004) *Abnormal folic acid-homocysteine metabolism as maternal risk factors for Down syndrome in Japan*, Eur. J. Nutr. 43: 285–287;
Van der Put N.M., F. Gabreels, E.M. Stevens, J.A.M. Smeitink (1998) *A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural tube defects?* Am. J. Hum. Genet. 62: 1044–1051;
Young S.S., B. Eskenazi, F.M. Marchetti, G. Block (2008) *The association of folate, zinc and antioxidant intake with sperm aneuploidy in healthy nonsmoking men,* Hum. Reprod. 23: 1014–1022;
Wang W., W. Xie, X. Wang (2007) *The relationship between polymorphism of gene involved in folate metabolism, homocysteine level and risk of Down syndrome*, Zhonghua Yi Xue Yi Chuan Xue Za Zhi 24: 533–537;
Wang S.S., F.Y. Qiao, L. Feng, J.J. Lv (2008) *Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome in China*, J. Zhejiang Univ. Sci. B 9: 93–99.

Institutional Affiliaton
1 – "Carol Davila" University of Medicine and Pharmacy, Department of Genetics, Bucharest
2 – Medical Genetics Laboratory, "Victor Babes" National Institute of Pathology, Bucharest
3 – Department of Genetics, Faculty of Biology, University of Bucharest

Corresponding address
"Carol Davila" University of Medicine and Pharmacy, Department of Genetics, Bucharest, Romania, Tel. 0721761267. Email: ruxandra_78@yahoo.com .
Date of manuscript submission
20/08/2010