MTHFR genetic polymorphism as a risk factor in Egyptian mothers with Down syndrome children

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Abstract. Recent reports linking Down syndrome (DS) to maternal polymorphisms at the methylenetetrahydrofolate reductase (*MTHFR*) gene locus have generated great interest among investigators in the field. The present study aimed at evaluation of *MTHFR* 677C/T and 1298A/C polymorphisms in the *MTHFR* gene as maternal risk factors for DS. Forty two mothers of proven DS outcomes and forty eight control mothers with normal offspring were included. Complete medical and nutritional histories for all mothers were taken with special emphasis on folate intake. Folic acid intake from food or vitamin supplements was significantly low (below the Recommended Daily Allowance) in the group of case mothers compared to control mothers. Frequencies of *MTHFR* 677T and *MTHFR* 1298C alleles were significantly higher among case mothers (32.1% and 57.1%, respectively) compared to control mothers (18.7% and 32.3%, respectively). Heterozygous and homozygous genotype frequencies of *MTHFR* at position 677 (CT and TT) were higher among case mothers than controls (40.5% versus 25% and 11.9% versus 6.2%, respectively) with an odds ratio of 2.34 (95% confidence interval [CI] 0.93–5.89) and 2.75 (95% CI 0.95–12.77), respectively. Interestingly, the homozygous genotype frequency (CC) at position 1298 was significantly higher in case mothers than in controls (33.3% versus 2.1% respectively) with an odds ratio of 31.5 (95% CI 3.51 to 282.33) indicating that this polymorphism may have more genetic impact than 677 polymorphism. Heterozygous genotype (AC) did not show significant difference between the two groups. We here report on the first pilot study of the possible genetic association between DS and *MTHFR* 1298A/C genotypes among Egyptians. Further extended studies are recommended to confirm the present work.

Keywords: Methylenetetrahydrofolate reductase (*MTHFR*), Down syndrome, polymorphism, *MTHFR* 677C/T polymorphism, *MTHFR* 1298A/C polymorphism, Egyptian

1. Introduction

Down syndrome is the most common genetic cause of human mental retardation, with a frequency of approximately 1 in 600 to 1000 live births [27]. It was estimated that 1 in 150 conceptions have trisomy 21

and that 80% of these are lost during early pregnancy [16]. The relationship between chromosomal non-disjunction and folate metabolism has drawn attention in recent years. Both folate and methylenetetrahydrofolate reductase (*MTHFR*) are involved in many complex biochemical reactions. *MTHFR* is the enzyme responsible for the reduction of methylenetetrahydrofolate, which is a key single-carbon donor that takes part in nucleotide synthesis; S-adenosylmethionine (SAM) synthesis; remethylation of homocysteine to methionine; and the methylation of DNA, proteins, neurotransmitters, and phospholipids [17]. Altered mater-

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nal folate status and homozygous mutation in *MTHFR* gene may promote chromosomal instability and non-disjunction resulting in fetal trisomy 21 [18]. DNA hypomethylation induces structural alterations in pericentromeric heterochromatin and abnormal chromosome segregation. Reduced *MTHFR* activity results in an increased requirement for folic acid to maintain normal homocysteine remethylation to methionine. In the absence of sufficient folic acid, intracellular homocysteine accumulates, methionine resynthesis is reduced and remethylation reactions *may be affected* [22]. Research in this field has accelerated in the recent years, examining the relationship between DS and the two known gene polymorphisms, *MTHFR* 677C/T and *MTRR* 66A/G taking role in the folate pathway [15]. Studies by [17] have demonstrated a link between DS and *MTHFR* 677C/T and *MTRR* 66A/G polymorphisms. Maternal heterozygosity (CT) or homozygosity (TT) for *MTHFR* and homozygosity (GG) for *MTRR* have been calculated to increase the risk of having a child with DS. A second polymorphism 1298A/C involving alanine to cytosine nucleotide substitution in *MTHFR* gene has also been reported [30]. The purpose of the present study is to evaluate the role of *MTHFR* 677C/T and 1298A/C polymorphisms in DS risk among Egyptian mothers.

2. Subjects and methods

2.1. Subjects

Forty two young mothers (less than 30 years old) with a previous history of bearing Down syndrome (DS) baby confirmed as regular trisomy 21 were selected from Children with Special Needs outpatient Clinic, National Research Center, Egypt. Forty eight control mothers, who resided in the same geographic area and almost the same social class and had given birth to at least two healthy children without history of any miscarriages or abnormal pregnancies, were included.

All subjects (mothers with DS children and control mothers) were subjected to:

1. Comprehensive history taking including pedigree analysis, family history of any genetic or non-genetic disorders, pregnancy history and obstetric history.
2. Dietary assessment: Quantitative daily consumption methods, consist recall and records designed to measure the quantity of the mother food and beverages consumed over 24 hours [12]. Dietary

history and food frequency questionnaire were completed for all mothers with special emphasis on folate intake through food or vitamin supplements during pregnancy. Adequacy of the diet was assessed by comparing the energy and nutrient intake of mothers with their recommended daily allowances [8]. Comparison between the requirement of each mother according to FAO and the nutritive value of her food intake within 24 hours was done using Food Composition Tables of the National Nutrition Institute of Egypt [9].

2.2. Sample collection

Venous blood samples were collected on EDTA from all mothers and preserved at 4°C till assayed. Total genomic DNA was extracted from whole blood using salting out technique.

a- Polymerase Chain Reaction Amplification: Amplification was performed in 50 µl reaction mixture containing approximately 100 ng genomic DNA, 200 µM dNTPS, 50 picomole of each forward and reverse primers, and 2 units of Taq DNA polymerase (Quia-gene, Frankfurt, Germany) in a 1X buffer supplied with the enzyme containing 1.5 mM MgCl₂.

For the polymorphic site (C→T) at 677 bp, primer sequences used were derived from intron sequence bracketing exon (4) according to Kowa et al. [19].

Forward primer: 5'-TGA AGG AGA AGG TGT CTG CGG GA-3'

Reverse primer: 5'-AGG ACG GTG CGG TGA GAG TG-3'.

The primer sequences used for amplification of exon (7) were derived from inside exon (7) surrounding the polymorphic site (A→C) at position 1298. The primer design was performed using Primer3 Output Software (primer3_www_results.cgi) and the sequences were as follows:

Forward primer: 5' GAA GAG CAA GTC CCC CAA AG 3'

Reverse primer: 5' ACA GGA TGG GGA AGT CAC AC 3'

Thermal cycling and product detection: Initial denaturation for one cycle at 95°C for 3 min. followed by 35 cycles each consisted of denaturation at 95°C for 30 seconds, annealing at 60°C for 45 seconds and extension at 72°C for one min. Amplification protocol was ended up with a final extension step at 72°C for 10 minutes. The PCR products were analyzed on 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The detection of a fragment of

Fig. 1. Mutational analysis of *MTHFR* 677 polymorphism in exon 4. The amplified product (198 bp) was digested with *Hinf* I and the digested products were resolved on 20 % polyacrylamide gel electrophoresis, stained with ethidium bromide and photographed on a photodocumentation system (Biometra, Germany). Lane 1 shows undigested 198 bp fragment. Lane 2 shows *Hinf* I digestion product of DNA from a mother of Down syndrome patient who is a homozygous for C to T (TT) polymorphism which displays doublet band at 175 bp besides 23 bp band (run off the gel). Lanes 4 displays 198 and 175 bp bands (heterozygous for C to T). Lane 5 shows a wild type (CC) with a doublet band at 198 bp. Band sizes were evaluated as a comparison with a molecular weight marker ØX174 Hae III digest (lane 3).

Fig. 2. Mutational analysis of *MTHFR* 1298 polymorphism in exon 7. The amplified product (221 bp) was digested with *Mbo*II enzyme and the digested products were resolved on 20% polyacrylamide gel electrophoresis, stained with ethidium bromide and photographed on a photodocumentation system (Biometra, Germany). Lane 2 and 3 show *Mbo*II digestion products of DNA from two mothers of Down syndrome patients who are heterozygous for A to C polymorphism which display two bands at 179 and 151 bp besides 30 and 28 bp bands (ran out of the gel). Lanes 1 and 4 display 179 and 151 bp bands respectively (homozygous mutant (CC) and homozygous wild type (AA) respectively). Band sizes were evaluated as a comparison with a molecular weight marker ØX174 Hae III digest (lane M).

198 bp indicates a successful amplification of exon 4 of human *MTHFR* gene (Fig. 1) and of a 221 bp band indicates amplification of exon 7 (Fig. 2).

b- Mutational analysis (Restriction fragment length polymorphism) of the digested PCR products: The digested products were resolved on 20% poly acrylamide gel. The poly acrylamide gel was stained with ethidium bromide and visualised with UV trans illuminator. The normal pattern (wild type) (CC) for exon (4) provides a single duplet band at 198 bp after digestion with *Hinf* I. While the heterozygous pattern (CT) creates a *Hinf* I restriction site leading to the digestion of the 198 bp, providing three bands at 198, 175, and 23 bp. The 23 bp, last band is undetectable and ran out the gel. The mutant pattern (TT) provides 2 bands at 175, and

23 bp. Only, one band at 175 bp is detectable while that of 23 bp ran out of gel (Fig. 1).

The 221 bp of exon (7) is reduced to 209 bp due to the presence of another *Mbo*II recognition site within the sequence of the forward primer which does not interfere with polymorphic site at 1298 bp, and that is used to detect whether the enzyme used is working or not. The normal pattern (wild type), (AA) for exon (7) provides 3 bands at 151, 30, and 28 bp, respectively after digestion with *Mbo*II. Only 151 bp band is visualized on 20% polyacrylamide gel while the other two bands ran out of the gel. While, the heterozygous pattern (AC), (contains both mutant and wild type allele) provides 4 bands at 179, 151, 30, and 28 bp. The only two bands visualized on gel were 179 and 151 bp while the other 2 bands ran out, as they are very small in size.

The mutant pattern (CC) provided 2 bands at 179 and 30 bp where only band at 179 bp was visualized on the gel (Fig. 2).

2.3. Statistical analyses

Odds ratios and 95% confidence intervals (95% CI) were calculated to estimate the risk of the different genotypes. Allele frequencies and genotype frequencies were calculated and the differences 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. All statistical analyses were done with SPSS software, version 9.0. P values were two tailed, and P was considered statistically different if ≤ 0.05 .

3. Results

Detailed nutritional history revealed that 93% of case mothers did not receive folic acid vitamin supplement. Dietary content of folate in 90% of mothers included in this group was below the Recommended Daily Allowance, which is 400 $\mu\text{g}/\text{d}$ [11], while the nutritional history taken from the control group showed folic acid supplementation (tablets of 5 mg once per day) in 13% of cases, multivitamin supplement in 53% of cases, while adequate intake of vegetables (good sources of folic acid) was recorded in 67% of cases (Table 1). Student T test was performed on folate intake of mothers with D.S. children and control mothers and showed that folic acid intake was significantly lower in the group of mothers with D.S. children than in the control group ($P < 0.01$).

The distribution of the *MTHFR* genotypes in the control population was found to be in Hardy-Weinberg equilibrium. Frequencies of *MTHFR* 677T and *MTHFR* 1298C alleles were significantly higher among case mothers (32.1% and 57.1%, respectively) compared to control mothers (18.7% and 32.3%, respectively) (Table 2).

The *MTHFR* 677C/T genotype CC frequency was significantly higher among control mothers compared to case mothers (68.8% and 47.6%, respectively). The frequencies of *MTHFR* 677C/T heterozygous and homozygous genotypes (CT and TT) were higher among case mothers (40.5% and 11.9%, respectively) than control mothers (25% and 6.2%, respectively) with an odds ratios of 2.34 (95% confidence interval [CI] 0.93–

5.89) and 2.75 (95% CI 0.95–12.77), respectively. On the other hand, sum of both variant genotypes (CT plus TT) showed significant increase in case mothers compared to control group (52.4% versus 31.2%, respectively) with an odds ratio of 2.42 (95% CI 1.02–5.72) (Table 3).

The frequency of *MTHFR* 1298 A/C genotype AA was higher among control mothers than case mothers with no statistically significant differences (37.5% versus 19.1%, respectively). However, the frequency of *MTHFR* 1298 A/C homozygous genotype (CC) was significantly higher among case mothers than control mothers (33.3% versus 2.1%, respectively) with an odds ratio of 31.5 (95% CI 3.51 to 282.33). Heterozygous genotype (AC) did not show significant difference between the two groups (47.6% versus 60.4% respectively) with an odds ratio of 1.55 (95% CI 0.57 to 4.26). Moreover, sum of both variant genotypes (AC plus CC) showed no significant difference between the case and control groups (80.9% versus 62.5% respectively) with an odds ratio of 2.55 (95% CI 0.97–6.71) (Table 4).

4. Discussion

In most cases of DS, the extra chromosome exists as a result of the failure of normal chromosome segregation during meiosis. The non-disjunction event is maternal in 95% of cases, occurring primarily during meiosis I in the maturing oocyte before conception [18]. Although mechanisms described recently that could account for the meiotic non-disjunction are diverse, not a single mechanism could be referred to as prevailing. The risk of meiotic nondisjunction may be dependent on the interaction between different maternal polymorphisms 677C/T and 1298A/C of *MTHFR*, and 66A/G *MTRR* genes together with nutrients interactions and lifestyle [20].

Nutritional history, as a possible risk factor, was considered in details to evaluate folate status of DS mothers in relation to control mothers. Folate acts as a co-factor for enzymes involved in DNA and RNA synthesis and in the supply of methyl group to the methylation cycle. Thus, folate deficiency can lead to defective cell proliferation and cell death [21]. Our results showed that folic acid intake was significantly lower than the Recommended Daily Allowance, which is 400 $\mu\text{g}/\text{d}$ [11]; in the group of case mothers compared to controls ($P < 0.01$).

MTHFR acts at a critical metabolic juncture in the regulation of cellular methylation reactions, catalyz-

Table 1
Main dietary findings of mothers with DS children and control mothers

Mothers	Use of Vitamin Supplements	Folate from Food	Number
Control Mothers	66%	67% > 400 µg/d	48
Case Mothers	7%	90% < 400 µg/d	42

Table 2
Allele Frequency of 677 C/T and 1298 A/C polymorphisms among case and control mothers

Genotype	Allele	Case mothers (n = 42) Alleles (%) (n = 84)	Control Mothers (n = 48) Alleles (%) (n = 96)	X ²	P
677	C	57 (67.9)	78 (81.3)	4.29	≤0.05
	T	27 (32.1)	18 (18.7)		
1298	A	36 (42.9)	65 (67.7)	11.24	≤0.001
	C	48 (57.1)	31 (32.3)		

Table 3
Genotype Frequencies of MTHFR 677 C/T polymorphism in case and control mothers

Genotype	Number (%) of case mothers (n = 42)	Number (%) of control mothers (n = 48)	X ²	Odds ratio	95% CI	P
CC	20 (47.6)	33 (68.8)	4.13	1.0		≤0.05
CT	17 (40.5)	12 (25.0)	2.46	2.34	0.93–5.89	NS
TT	5 (11.9)	3 (6.2)	0.88	2.75	0.59–12.77	NS
Combined mutant (CT plus TT)	22 (52.4)	15 (31.2)	4.13	2.42	1.02–5.72	≤0.05

NS: non-significant.

Table 4
Genotype frequencies of MTHFR 1298 A/C polymorphism in case and control mothers

Genotype	Number (%) of case mothers (n = 42)	Number (%) of control mothers (n = 48)	Odds ratio	95% CI	X ²	P
AA	8 (19.1)	18 (37.5)	1.0		3.71	NS
AC	20 (47.6)	29 (60.4)	1.55	0.57–4.26	1.48	NS
CC	14 (33.3)	1 (2.1)	31.5	3.51–282.33	15.75	≤0.001
Combined mutant (C plus CC)	34 (80.9)	30 (62.5)	2.55	0.97–6.71	3.71	NS

NS: non-significant.

ing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor for the remethylation of homocysteine to give methionine [4].

Frequent mutations may modify enzymatic activity. The specific activity of *MTHFR* is low in the heterozygous genotype and in the homozygous mutant genotype. Reviewing the literature, many polymorphisms have been identified in *MTHFR* gene. However, two most common genetic polymorphisms associated with a reduced *MTHFR* activity have been identified. One is located in exon 4 at the folate-binding site (677C/T). The cytosine to thymine transition mutation at position 677 within the *MTHFR* gene (677C/T) causes an alanine to valine substitution in the *MTHFR* protein and reduced enzyme activity [10]. In homozygous TT subjects, this mutation results in approximate-

ly 50% of enzyme activity compared to those without mutation and leads to decreased synthesis of 5-methyltetrahydrofolate. Among healthy subjects, the homozygous TT genotype is associated with a significantly higher homocysteine and low red cell folate levels than in heterozygotes or individuals with wild type C alleles. The (1298A/C) polymorphism is located in exon 7 within the presumptive regulatory domain [13]. It involves adenosine to cytosine nucleotide substitution in *MTHFR* gene resulting in a glutamine to alanine substitution [30]. The mutation destroyed an *MboII* recognition site and had an allele frequency of 0.33. The 1298A→C mutation results in decreased *MTHFR* activity, which was more pronounced in the homozygous than heterozygous state [29].

T-allele frequency for 677C/T mutation of *MTHFR* gene in the Egyptian control group established by the present study (18.7%) is much similar to that reported in UK (18.6%), lower compared to USA (32.2%) and higher when compared to Sri Lanka (4.9%) and South India (10%) [7,26]. Moreover, we found that frequency of *MTHFR* 677T allele was significantly higher among case mothers (32.1%) compared to control mothers. The present study showed that frequencies of *MTHFR* 677C/T; either heterozygous (CT) or homozygous (TT) genotypes were higher among case mothers (40.5% and 11.9% respectively) than control mothers (25% and 6.2% respectively) with an odds ratios of 2.34 and 2.75, respectively. The sum of both variant genotypes (CT plus TT) showed significant increase in case mothers compared to control mothers (52.4% versus 31.2% respectively). Many studies pointed to the role of maternal *MTHFR* 677C/T polymorphism as a risk factor for Down syndrome. James et al. [18] found a higher frequency of both the *MTHFR* CT and TT genotype in the mothers of children with DS as compared to control mothers which is consistent with our results. Another subsequent similar study by Hobbs et al. [17] have shown data consistent with the preliminary observation of *MTHFR* 677C/T polymorphism in mothers with DS children than among controls with an odd ratio of 1.91. Moreover Acacio [1] in Brazil found that the frequency of joint heterozygotic polymorphism 677C/T of *MTHFR* gene was significantly higher in 70 women with children affected by trisomy 21 than in 88 controls with normal offspring. In a study from India, where 677T allele frequency is quite low in the population Rai et al. [23] have shown a strong association of this SNP, especially the T homozygote, with DS mothers. Interestingly, the T homozygous case mothers were all younger than 30 years of age. These authors have attributed 677TT as a genetically predisposing factor for susceptibility of young mothers to DS in this population.

Other studies done on Italian and French mothers did not support the presence of an increased risk of DS birth in mothers with 677T allele of *MTHFR* gene [4,22,28]. They reported that, the high intake of food folate in France and Italy may neutralize the metabolic impact of the *MTHFR* polymorphism. Moreover Tanamandra et al. [31] reported no correlation of *MTHFR* mutant 677TT homozygosity or mutant 677T allele frequency with prenatal Down syndrome Caucasian cases. A recent study done by Copped et al. [5] on 80 Italian mothers of DS individuals and 111 Italian control mothers showed that *MTHFR* 677C/T polymorphism was an in-

dependent risk factor for a DS offspring at a young maternal age; however, a role for the combined *MTHFR* folate carrier gene (*RFC-1*) genotypes in the risk of DS pregnancies among young Italian women could not be excluded. They reported that *MTHFR* 1298A/C genotype frequencies did not significantly differ between DS mothers and control mothers when polymorphisms were considered alone, however, the combined *MTHFR* AA/*RFC-1* 80(GA or AA) genotypes was inversely associated with the risk of DS pregnancies among young Italian women.

In the present study, the frequency of the 1298C allele frequency in the Egyptian control group was 32.3% which is relatively lower than those reported in the Japanese and Africans who had 79% and 91% frequencies respectively [24]. Moreover, our results showed that the frequency of *MTHFR* 1298C allele was significantly higher among case mothers (57.1%) compared to controls (32.3%).

Regarding the *MTHFR* 1298A/C polymorphism in the present study; frequency of homozygous genotype (CC) was significantly higher among case mothers than controls (33.3% versus 2.1% respectively). Heterozygous genotype (AC) did not show statistically significant difference between the two groups. The sum of both variant genotypes (AC and CC) was higher among case group compared to control group with no significant difference between the two groups (80.9% versus 62.5% respectively). Similar results were reported by Grillo et al. [14], who evaluated the frequency of the *MTHFR* 1298A→C mutations in 36 mothers of children with DS and in 200 controls. They reported that the *MTHFR* 1298A→C mutations were more prevalent among mothers of children with DS than controls. Moreover Scala et al. [25] investigated 94 mothers of DS offspring and 264 control women from Campania, Italy. They reported an increased risk of DS associated with *MTHFR* 1298C allele and the *MTHFR* 1298CC genotype. However, the study done by da Silva et al. [6] on Brazilian mothers and Boduroglu et al. [2] on Turkish mothers did not find any association between *MTHFR* 1298A/C polymorphism and the risk for Down syndrome.

Castro et al. [3] suggested that the 1298CC *MTHFR* genotype, independently of folate availability, might be potential risk factors for disease states associated with DNA hypomethylation status including cancer. Moreover, The involvement of the 1298A>C variant in the development of neural tube defects has only been considered in a few studies which report a tendency towards a higher frequency in neural tube defective children [30].

Hassold et al. [15] reported that polymorphism variability in the folate pathway is not a significant contributor factor for human meiotic nondisjunction. They suggested that factors involving, both genotype and nutrition may underlie susceptibility to nondisjunction involving chromosome 21 but the same may not be true for other autosomal trisomies. Therefore genetic-environmental interactions will continue to be unmasked as an increasing number of genes involved in folate metabolism is cloned. We concluded that *MTHFR* gene polymorphisms may be associated with maternal risk for DS. For adequate functioning of *MTHFR* enzyme in mutants; both in heterozygous and homozygous conditions; folate supplementation would be helpful.

References

- [1] G.L. Acacio, R. Barini, C.S. Bertuzzo, E.C. Couto, J.M. Annichino-Bizzacchi and W.P. Junior, Methylenetetrahydrofolate reductase gene polymorphisms and their association with trisomy 21, *Prenat Diagn* **25**(13) (2005), 1196–1199.
- [2] K. Boduroglu, Y. Alanay, B. Koldan and E. Tuncbilek, Methylenetetrahydrofolate Reductase Enzyme Polymorphisms as Maternal Risk for Down Syndrome Among Turkish Women, *American Journal of Medical Genetics A* **127** (2004), 5–10.
- [3] R. Castro, I. Rivera, P. Ravasco, M.E. Camilo, C. Jakobs, H.J. Blom and I.T. de Almeida, 5,10-Methylenetetrahydrofolate reductase (MTHFR) 677C-T and 1298A-C mutations are associated with DNA hypomethylation, *J Med Genet* **41** (2004), 454–458.
- [4] B. Chadeaux-Vekemans, M. Coudé, F. Muller, J.F. Oury, A. Chabli, J.P. Jaïs and P. Kamoun, Methylenetetrahydrofolate Reductase Polymorphism in the Etiology of Down Syndrome, *Pediatric Research* **51**(6) (2002), 766–767.
- [5] 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**(10) (2006), 1083–1091.
- [6] 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**(3) (2005), 263–267.
- [7] A.R. Devi, V. Govindaiah, G. Ramakrishna and S.M. Naushad, Prevalence of methylene tetrahydrofolate reductase polymorphism in South Indian population, *Current Science* **86**(3) (2004), 440–443.
- [8] FAO/WHO/UNU (1985), Energy and protein requirements. Report of a joined FAO/WHO/UNU Expert Consultation. World Health Organization. Geneva.
- [9] Food Composition Tables of National Nutrition Institute of Egypt, 1993: Based on local food analysis and food composition tables in the Middle East.
- [10] P. Frosst, H.J. Blom, R. Milos, P. Goyette, C.A. Sheppard, R.G. Matthews, G.J.H. Boers, M. den Heijer, L.A.J. Kluijtmans, L.P. van den Heuvel and R. Rozen, A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase, *Nature Genet* **10** (1995), 111–113.
- [11] S. Gerrior and C. Zizza, Nutrient content of the U.S. Food Supply, Home Economics Research Report No.52., Department of Agriculture, Washington, USA, 1994, 88.
- [12] S.R. Gibson, *Principle of Nutritional Assessment*, New York, Oxford; Oxford University Press, 1990.
- [13] P. Goyette, P. Aditya, M. Renate, P. Frosst, P. Tran and Z. Chen, Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR), *Mammalian Genome* **9** (1998), 652–656.
- [14] L.B. Grillo, G.L. Acacio, R. Barini, W. Pinto Jr. and C.S. Bertuzzo, Mutations in the methylene-tetrahydrofolate reductase gene and Down syndrome, *Cad Saude Publica* **18**(6) (2002), 1795–1797.
- [15] T.J. Hassold, L.C. Burrage, E.R. Chan, L.M. Judis, S. Schwartz, S.J. James, P.A. Jacobs and N.S. Thomas, Maternal folate polymorphisms and the etiology of human nondysjunction, *Am J Hum Genet* **69** (2001), 434–439.
- [16] D. Hernandez and E.M.C. Fisher, Down syndrome genetics: unravelling a multifactorial disorder, *Hum Mol Genet* **5** (1996), 1411–1416.
- [17] 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.
- [18] 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.
- [19] H. Kowa, K. Yasui, T. Takeshima, K. Urakami, F. Sakai and K. Nakashima, The homozygous C677T mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for migraine, *Am J Med Genet* **96**(6) (2000), 762–764.
- [20] M.L. Martinez-Frias, B. Perez, L.R. Desviat, M. Castro, F. Leal, L. Rodriguez, E. Mansilla, M.L. Martinez-Fernandez, E. Bermejo, E. Rodriguez-Pinilla, D. Prieto and M. Ugarte, ECEMC Working Group (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**(9) (2006), 987–997.
- [21] M. Mooij, T. Steegers, C. Thomas, W. Doesburg and T. Eskes, Periconceptional vitamin profiles are not suitable for identifying women at risk for neural tube defects, *J Nutri* **123** (1993), 197–203.
- [22] V.B. O’Leary, A. Parlie-McDermott, A.M. Molloy, P.N. Kirke, Z. Johnson and M. Colney, MTRR and MTHFR polymorphism; Link to Down syndrome, *Am J Med Genet* **107** (2002), 151–155.
- [23] 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.
- [24] 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.
- [25] 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**(7) (2006), 409–416.
- [26] J.A. Schneider, D.C. Rees, Y.T. Liu and J.B. Clegg, Worldwide distribution of a common methylenetetrahydrofolate reductase mutation, *Am J Hum Genet* **62**(5) (1998), 1258–1260.
- [27] G. Smith and J. Berg, Down's anomaly. 2nd ed. Churchill Livingstone, Edinburgh and New York.
- [28] 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.
- [29] N.M. van der Put, F. Gabreels, E.M. Stevens, J.A.M. Smeitink, F.J.M. T.K.A.B. Trijbels, T.K.A.B. 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.
- [30] I. Weisberg, P. Tran, B. Christensen, S. Sibanis and R. Rozen, A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity, *Mol Genet Metab* (1998), 169–172.
- [31] K. Yanamandra, J.A. Bocchini Jr. and T.F. Thurmon, Absence of association of fetal MTHFR C677T polymorphism with prenatal Down syndrome pregnancies, *Europ J Hum Genet* **11** (2003), 5.