

## Association between 5,10-Methylenetetrahydrofolate Reductase C677T and A1298C Polymorphisms and Conotruncal Heart Defects

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**Reports related some polymorphisms of the 5,10-methylenetetrahydrofolate reductase (MTHFR) to folate-dependent neural tube defects. In view of the common origin of the cells involved both in neural tube closure and heart septation, we analyzed the MTHFR C677T and A1298C polymorphisms in mothers of children with conotruncal heart defect (CD) and in their offspring to evaluate the association between the MTHFR genotype and the risk of CD. We genotyped 103 Italian mothers with CD offspring, 200 control mothers, 103 affected children and their fathers by restriction fragment length polymorphism analysis. No increased risk was observed for the prevalence of the 677TT genotype by itself in affected children and in their mothers. The combined maternal 677TT/1298AA and 677CC/1298CC genotypes have odds ratio of 1.73 and 1.85, respectively. The prevalence of 1298CC genotype in the affected children gives odds ratio of 1.90, that becomes 2.31 for the 677CC/1298CC genotype. However, none of the odds ratios was statistically significant. We observed a higher frequency of the 677T allele in Italy than in other European countries. No association has been demonstrated between the 677TT MTHFR genotype and CD.** Clin Chem Lab Med 2003; 41(3):276–280

**Key words:** Conotruncal heart defects; Methylenetetrahydrofolate reductase; Gene polymorphisms; Restriction fragment length polymorphism.

**Abbreviations:** CD, conotruncal heart defects; Hcy, homocysteine; MTHFR, methylenetetrahydrofolate reductase; NTD, neural tube defects.

### Introduction

Numerous congenital heart defects have a multifactorial etiology, resulting from the interaction between genetic predisposition and environmental factors, such

as nutritional deficiency or exposure to teratogenic substances (1). Conotruncal heart defects (CD), a group of severe heart malformations involving the cardiac outflow tract, aortic arch, *ductus arteriosus*, and proximal pulmonary arteries, account for 20% to 30% of all congenital heart disease (2) and include transposition of the great arteries, tetralogy of Fallot, double-outlet right ventricle, persistent truncus arteriosus, and aortic arch anomalies. A specific neural crest cell population, named cardiac neural crest, is responsible for the morphogenesis of the outflow region of developing heart, which originates in a site conterminus with the site of neural tube closure (3). Studies of DiGeorge syndrome and other CD in humans always assume a neural crest origin of the defects. The cardiac neural crest is required for normal cardiovascular morphogenesis (4).

A periconceptual multivitamin therapy, including folic acid, reduces the risk of congenital malformations, such as cardiovascular defects, urinary tract defects (5), and neural tube defects (NTD) such as spina bifida (6). The positive effects of multivitamin use on the risk of CD have been also assessed (7). Although the mechanism by which folic acid exerts its protective effect is unclear, the teratogenic process that results from folate insufficiency may be related to hyperhomocysteinemia. The interest for a disturbed homocysteine (Hcy) metabolism in relation to NTD was raised by the observation of elevated Hcy levels in mothers of a NTD child (8). Moreover, maternal hyperhomocysteinemia may be a risk factor for congenital heart defect too (9).

An important enzyme involved in the Hcy metabolism is the methylenetetrahydrofolate reductase (MTHFR); the *MTHFR* gene is located on chromosome 1 at 1p36.3 (10). The major product of the *MTHFR* gene is a catalytically active 77 kDa protein, catalyzing the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the major circulating form of folate. Two common genetic polymorphisms associated with a reduced MTHFR activity have been identified. The C677T polymorphism is located on exon 4 at the folate-binding site and results in an alanine-to-valine substitution (11). In healthy homozygous subjects, 677TT genotype is associated with higher total Hcy and lower folate plasma level. The other polymorphism (A1298C) is on exon 7 within the presumptive regulatory domain and results in a glutamate-to-alanine change. Heterozygosity and homozygosity are associated neither with higher total Hcy nor with lower folate plasma concentration (12).

The 677TT mutation has been considered one of the first common genetic risk factors for NTD (12, 13), even though some authors failed to find a significant association (14). MTHFR 1298CC genotype has been already

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described as a risk factor for NTD (12) also in Italian population (15) even though the results were controversial (16). To our knowledge, few studies have addressed the association between MTHFR genotypes and the risk of congenital anomalies other than NTD. Maternal C677T polymorphism has been investigated for its associations with human meiotic nondisjunction with controversial results (17, 18). A significant association with the development of structural congenital heart malformation has been also reported (19, 20).

In view of the common origin of the cells involved both in neural tube closure and heart septation, we hypothesized that the MTHFR genotype, already associated with NTD, might be also a risk factor for CD. To assess the association between these polymorphisms and CD insurgence, we analyzed the MTHFR C677T and A1298C polymorphisms in mothers of children affected by CD and in the babies themselves. Fathers of affected children were also genotyped.

## Materials and Methods

### Patients and controls

One hundred and three CD-affected children (cases) and their parents (case mothers and case fathers), all Caucasian Italian individuals from all Italy, were selected as a consecutive series, attending the "G. Pasquinucci" Hospital (Massa, Italy) and the Department of Medical Genetics (Ferrara, Italy). These individuals were enrolled in our study between June 2000 and December 2001. Table 1 reports the specific conotruncal congenital heart disease in the studied cases: 11 of them presented the 22q11.1 deletion, which is related to CD (1). The other cases were not affected by any chromosomal anomalies or relevant clinical features other than CD. Mean age of the case mothers was  $30 \pm 4$  years; the control mothers (controls) consisted of 200 healthy Caucasian Italian women (mean age

**Table 1** Conotruncal heart defects affecting the studied children.

Pathology	Subjects (no.)
Malalignment ventricular septal defect	6
Tetralogy of Fallot	30
Double-outlet right ventricle	14
Pulmonary atresia and ventricular septal defect	17
Transposition of great arteries	24
Persistent truncus arteriosus	6
Double-outlet right ventricle and transposition of great arteries	1
Tetralogy of Fallot and right aortic arch	1
Interrupted aortic arch type A and residual ventricular septal defect	1
Tetralogy of Fallot and right aortic arch and dextrocardia	1
Single ventricle and transposition of great arteries and ventricular septal defect	1
Aortic coarctation and malalignment ventricular septal defect	1
Total	103

$31 \pm 5$  years) who had children unaffected by congenital disease (diagnosis made at birth). Other environmental factors were not available.

Since some studies suggested that the 677T variant is a genetic risk factor for pre-eclampsia and other maternal obstetric complications (21), women who had had pre-eclampsia during the pregnancy were excluded from the study.

Written informed consent was obtained from each subject (parents gave it for minor children). The study was approved by the local Ethical Committee.

### Polymorphism detection

Peripheral blood samples were collected from cases and controls, and DNA was extracted using the Nal method (22). The genotyping protocol used for detection of the MTHFR C677T polymorphism was previously described by Skibola *et al.* (23) and followed by several modifications. Briefly, 50 ng of DNA was amplified with 62.5 ng of each specific primer (M-Medical Genenco, Firenze, Italy) in a final volume of 25  $\mu$ l. PCR thermal cycling conditions were: 2 min denaturation period at 95 °C and 30 cycles at 95 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s, followed by a 10 min extension at 72 °C. The 198 bp PCR products were verified on 2% agarose-0.003% ethidium bromide gel. PCR reaction underwent *Hinfl* (Pharmacia Biotech Italia, Milano, Italy) enzymatic restriction. The presence of a thymine in the 677 nucleotide creates a *Hinfl* restriction site. Digestion products were verified on a 6% polyacrylamide gel stained with ethidium bromide. Wild-type (677 CC) produced a single band of 198 bp, heterozygotes (677 CT) produced 198, 175, and 23 bp fragments, while homozygous mutants (677 TT) produced 175 and 23 bp fragments.

In order to analyze the A1298C polymorphism, 50 ng of DNA was amplified with 62.5 ng of each specific primer (M-Medical Genenco, Firenze, Italy) as reported by van der Put *et al.* (12). The 241 bp PCR products were verified on 2% agarose-0.003% ethidium bromide gel. The glutamate-to-alanine substitution abolishes an *MbolI* restriction site. *MbolI* (Pharmacia Biotech Italia, Milano, Italy) digestion products were separated on a 6% polyacrylamide gel stained by ethidium bromide. Wild-type (1298 AA) produced two fragments, one consisting of 204 bp and one of 37 bp. For the homozygous mutant genotype (1298 CC), only the 241 bp fragments were obtained, while for the heterozygous genotype (1298 AC) all three were found.

### Statistical analyses

Allele frequencies were calculated for each polymorphism. Differences in allele frequencies and genotype distribution between case mothers, case fathers, cases, and controls respectively were calculated using a chi-square test by Statview Software (SAS Institute Inc., Cary, NC, USA, Second Edition 1998). In order to measure the association between MTHFR genotype and a CD affected pregnancy, odds ratios of all genotypes, as compared with the wild-types, were calculated by the Statview Software.

## Results

### 677T and 1298C allele frequencies

The distribution of the MTHFR C677T and A1298C alleles in controls was compatible with the Hardy-Weinberg equilibrium. Table 2 indicates the C677T and A1298C allele frequencies for CD cases, their parents, and controls, respectively. The percentages of observed fre-

quency for 677T allele were 46% for CD cases, 48% for their mothers and fathers, and 47% for controls. The frequency of 1298C allele was 33% for CD patients, 30% for their mothers, 29% for their fathers, and 28% for controls. No statistically significant differences were observed in the distribution of the mutated allele in CD patients or in their parents when compared with controls.

#### C677T and A1298C genotype frequencies

Table 3 indicates the distribution of MTHFR genotypes in CD cases, their parents and controls, respectively. The percentages of observed frequency for 677TT genotype were 20% for CD patients, 22% for their mothers, 18% for their fathers, and 20% for controls, respectively. The frequency of the 1298CC genotype was

**Table 2** 677T allele and 1298C allele frequencies in cases, case parents, and controls.

	CD case mothers % (no.)	CD cases % (no.)	CD case fathers % (no.)	Controls % (no.)
<i>C677T</i>				
C	52 (107)	54 (111)	52 (104)	53 (212)
T	48 (99)	46 (95)	48 (96)	47 (188)
<i>A1298C</i>				
A	70 (144)	67 (137)	71 (143)	72 (288)
C	30 (62)	33 (69)	29 (57)	28 (112)

**Table 3** Prevalence of the MTHFR C677T and A1298C polymorphisms in CD cases, their parents, and controls.

	CD case mothers % (no.)	CD cases % (no.)	CD case fathers % (no.)	Controls % (no.)
<i>C677T genotype</i>				
CC	26 (27)	27 (28)	22 (22)	26 (52)
CT	52 (53)	53 (55)	60 (60)	54 (108)
TT	22 (23)	20 (20)	18 (18)	20 (40)
<i>A1298C genotype</i>				
AA	48 (49)	43 (45)	50 (50)	50 (101)
AC	45 (46)	46 (47)	43 (43)	43 (86)
CC	7 (8)	11 (11)	7 (7)	7 (13)
<i>Combined genotypes</i>				
677CC/1298AA	3 (3)	3 (3)	3 (3)	4 (9)
677CC/1298AC	15 (16)	15 (15)	12 (12)	15 (30)
677CC/1298CC	8 (8)	10 (10)	7 (7)	7 (13)
677CT/1298AA	22 (23)	21 (22)	30 (30)	26 (52)
677CT/1298AC	29 (30)	32 (33)	30 (30)	28 (56)
677TT/1298AA	22 (23)	19 (20)	18 (18)	20 (40)
Total (no)	103	103	100	200

11% for CD patients and 7% for their mothers, in case fathers, and in controls, respectively. No statistically significant differences were found in these frequency distributions for CD patients or their parents when compared with controls.

#### C677T and A1298C combined genotype frequencies

The prevalence of MTHFR C677T/A1298C combined genotypes in CD patients, their parents, and controls are reported in Table 3. The three genotypes 677CT/1298CC, 677TT/1298AC, and 677TT/1298CC were not found. The frequency of the 677TT/1298AA genotype was 19% in cases, 22% in case mothers, 18% in case fathers, and 20% in controls, respectively. In agreement with other authors, we observed that an individual with 677TT genotype always had a 1298AA genotype (12) but not *vice versa*, and that an individual with the 1298CC genotype always had a 677CC genotype but not *vice versa*. No statistically significant differences were observed in the combined genotype distribution for CD patients and their parents when compared with controls.

None of the calculated odds ratios were statistically significant.

## Discussion

We evaluated the possible association between the MTHFR C677T and A1298C polymorphisms and the development of CD through the analysis of 103 affected children and their parents. Particularly, our interest was focused on maternal genotype. This is because it has been reported that maternal genetic effects occur when a genetically mediated maternal phenotype influences the phenotype of offspring. With respect to offspring, maternal genetic effects behave as environmental risk factors (24).

In agreement with other studies (25, 26), we found that both allelic and genotypic frequencies of the C677T polymorphism are higher in Italy than in other European countries.

The calculated odds ratios (Table 4) show that no higher risk of having a CD offspring is associated with maternal 677TT MTHFR genotype, while mothers with the 1298CC genotype have an odds ratio of 1.27 (CI 95%: 0.49–3.26). Besides, considering the interaction between the two genotypes, the combined maternal 677TT/1298AA genotype gives an odds ratio of 1.73 (CI 95%: 0.42–7.02). The 1298CC genotype in children gives an odds ratio of 1.90 (CI 95%: 0.79–4.56), while the heterozygous 1298AC by itself and in combination with the 677CC or CT gives an odds ratio > 1 although it is not statistically significant. The odds ratio is 2.31 (CI 95%: 0.49–10.82) when the genotype is 677CC/1298CC.

It is important to note that all the observed odds ratios do not reach statistical significance. A possible explanation could be the relatively small size of the different categories of genotypes as well as the small number of "wild-type" alleles (677CC/1298AA genotype frequency is only 4%).

**Table 4** Calculated odds ratios with 95% CI of the MTHFR polymorphisms in the investigated subjects.

	CD case mothers		CD cases		CD case fathers	
	OR	CI 95%	OR	CI 95%	OR	CI 95%
C677T genotype						
CC (1)	–	–	–	–	–	–
CT	0.95	0.54–1.67	0.95	0.54–1.66	1.31	0.73–2.37
TT	1.11	0.55–2.21	0.93	0.46–1.88	1.06	0.50–2.25
A1298C genotype						
AA (1)	–	–	–	–	–	–
AC	1.10	0.67–1.81	1.23	0.74–2.02	1.01	0.61–1.66
CC	1.27	0.49–3.26	1.90	0.79–4.56	1.09	0.41–2.90
Combined genotypes						
677 CC/1298AA (1)	–	–	–	–	–	–
677 CC/1298AC	1.60	0.38–6.76	1.50	0.35–6.37	1.20	0.28–5.21
677 CC/1298CC	1.85	0.38–8.93	2.31	0.49–10.82	1.62	0.33–7.98
677 CT/1298AA	1.33	0.33–5.36	1.27	0.31–5.14	1.73	0.44–6.89
677 CT/1298AC	1.61	0.40–6.39	1.77	0.45–7.00	1.61	0.40–6.39
677 TT/1298AA	1.73	0.42–7.02	1.50	0.37–6.16	1.35	0.33–5.59

(1) Reference category

However, a further factor must be taken into account in explaining these findings: in the Mediterranean populations, the detrimental action of 677T allele on the total phenotype may be attenuated, or even eliminated, by a large intake of folate throughout life. A study in Spanish population showed that early folate treatment for all pregnant women seems to influence the MTHFR genotype frequencies (27). In effect, the Mediterranean diet may influence differences in genotype frequencies between northern and southern Europe. The association between the risk of NTD and 677TT genotype was revealed first in the Dutch population. In Italy, the frequency of NTD is not higher than in other countries, such as The Netherlands and France (28), in spite of the increased frequency of the C677T MTHFR polymorphism. Therefore, the Mediterranean diet, which ensures greater intake of folate than other nutritional styles, may act as a protective factor, masking in some way the negative effect of this polymorphism on the MTHFR activity. Unfortunately, our study is limited by the lack of data on folate and Hcy plasma levels.

Finally, there was no association between the 677TT MTHFR genotype and CD. To clarify whether the altered metabolism of Hcy could be a risk factor for CD, further studies of other enzymes involved are required.

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