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Association between *MTHFR* C677T variant and risk for congenital heart defects in Egyptian children: a case–control study including meta-analysis based on 147 cases and 143 controls

Nora N. Esmaiel[1*], Engy A. Ashaat[2], Ghada M. Al-Ettribi[4], Alaaeldin Fayez[1], Sonia A. Alsaiedi[3] and Mona O. El Ruby[2]

Abstract

Background Stratification analysis studies showed that ethnicity has a significant association regarding *MTHFR* C677T variant and congenital heart diseases (CHDs) risk, and many published studies have controversial conclusions toward this association.

Methods In this study, the association between the *MTHFR* C677T variant and the risk for CHDs was evaluated in 91 children with CHD and 95 healthy controls, as new cases, by using restriction fragment length polymorphism (RFLP) technique. Besides that, 2 case–control studies in the Egyptian population published before 2021 were included in this meta-analysis. The association was assessed by the odds ratio (OR) with a 95% confidence interval (CI) based on 294 alleles in CHD cases and 286 alleles in controls.

Results The overall meta-analysis showed a significant association between *MTHFR* C677T variant and CHDs risk in Egyptian children with heterogeneity (Heterogeneity = 0.001) in all the genetic models with the highly significant association in T versus C allele (pooled OR 1.89, 95% CI 1.31–2.74; p value < 0.0004). The consistency of the genotypes was detected by Hardy–Weinberg equilibrium (HWE).

Conclusions Our results support the *MTHFR* -677T allele as a susceptibility factor for CHDs in the Egyptian pediatric patients.

Keywords Congenital heart defects, *MTHFR* C677T, Meta-analysis

Background

A congenital heart defect is a common heart disease leading to a high mortality rate in newborns [1]. With an estimated prevalence rate of 8.6–10.3 per 1000 live births and growing, congenital heart disease (CHD) is the most prevalent birth defect [2]. Folic acid considers a key player in the development of the cardiovascular system, where reduced maternal folic acid leads to elevated homocysteine in the blood as a toxic material, which has been described as a potential risk factor for CHDs [3–5].

*Correspondence:
Nora N. Esmaiel
besmkallahom@hotmail.com
[1]Molecular Genetics and Enzymology Department, Human Genetics and Genome Research Institute, National Research Centre, Giza, Egypt
[2]Clinical Genetics Department, Human Genetics and Genome Research Institute, National Research Centre, Giza, Egypt
[3]Pediatric Cardiology Department, Cairo University, Giza, Egypt
[4]Medical Molecular Genetics Department, Human Genetics and Genome Research institute, NRC: National Research Centre, Giza, Egypt

The 5,10-methylenetetrahydrofolate reductase (*MTHFR*), encoded by the *MTHFR* gene, is an important enzyme in homocysteine metabolism, converting 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate in the process of methyltetrahydrofolate, and the *MTHFR* C677T variant reduces the activity of the *MTHFR* enzyme increasing plasma homocysteine level. The homozygous 677TT and heterozygous 677CT genotypes lead to approximately 30% and 65% of the enzyme activity reduction in *MTHFR*, respectively, compared with the 677CC genotype [6].

Although there are enormous studies that have been pointed to a relationship between C677T and the risk of CHD, the conclusion is still inconsistent, especially regarding the participants' ethnicity, sample size, and limited statistical analysis. On the Egyptian population, there are three distinct case–control studies that were done and published on the PubMed database [7–9]. Thus, to provide more consistency and comprehensive results, an updated meta-analysis approach was applied in the current study for all published data (until Jan 2021) and our cases data of Egyptian conotruncal heart and cardiac septal defects patients including 900 alleles (454 patients alleles and 446 controls one) to evaluate the association between *MTHFR* C677T variant and CHD susceptibility.

Methods

Samples collection

Our prospective study included 91 children with CHDs and 95 controls. Participants were recruited from the Clinical Genetics Clinic, National Research Centre. The study was approved by the Ethics Committee of National Research Centre (NRC) under Decision No. 19257, and a written informed consent was obtained from the parents. Patients with known syndromic CHDs were excluded. All participants were examined for general appearance and genetic syndromes. Full anthropometric measurements including weight, length, and skull circumference were recorded. Full clinical and cardiac examinations were conducted for each case including personal and family history, pedigree analysis, and clinical examination for all systems with special emphasis on heart evaluation. Cardiac investigations were done including chest x-ray, Echocardiogram, and ECG for each case. Conotruncal heart and cardiac septal defects were diagnosed based on clinical evaluation and cardiac investigations. Our control group was selected as healthy children who were not appearing observational syndromic features by clinicians' examination or cardiac anomalies by ECHO investigation.

MTHFR C677T genotyping by PCR–RFLP

Venous blood was collected into EDTA vacutainer tubes and was stored in − 80C. DNA was extracted by iNtRON G-spin total DNA extraction kit (50 preps), catalogue number 17045, Korea (https://www.intronbio.com/eg/). The C677T polymorphism was genotyped by PCR–RFLP. To determine the genotype of the *MTHFR* gene, genomic DNA was amplified through PCR by using specific primers. The *MTHFR* C677T primer sequences are as follows: forward, 5′-CATCCCTATTGGCAGGTTACCC -3' and reverse, 5'- GGGAAGAACTCAGCGAACTCAG -3'. The PCR amplifications were performed in a total volume of 35 µL, which consisted of 10 pmol of each primer, Master Mix and template DNA. Thermal cycling conditions were 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 3 min. The PCR products were digested with 2–4 units of restriction enzyme, Hinf I at 37°C for 1 h in 10 × Fast Digest Buffer at a final volume of 30 µL. The restriction patterns of the PCR products were determined via separation on 3% ethidium bromide agarose gels according to Table 1.

Identification and eligibility of relevant studies

We conducted an electronic search for relevant articles published before Jan 2021 in PubMed databases with the combination of the following terms: "congenital cardiac/heart defects," "conotruncal heart defects" "MTHFR," "polymorphism/mutation or variant," "C677T" and "Egypt or Egyptian."

Data extraction of the eligible studies

All data were extracted independently by two authors (Fayez, Alaaeldin, and Esmaiel, Nora). And a third investigator reviewed the result. The extraction of eligible study data included the following: a first author, publication year, Egypt country of origin, Arabian ethnicity, number of cases and controls, genotype frequency, counts of alleles in case and control groups, Hardy–Weinberg equilibrium (HWE), PCR–RFLP method only to eliminate method effect, and case–control studies from 2010 to 2021. Animal studies, reviews, short communication, case reports, and mixed population were

Table 1 The sizes of DNA fragments formed after a restriction digestion with Hinf I enzyme

Genotype	CC (normal allele) bps	CT (heterozygous allele) bps	TT (homozygous allele) bps
Digested pattern	318	318, 227, 91	227, 91

excluded. To support our results reliability, the studies with deviated genotype frequency distribution according to recalculated HWE score were not eligible to pass in the further statistical analysis.

Statistical analysis

In each of the included studies, we calculated again Hardy–Weinberg equilibrium (HWE) in controls of each included study using a Chi-square goodness of fit test in order to test the hypothesis that the observed genotype frequencies do not differ from their expected values, $P \leq 0.05$ as considered significant level. We calculated odds ratios (ORs) and its 95% confidence intervals (95% CIs) to estimate the associations between *MTHFR* polymorphisms and CHDs susceptibility. The pooled ORs and 95% CIs were calculated in five genetic models: allele model (T versus C), heterozygote model (TC versus CC), homozygote model (TT versus CC), dominant model (TT + TC versus CC), and recessive model (TT versus TC + CC). The significance of the pooled OR was considered statistically significant at $P \leq 0.05$. Heterogeneity across the eligible studies was tested using x2-based Cochran' Q-test in which heterogeneity was considered significant at $P < 0.1$; When the effects were assumed to be homogeneous, the fixed-effects model was used; otherwise, the random-effects model was more appropriate. All analyses were performed using SPSS software, release 18.0.0

Table 2 The cardiac diagnosis of our clinically examined cases

CHD type* (*n* = 91)	Number	Approx. percent (%)
AVSD	8	8.8
ASD	10	11
VSD	9	10
Complete A-V canal	6	6.5
Complete A-V canal, ASD or VSD	9	10
TGA, ASD	7	7.6
DORV, ASD or VSD	8	8.8
ASD, valvular aortic stenosis	3	3.3
TOF	21	23
VSD, PS	10	11
Total	91	100

* *AVSD* atrioventricular septal defect, *ASD* atrial septal defects, *VSD* ventricular septal defects, *A-V* canal; atrioventricular canal, *TGA* transposition of the great arteries, *DORV* double outlet right ventricle, *TOF* Fallot's tetralogy, *PS* pulmonary stenosis

Results

Characteristics of the included participants and studies

The cardiac diagnosis of our clinically examined participants is listed in Table 2. Our literature search identified 3 publications according to the inclusion term criteria. According to recalculated HWE scores as shown in Table 3, we removed Zidan et al. [8] study because its genotype distributions in the control group were deviating from HWE law. Therefore, two out of three retrieved publications were considered in the followed meta-analysis work. Thus, the participants in this meta-analysis study involved pooled 147 (294 alleles) cases and 143 (286 alleles) controls. The included cases involve two categories of CHDs (conotruncal heart defects with septal defects (mixed) and conotruncal heart defects only). In El-Abd et al. [9] and Kotby et al. [7], the equilibrium of the genotype distributions in controls was detected according to Hardy–Weinberg Equilibrium (HWE) test, and all values are shown in Table 3. Consideration of patients' sex and age comparison was not permitted because that the descriptive data of the study population regarding sex and age were not completely cited in all included publications.

Table 3 The detailed characteristics of cases in the eligible studies

Author/participants	Year	CASES					CONTROL					HWE test*	
		CC	CT	TT	C	T	CC	CT	TT	C	T	Chi-square	*P* value**
El-Abd et al./Mixed***	2012	7	12	7	26	26	13	5	0	31	5	0.47	0.49
Zidan et al./Mixed	2013	18	21	41	57	103	32	21	27	85	75	17.04	**0.000**
Kotby et al./ CHD****	2012	12	14	4	38	18	20	8	2	48	12	0.852	0.653
The current study/Mixed	2021	42	41	8	125	57	55	34	6	144	46	0.046	0.977
Pooled studies *****	–	61	67	19	189	105	88	47	8	223	63	0.250	0.882

*Hardy–Weinberg equilibrium (HWE) in controls was assessed using a Chi-square goodness of fit test

**$P \leq 0.05$ considered statistically significant. The bold *p* values are significant as appeared

***"Mixed" means that the authors' subjects include conotruncal heart and cardiac septal defects in one study

****CHD refers here to Conotruncal Heart Defect

*****The pooled study includes all except Zidan et al. study

The association between *MTHFR* C677T variant and CHDs risk; T allele is a risk factor for CHDs

Three digestion patterns of the amplified *MTHFR* C677T fragment in our cases shown in Fig. 1. Through extracted datasets from our participants and 2 studies of the *MTHFR* C677T polymorphism in Egyptian CHDs children and underestimated T versus C frequency, significant heterogeneity was detected ($P_{heterogeneity} < 0.01$), thus the random-effects model was employed. Compared to the C allele, the T allele conferred a pooled OR of 1.89 (95% CI 1.31–2.74 at p value = 0.0004) in the allelic model (Fig. 2). To assess the effect of each study on the pooled OR estimate, a sensitivity analysis was applied. The result showed the pooled ORs for the allelic model were similar before and after elimination of each study (Table 4). Investigating the potential source of heterogeneity was not done to limit the included publications and their characteristic data. Nevertheless, significant associations between *MTHFR* C677T polymorphism and the risk of CHDs were observed as shown in Table 5.

Discussion

CHDs are serious genetic conditions with almost unknown etiology, and there are no established strategies for reducing their public health impact. Single nucleotide polymorphisms (SNPs) in several genes, such as *Notch1*, *GATA4*, *NKX2-5*, *TBX5*, and others, have been shown to be significantly linked to the risk of CHDs. However, the *MTHFR* gene is one of the commonly associated potential genes with the risk of CHDs [2].

MTHFR gene encodes a active catalytic 77 kDa protein converting 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the major circulating form of folate [6]. So, the *MTHFR* enzyme activity regulates 5-methyltetrahydrofolate synthesis, and hence, the process of remethylation of homocysteine to methionine is affected inducing hyperhomocysteinemia. Specifically, the *MTHFR* C677T variant leads to increase plasma homocysteine [10, 11], where C677T is one of the common variants in *MTHFR* gene located at the folate binding site [12]. The folate–homocysteine metabolic pathway has role in neural tube formation and cardiogenesis, where low folate and high homocysteine levels are a closely related to congenital heart defects traits [13]. Therefore, we performed a case–control study including 186 participants and an updated meta-analysis on Egyptian CHD patients. Our selection to encompass our original study with subsequent meta-analysis due to being meta-analysis integrates results from the original and subsequent replication studies increasing the statistical power to detect gene–disease associations.

Kapusta et al. [14] concluded a first report that maternal hyperhomocysteinemia is correlated with an increased risk of CHDs. Hobbs et al. [15] studied mothers whose pregnancies were affected by congenital heart defects, and they found that homocysteine,

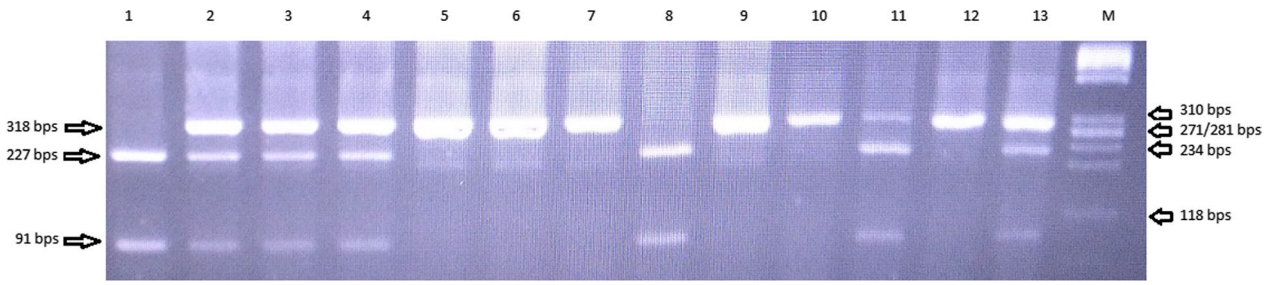

Fig. 1 Digestion patterns of 318 bps amplicon containing C677T site showing (i) normal allele pattern (677CC) with intact 318 bps fragment in cases 5, 6, 7, 9, 10, and 12, (ii) heterozygous mutant allele pattern (C677T) with 318, 227, and 91 bps fragments in cases 2, 3, 4, 11, and 13, (iii) homozygous mutant allele pattern (677TT) with 227 and 91 bps fragments in cases 1 and 8. (M) lane represents ΦX174 DNA/HaeIII Marker

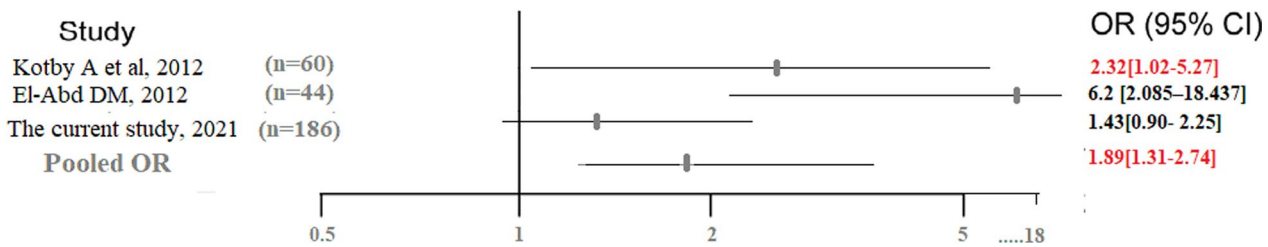

Fig. 2 The ORs with its 95% CIs for the MTHFR C667T in children for congenital heart defects according to allele frequency T versus C. Random-effects pooled OR = 1.89, 95%CI = 1.31–2.74, $P = 0.0004$, $P_{heterogeneity} = 0.001$, $I^2 = 97.4\%$

Table 4 Association between *MTHFR* C677T variant and CHDs Risk across the selected cases

Author	**El-Abd et al.**		**Kotby et al.**		**The current study**	
CHDs population	**Mixed***		**Conotruncal heart defects**		**Mixed**	
Models of inheritance	**Cases** ***N*** **= 26**	**Controls** ***N*** **= 18**	**Cases** ***N*** **= 30**	**Controls** ***N*** **= 30**	**Cases** ***N*** **= 91**	**Controls** ***N*** **= 95**
CT + TT CC Dominant model	19 7	5 13	18 12	10 20	49 42	40 55
OR [CI 95%] *P* value	7.06 [1.83–27.15] 0.005		3.00 [1.05–8.60] 0.0410		1.60 [0.90–2.86] 0.1099	
CT CC Heterozygote model	12 7	5 13	14 12	8 20	41 42	34 55
OR[CI 95%] *P* value	4.46 [1.11–17.90] 0.0351		2.92 [0.95–8.99] 0.0623		1.58 [0.86–2.90] 0.1399	
TT CT + CC Recessive model	7 19	0 18	4 26	2 28	8 83	6 89
OR[CI 95%] *P* value	14.23 [0.76–267.20] 0.0759		2.15 [0.36–12.76] 0.3980		1.42 [0.48–4.29] 0.5241	
TT CC Homozygote model	7 7	0 13	4 12	2 20	8 42	6 55
OR[CI 95%] *P* value	27.00 [1.35–541.60] 0.0312		3.33 [0.53–21.03] 0.2002		1.75 [0.56–5.42] 0.3346	
T C	26 26	5 31	18 38	12 48	57 125	46 144
OR [CI 95%] *P* value	6.2 [2.08–18.44] 0.001		2.32 [1.02–5.27] 0.05		1.43 [0.90–2.25] 0.126	
Phet	0.001		0.002		0.001	

*"Mixed" means the authors' subjects include conotruncal heart and cardiac septal defects in one study

Table 5 Stratified analyses of the *MTHFR* C667T polymorphism in association with CHDs risk under the allelic model

Models of inheritance	**2012–2021**		**Association test**	**Heterogeneity test**			**Effect model**
	Cases ***N*** **= 147**	**Control** ***N*** **= 143**	**OR[CI 95%]** ***P*** **value**	***Q***	P_{het}	I^2* (%)	
CT + TT CC Dominant model	86 61	55 88	2.26 [1.41–3.61] 0.0003	27.00	< 0.01	96.3	Random
CT CC Heterozygote model	67 61	47 88	2.01 [1.25–3.38] 0.0021	26.00	< 0.01	96.2	Random
TT CT + CC Recessive model	19 128	8 135	2.50 [1.06–5.92] 0.018	11.00	< 0.01	91	Random
TT CC Homozygote model	19 61	8 88	3.42 [1.41–8.33] 0.0033	11.00	< 0.01	91	Random
T C	101 189	63 223	1.89 [1.31–2.74] 0.0004	38.00	< 0.01	97.4	Random

*$I^2 = 100\% * (Q - df)/Q$

S-adenosylhomocysteine, and methionine are most important biomarkers predictive of case or control status. Recently, Raina et al. [16] pointed that a significant role of MTHFR C677T in increased risk of CHD. It is importantly that Newborns can be protected from various congenital abnormalities by maternal uptake folic acid supplement, and this including decrease CHD risk by 40–60% [6].

Our recent meta-analysis demonstrated that the *MTHFR* C677T variant is associated with the risk of congenital heart defects in Egyptian neonatal patients. Across Egyptian CHD patients, several case–control studies were performed on the association of *MTHFR* variants with CHDs susceptibility, out of the three studies recruited Egyptian CHDs children [7–9], showing uninformative results. Although the genotyping bias was detected in two studies out of them, associations between infant *MTHFR* C677T variant and the risk of CHDs were detected across all genetic inheritance models. The mode of action of the *MTHFR* C677T variant might be different across ethnicities according to what was reported in Wang [17].

To elucidate the differential effect of C677T variant across multiple ethnicities, we retrieved the recent relevant publications across different ethnicities. Elizabeth et al. [18] reported that an association between the *MTHFR* C677T variant is high risk in Indians CHD patients especially in South Indians, whereas Raina et al. [16] concluded lack of association for MTHFR C677T with risk of CHD in Jammu & Kashmir Indians. Mamasoula [19] analyzed *MTHFR* C677T genotyping on 5814 CHD cases and 10,056 controls in the European and Australian populations across meta-analysis study; they found no significant effect of *MTHFR* C677T genotyping on CHD risk. Wang et al. [20] and Shi et al. [21] concluded that maternal MTHFR C677T increase in risk of given birth of a CHDs children and frequency of maternal TT allele was significantly higher in affected group than control one. In Iranian CHD patients, Noori et al. [22] found that TT allele was considered highest risk for CHDs especially VSD phenotype (OR 10, 95% CI 1–92.2, $P=0.04$).

Herein, we intend to include Egyptian children only to exclude the ethnicity effect. Regarding that *MTHFR* C677T variant is relevant to increase the risk of CHD as concluded in the current study, the previous studies showed that *MTHFR* C677T variant may lead to hyperhomocysteinemia and consequently reduced folic acid level causing high risk factor for CHDs [23–25]. Some studies reported that the *MTHFR* C677T variant was just associated with a specific phenotype of CHDs [26, 27], suggesting that the results of the current meta-analysis should be interpreted cautiously.

Based on our meta-analysis, we found that the T allele of the *MTHFR* C677T variant is relevant to increasing the risk of CHDs in all genetic inheritance models, and the association was more significant in the dominant model than the recessive one.

Our selection to gather conotruncal heart and cardiac septal defects in one patients group due to our observation that mixed conotruncal heart and cardiac septal defects patients is comparable with controls in different studies, where in Xuan et al. [28], the authors carried out a meta-analysis for 9329 cases and 15,076 controls which were extracted from 35 publications from year 2001 to 2014 involving different ethnicities. It is worth to mention that our observation revealed that 28 out of 35 publications had been included mixed patients from conotruncal heart and cardiac septal defects; however, the pooled children' genotypes and alleles showed significant association in all C677T model inheritance.

It well known that deviated HWE scores in a human control population can be caused by multiple reasons such as technical problems in genotyping, inbreeding caused by consanguinity, population size effect, natural factors, and hidden population structure, and consistent HWE supports to a real link between the targeted genotypes and the corresponding trait [29]. Herein, except Zidan et al. [8] study, all recalculated HWE scores were consistent supporting potential reliable link between *MTHFR* C677T and CHD.

Conclusions

Our meta-analysis demonstrated a potentially significant association of the MTHFR C677T variant with CHD risk. The current meta-analysis study has limitations as (1) all 3 studies were collected only from the PubMed database, so other relevant studies might be cited in other databases, (2) the current study included the Egyptian CHDs children only without their mothers, (3) all 3 studies have limited information regarding cases characterization, (4) several gene variants may act together as haplotype block or protein–protein interaction with MTHFR C677T variant, and finally, (5) there are some hidden factors, such as environmental conditions, lifestyle or gender may affect.

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Author contributions

NN and AG contributed to the study conception and design. EA collected the clinical data and conducted the investigations. MO and SA supervised the full clinical and manuscript revisions. NN and GM done the Genotyping work. NN and AG performed Material preparation, data collection and analysis. AG wrote the first draft of the manuscript. All authors read and approved the final manuscript. All authors have approved the manuscript for submission.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are available upon request.

Declarations

Ethics approval and consent to participate
This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of National Research Centre (Date December 2020../No20177). Written informed consent was obtained from the parents.

Consent for publication
Not applicable.

Competing interests
The authors have no relevant financial or non-financial interests to disclose.

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