Association Between C677T Polymorphism of Methylene Tetrahydrofolate Reductase and Congenital Heart Disease

Meta-Analysis of 7697 Cases and 13125 Controls

Chrysovalanto Mamasoula, MSc; R. Reid Prentice, PhD; Tomasz Pierscionek, MB, ChB; Faith Pangilinan, PhD; James L. Mills, MD, MS; Charlotte Druschel, MD, MPH; Kenneth Pass, PhD; Mark W. Russell, MD; Darroch Hall, PhD; Ana Töpf, PhD; Danielle L. Brown, MD; Diana Zelenika, PhD; Jamie Bentham, DPhil; Catherine Cosgrove, PhD; Shoumo Bhattacharya, PhD; Javier Granados Riveron, PhD; Kerry Setchfield, BSc; J. David Brook, PhD; Frances A. Bu’Lock, MD; Chris Thornborough, RGN; Thahira J. Rahman, PhD; Julian Palomino Doza, PhD; Huay L. Tan, PhD; John O’Sullivan, MD; A. Graham Stuart, MD; Gillian Blue, PhD; David Winlaw, MD; Alex V. Postma, PhD; Barbara J.M. Mulder, MD; Aelko H. Zwinderman, PhD; Klaartje van Engelen, MD; Antoon F.M. Moorman, PhD; Anita Rauch, PhD; Marc Gewillig, MD; Jeroen Breckpot, PhD; Koen Devriendt, MD; G. Mark Lathrop, PhD; Martin Farrall, FRCPath; Judith A. Goodship, MD; Heather J. Cordell, PhD; Lawrence C. Brody, PhD; Bernard D. Keavney, DM

Background—Association between the C677T polymorphism of the methylene tetrahydrofolate reductase (MTHFR) gene and congenital heart disease (CHD) is contentious.

Methods and Results—We compared genotypes between CHD cases and controls and between mothers of CHD cases and controls. We placed our results in context by conducting meta-analyses of previously published studies. Among 5814 cases with primary genotype data and 10056 controls, there was no evidence of association between MTHFR C677T genotype and CHD risk (odds ratio [OR], 0.96 [95% confidence interval, 0.87–1.07]). A random-effects meta-analysis of all studies (involving 7697 cases and 13125 controls) suggested the presence of association (OR, 1.25 [95% confidence interval, 1.03–1.51]; P=0.022) but with substantial heterogeneity among contributing studies (I²=64.4%) and evidence of publication bias. Meta-analysis of large studies only (defined by a variance of the log OR <0.05), which together contributed 83% of all cases, yielded no evidence of association (OR, 0.97 [95% confidence interval, 0.91–1.03]) without significant heterogeneity (I²=0). Moreover, meta-analysis of 1781 mothers of CHD cases (829 of whom were genotyped in this study) and 19861 controls revealed no evidence of association between maternal C677T genotype and risk of CHD in offspring (OR, 1.13 [95% confidence interval, 0.87–1.47]). There was no significant association between MTHFR genotype and CHD risk in large studies from regions with different levels of dietary folate.

Conclusions—The MTHFR C677T polymorphism, which directly influences plasma folate levels, is not associated with CHD risk. Publication biases appear to substantially contaminate the literature with regard to this genetic association.

Key Words: congenital heart disease ■ folate ■ genetic association ■ Mendelian randomization ■ MTHFR

Congenital heart disease (CHD) is the most common birth defect. It affects 7/1000 live births and is a major cause of childhood morbidity and mortality worldwide.1 Folic acid has long been hypothesized to be protective against CHD, and folate deficiency is suspected to be a CHD risk factor, but the evidence remains inconclusive.2 Several retrospectively conducted observational epidemiology studies suggest a beneficial effect of periconceptual folate supplementation on CHD risk, but retrospective studies of adverse pregnancy outcomes may be susceptible to recall bias and confounding.3–6 There is only

Received February 26, 2013; accepted July 16, 2013.

The current affiliation for Dr Prentice is Illumina Inc., San Diego, CA.
The online-only Data Supplement is available at http://circgenetics.ahajournals.org/lookup/suppl/doi:10.1161/CIRCGENETICS.113.000191/DC1.

Correspondence to Bernard D. Keavney, DM, British Heart Foundation Professor of Cardiology, Institute of Cardiovascular Sciences, The University of Manchester, 46 Grafton St, Manchester M13 9NT, United Kingdom. E-mail bernard.keavney@manchester.ac.uk or Lawrence C. Brody, PhD, Chief and Senior Investigator, Molecular Pathogenesis Section, Genome Technology Branch, Bldg 50, Room 5306, 50 S Dr, MSC 8004, Bethesda, MD 20892–8004. E-mail lbrody@mail.nih.gov

© 2013 American Heart Association, Inc.

Circ Cardiovasc Genet is available at http://circgenetics.ahajournals.org

DOI: 10.1161/CIRCGENETICS.113.000191

347
The investigation was conducted according to the principles of the Declaration of Helsinki.

### Populations Studied

#### European White Cohort

Cases of CHD were collected from United Kingdom CHD units in Bristol, Leeds, Liverpool, Leicester, Newcastle, Oxford, and London, and from centres in Amsterdam (the Netherlands), Leuven (Belgium), Erlangen (Germany), and Sydney (Australia). All cases were of European white ancestry. Patients with known genetic causes of CHD (eg, Down syndrome, 22q11 deletion syndrome, Noonan syndrome) or known in utero teratogen exposure were excluded from analysis. We did not include families in whom CHD appeared to be segregating as a Mendelian trait. Because any effect of MTHFR genotype on risk of CHD could be mediated by the early in utero environment, which might well be determined chiefly by the mother’s MTHFR genotype, we also collected, where possible, mothers of cases. Publicly available genotypes for 3800 healthy white individuals at MTHFR C677T (rs1801133) were obtained from the Wellcome Trust Case-Control Consortium (WTCCC2) common control panel (http://www.wtccc.org.uk). Additionally, we included 368 healthy European white controls free of CHD ascertained as previously described who were genotyped on both platforms used in this cohort (see below).

#### New York Cohort

This was a population-based, nested case–control study that included all cases born in the State of New York with a CHD during 1997 and 1998. Cases were identified using the New York State Congenital Malformations Registry. In New York, physicians and hospitals are mandated by law to report birth defect cases that come to their attention if the child is under 2 years of age and was born, or resides, in New York State. Cases were selected if they were listed as having a CHD using a modified version of the British Paediatric Cardiac Association code system. Cases with chromosomal abnormalities or other malformations in addition to CHDs were excluded. Controls born in the same interval but free of CHDs were matched to cases on race/ethnicity and sex. Two controls were selected for each case. Information extracted from the Congenital Malformations Registry was linked to the records of the New York State Newborn Screening Program for retrieval of archived residual dried blood spots. DNA was available on >80% of cases listed in the Registry.

### Genotyping

In the European white cohort, MTHFR C677T (rs1801133) was genotyped either on an Applied Biosystems 7900HT Fast Real-Time PCR System (TaqMan) using Sequence Detection System v.2.3 or using the Illumina 660 W-Quad array, which features rs1801133. Genotypes in the WTCCC2 panel of controls were assigned using gene chip technology. To rule out any systematic error from the use of different platforms, 368 additional healthy controls were genotyped using both methodologies to ensure comparability of genotypes between platforms—no discrepancies were observed between TaqMan and array-derived genotypes. In the New York cohort, MTHFR C677T (rs1801133) was genotyped by detection of allele-specific primer extension using matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry (Sequenom, San Diego, CA). In both cohorts, ≥10% of samples were randomly plated a second time and regenotyped. The concordance rate between these replicates was >95%.

### Literature Search

The methods for the literature search are described in the online-only Data Supplement.

### Statistical Analysis

In the principal analyses, we estimated ORs for CHD risk with T/T genotype compared to (C/T+C/C) genotypes, and their 95% CIs, using logistic regression for each study. In subsidiary analyses, chiefly
to facilitate comparison of our results with previous meta-analyses, we considered the allelic model (where C/T genotype would confer intermediate risk between T/T and C/C genotypes), and we also compared T/T with C/C genotypes without consideration of C/T heterozygotes. We considered offspring genotypes and maternal genotypes in separate analyses. We decided a priori to calculate pooled ORs and 95% CIs using the DerSimonian and Laird random-effect model, as we anticipated substantial heterogeneity between the studies, possibly related to interpopulation variability in folate intake or to the previously described heterogeneity in C677T genotype frequencies between different populations. In subsidiary analyses, we used the Mantel–Haenszel method to calculate fixed-effects ORs. We assessed between-study heterogeneity using Cochran Q and also quantified heterogeneity using the I² statistic, which describes the percentage of variation across studies that is due to heterogeneity rather than chance.16 Values of I² of 25%, 50%, and 75% are typically considered to indicate low, moderate, or high levels of heterogeneity. Publication bias was assessed visually using funnel plots of log(OR) against standard error of the OR and formally tested using Egger and Begg tests. To address the possibility that particular CHD phenotypes might be differentially susceptible to any effect of MTHFR C677T genotype, we carried out subgroup analyses among the patients in whom we had primary genotype data in 3 diagnostic subgroups: septal defects (atrial septal defect, ventricular septal defect, and atrioventricular septal defect), conotruncal lesions (chorda terytoria of Fallot, pulmonary stenosis with VSD, pulmonary atresia, and transposition of the great arteries), and left-sided lesions (chorea coarctation of the aorta, aortic stenosis, aortic atresia, patent ductus arteriosus, and left heart hypoplasia). Within these groups, if multiple lesions were present, patients were assigned based on their clinically dominant defect. Cases who could not be classified into 1 of these 3 groups were designated Other—examples of defects so classified would include laterality defects and anomalous drainage of the pulmonary veins. In the subgroup analyses, cases were compared with randomly selected individuals from the control population, in the ratio of 2 controls per case in each subgroup. To make some allowance for multiple testing, we calculated 99% (rather than 95%) CIs for the ORs in these subgroup analyses (ie, imposed a significance threshold of 0.01 rather than 0.05).

We explored sources of heterogeneity, in particular examining the importance of study size, using 2 statistical approaches. First, we used the trim-and-fill method, which assumes funnel plot symmetry to estimate and model the studies missing from the analysis due to publication bias. Second, we used the selection model of Copas, which assumes a relationship between publication probability and the standard error of the estimated OR.17 We examined whether there was a relationship between any risk of CHD associated with TT genotype and folate status in low, medium, and high folate groups of studies and formally tested using Egger and Begg tests. To address the possibility that particular CHD phenotypes might be differentially susceptible to any effect of MTHFR C677T genotype, we carried out subgroup analyses of offspring genotypes.

### Table 1. MTHFR C677T Polymorphism and Risk of CHD in Cases and Controls Genotyped in This Study

<table>
<thead>
<tr>
<th>Population</th>
<th>OR (95% CI), T vs C</th>
<th>OR (95% CI), TT vs CC</th>
<th>Allele Frequencies, Cases</th>
<th>Allele Frequencies, Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>for C</td>
<td>for T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>EU White</td>
<td>1.06 (0.98, 1.13)</td>
<td>1.00 (0.86, 1.16)</td>
<td>1.244</td>
<td>1338</td>
</tr>
<tr>
<td>NY White</td>
<td>0.94 (0.86, 1.03)</td>
<td>0.91 (0.76, 1.10)</td>
<td>676</td>
<td>716</td>
</tr>
<tr>
<td>NY Black</td>
<td>0.90 (0.74, 1.10)</td>
<td>0.97 (0.51, 1.85)</td>
<td>508</td>
<td>532</td>
</tr>
<tr>
<td>NY Hispanic</td>
<td>0.93 (0.79, 1.10)</td>
<td>0.97 (0.72, 1.31)</td>
<td>200</td>
<td>172</td>
</tr>
<tr>
<td>NY Other</td>
<td>0.91 (0.70, 1.20)</td>
<td>0.89 (0.47, 1.71)</td>
<td>131</td>
<td>72</td>
</tr>
<tr>
<td>Total</td>
<td>1.00 (0.96, 1.05)</td>
<td>0.96 (0.87, 1.07)</td>
<td>2759</td>
<td>2430</td>
</tr>
</tbody>
</table>

C indicates cytosine; CHD, congenital heart disease; CI, confidence interval; EU, European; NY, New York; OR, odds ratio; and T, thymidine.
Cochran Q). We considered publication bias as a possible explanation for the high heterogeneity observed among the studies of offspring genotype; this seemed particularly important to explore as random-effects models can give undue weight to individuals in smaller studies, and our subsidiary fixed-effect meta-analysis of offspring genotypes showed no evidence of association (summary OR, 1.06 [95% CI, 0.97–1.15]). A funnel plot of the studies contributing to the meta-analysis of offspring genotype was indeed highly suggestive of publication bias (Figure 3 left), and formal tests for publication bias were significant (Begg \( P = 0.05 \); Egger \( P = 0.03 \)). By contrast, there was no evidence of publication bias among the studies contributing to the meta-analysis of maternal genotype (Figure 3 right). We used 2 statistical approaches to attempt to correct for publication bias in the studies of offspring genotype. The trim-and-fill method suggested 7 missing studies, and the filled data yielded an estimated OR free of publication bias of 0.97 (95% CI, 0.79–1.20). This result was corroborated by the Copas selection model, which yielded an estimated OR free of publication bias of 1.00 (95% CI, 0.84–1.20; Figure II in the online-only Data Supplement). Finally, we followed the approach of previous investigators by designating studies in which the variance of the log OR was <0.05 as large. Using these criteria, we identified 3 studies (the present European white and New York studies and the previous study of Xu et al, 2010; these were also the only studies that included >500 cases). Among these studies, which included 83% of all CHD cases in the meta-analysis (6416 of 7697 cases), the summary OR was 0.97 (95% CI, 0.91–2.21), further reinforcing the role of publication bias.

We conducted analyses comparing the risk of CHD by C677T genotype in 4 diagnostic subgroups: septal defects (2723 cases and 5022 controls), conotruncal defects (1718 cases and 3168 controls), left-sided lesions (389 cases and 717 controls), and other defects (623 cases and 1149 controls; Figure 4). In the other defects subgroup, the OR for TT genotype was 0.68 (95% CI, 0.49–0.95; \( P = 0.021 \)). However, the 99% CIs we prespecified to make allowance for multiple testing overlapped unity (99% CI, 0.45–1.05), and adopting an alternative approach to multiple testing by applying a Bonferroni correction for 4 subgroup analyses likewise rendered that result nonsignificant (corrected \( P = 0.084 \)). Moreover, a test for interaction was nonsignificant (\( \chi^2 = 0.97; P = 0.32 \)), indicating no evidence of difference between the ORs in the different subgroups. Finally, the other defects subgroup was of small size, and hence the result in that subgroup might be particularly susceptible to the play of chance.

**Effect of Prevailing Level of Folate Intake**

We grouped studies into low, medium, and high folate groups with the intent of exploring any effect of prevailing folate levels on the risk of CHD associated with MTHFR genotype. Although meta-regression including all studies suggested a borderline significant effect of prevailing levels of plasma folate on the association, with a trend toward an increased OR in studies with lower folate (\( \beta = 0.33; P = 0.02 \)), there was marked heterogeneity among the low folate group of Asian studies largely responsible for the significant result (I\(^2\)=89.3%), whereas there was no significant heterogeneity (I\(^2\)=0%) in the high folate group of studies. The low folate group included 3 small studies with extreme ORs (between 2.10 and 3.44) and 1 large study with a null result (OR 0.85), all conducted in China. Therefore, publication bias appears to be confounded with region of study origin, and hence folate status, in our data. In view of this, we carried out a meta-regression restricted to the 3 large studies identified as above, which by chance represented each of the 3 folate status groups. This analysis yielded no relationship between folate status and CHD risk associated with MTHFR genotype (\( \beta = 0.078; P = 0.49 \)).

**Alternative Genetic Models**

Given the clear indication of publication bias in the dataset, we restricted analysis of alternative genetic models (C allele...
versus T allele and C/C versus T/T genotype) to the 3 large studies only. The allele model yielded an OR of 0.97 (95% CI, 0.87–1.08), and comparison of T/T and C/C genotypes yielded an OR of 1.06 (95% CI, 0.92–1.23).

Discussion

This is the largest study to date of genetic influences on CHD. We analyzed primary genotyping data on 5814 CHD cases and 10056 controls, together with meta-analysis of a further 1883 cases and 3103 controls, and we found no significant effect of MTHFR C677T genotype on CHD risk. Among the 3 largest studies, which contributed 83% of the genotyped cases, the CIs were narrow around the null (OR, 0.97 [95% CI, 0.91–1.03]). In subgroup analyses, no effect of genotype was observed when we grouped CHD cases by the type of defect. Additionally, primary genotyping data on 829 mothers of CHD cases and 4348 healthy controls, together with meta-analysis of a further 952 mothers of cases and 15513 healthy controls, provided no support for an effect of maternal MTHFR genotype on CHD risk.

Our analyses showed a substantial effect of publication bias that appeared to be confounded with study region of origin. Consideration of large studies only yielded no evidence that MTHFR genotype had a differential effect on CHD risk dependent on prevailing levels of folate intake. Because MTHFR genotype directly influences plasma folate levels, using the principles of Mendelian randomization, these data provide no support for the notion that plasma levels of folate influence CHD risk.

Four previous meta-analyses of this question had reached conflicting conclusions, with early meta-analyses suggesting no effect of MTHFR genotype and more recent meta-analyses suggesting the presence of an effect, possibly more marked in white populations (Table II in the online-only Data Supplement). 20–23 The present study approximately trebles the number of cases investigated in published studies to date and conclusively rules out even a small effect of genotype on CHD risk. Analyses using the principles of Mendelian randomization are typically limited by the power of the genetic instrument used. We, therefore, estimated the magnitude of the effect of MTHFR genotype on CHD risk that we were likely to have observed if lower levels of plasma folate caused CHD using previously published epidemiological data (in the online-only Data Supplement). The upper 95% CI of 1.03 around our estimate robustly excludes an effect of the anticipated magnitude of ≈18% and suggests that, among the populations we studied,
any effect of plasma folate level on CHD risk is at most minimal. Moreover, we found no evidence of association between MTHFR genotype and being a mother of a case of CHD (such an association has been robustly demonstrated for neural tube defect, in keeping with a likely important contribution of maternal MTHFR genotype to fetal folate bioavailability during organogenesis). Our maternal genotype analyses approximately double the amount of information available on this question.

Our study has certain limitations. Although we attempted to exclude patients with recognized syndromes, not all such patients are diagnosed in childhood (eg, Noonan syndrome, the second most common syndromic cause of CHD after Down syndrome, may not infrequently be diagnosed in later life). Inadvertent inclusion of such patients, who have specific genetic causes of their CHD, among our cases could have biased our results toward the null. However, it is unlikely that our sample contains significant numbers of undiagnosed Down syndrome patients, and the prevalence of other syndromes (eg, Noonan: 1/1000–1/2500) is sufficiently high to have materially affected our conclusions. Our subgroup analyses were guided by diagnostic information and by the numbers of patients available in each subgroup. We cannot exclude a role of the MTHFR gene in individual diagnostic categories, which were too small to be analyzed individually in our sample (eg, Ebstein anomaly). Because we did not preferentially ascertain multiplex families, we cannot comment on whether MTHFR genotype may act as a modifier in the presence of particular high-risk alleles responsible for highly familial CHD. We focused on CHD conditions typically presenting in childhood; therefore, we have not addressed the relationship between MTHFR genotype and bicuspid aortic valve. Further studies focused on bicuspid aortic valve, the most common cardiovascular malformation, would be of interest.

Our results should not be interpreted as an argument against mandatory folate fortification, which substantially reduces the risk of neural tube defect. However, we found no evidence for a relationship between CHD and the MTHFR 677TT genotype, which is known to reduce plasma folate, in the largest genetic study of CHD thus far conducted. More generally, our data add to the results of previous investigations showing the substantial degree to which publication bias may influence the results of genetic meta-analyses.

Acknowledgments
The principal acknowledgment is to the CHD patients, their families, and healthy people who participated in the research. We also acknowledge the expert assistance of Sr Linda Sneddon and Rafiqul Hussein.

Sources of Funding
This study was funded by the British Heart Foundation, the Wellcome Trust (grant BH100708), the European Union FP7 Program CHeartED (HEALTH-F2-2008–223040), the Netherlands Heart Foundation, Heart Research UK, the Intramural Research Programs of the National Human Genome Research Institute, and the Eunice Kennedy Shriver National Institute of Child Health and Human Development. This study makes use of data generated by the Wellcome Trust Case-Control Consortium 2, funded by the Wellcome Trust under award 085475. The study sponsors had no role in the study design; collection, analysis, and interpretation of data; manuscript writing; or decision to submit the paper for publication.

Disclosures
None.

Appendix
From the Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom (C.M., T.P., D.H., A.T., D.L.B., T.J.R., J.P.D., H.L.T., J.A.G., H.J.C., B.D.K.); Molecular Pathogenesis Section, Genome Technology Branch, National Human Genome Research Institute (F.P., R.R.P., L.C.B.); Division of Epidemiology, Statistics and Prevention Research, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD (J.L.M.); Congenital Malformations Registry, New York State Department of Health, Troy, and Department of Epidemiology and Biostatistics, University at Albany School of Public Health, Rensselaer, NY (C.D.); Wadsworth Center, New York State Department of Health, Albany, NY (K.P.); Department of Pediatrics and Communicable Diseases, The University of Michigan Medical School, Ann Arbor (M.W.R.); Commissariat à l’énergie Atomique (CEA), Institut Genomique, Centre National de Genotypage, Evry, France (D.Z., G.M.L.); Fondation Jean Dausset, Centre d’Etude du Polymorphisme Humain, Paris, France (D.Z., G.M.L.); McGill University and Genome Quebec Innovation Centre, Montreal, Canada (G.M.L.); Department of Cardiovascular Medicine, Oxford University, Oxford, United Kingdom (J.B., C.C., S.B., M.F.); Institute
of Genetics, Nottingham University, Nottingham, England (J.G.R.,
K.S., J.D.B.); East Midlands Congenital Heart Centre, University
Hospitals of Leicester NHS Trust, Leicester, England (F.A.B.L., C.T.);
Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle
upon Tyne, United Kingdom (J.O.S.); Bristol Royal Hospital
for Children, Bristol, United Kingdom (A.G.S.); The Children’s Hospital
at Westmead, Westmead, Australia (G.B., D.W.); Academic Medical
Center, Amsterdam, The Netherlands (A.Y.P., B.J.M.M., A.H.Z.,
K.v.E., A.F.M.M.); Institute of Medical Genetics, University of
Zurich, Zurich, Switzerland (A.R.); Pediatric Cardiology (M.G.),
Center for Human Genetics, University of Leuven, Leuven, Belgium
(J.B., K.D.); and Institute of Cardiovascular Sciences, The University
of Manchester, Manchester, United Kingdom (B.D.K.).

References
American Heart Association Council on Cardiovascular Disease in the
Young. Noninherited risk factors and congenital cardiovascular defects:
current knowledge: a scientific statement from the American Heart Asso-
ciation Council on Cardiovascular Disease in the Young: endorsed by the
3. Botto LD, Khoury MJ, Mulinear J, Erickson JD. Periconceptional multi-
vitamin use and the occurrence of conotruncal heart defects: results from
4. Botto LD, Mulineal J, Erickson JD. Occurrence of congenital heart
5. Shaw GM, O’Malley CD, Wasserman CR, Tolarova MM, Lammer EJ.
Maternal periconceptional use of multivitamins and reduced risk for
6. Werler MM, Hayes C, Louik C, Shapiro S, Mitchell AA. Multivi-
1999;150:675–682.
7. Czeizel AE. Periconceptional folic acid containing multivitamin supple-
8. Ionescu-Ittu R, Marelli AJ, Mackie AS, Pilote L. Prevalence of severe con-
genital heart disease after folic acid fortification of grain products: time
trend analysis in Quebec, Canada. BMJ. 2009;338:b1673.
A candidate genetic risk factor for vascular disease: a common mutation
10. Yang QH, Botto LD, Gallagher M, Friedman JM, Sanders CL, Koontz
D, et al. Prevalence and effects of gene-gene and gene-nutrient interac-
tions on serum folate and serum total homocysteine concentrations in the
United States: findings from the third National Health and Nutrition Ex-
11. Smith GD, Timpson N, Ebrahim S. Strengthening causal inference in car-
ternal MTHFR C677T polymorphism with susceptibility to neural tube
2012;7:e41689.
13. Van den Berge M, Lima MA, Castilla EE, Orozio IM. Non-Latin European de-
script could be a requirement for association of NTDs and MTHFR variant
14. Botto LD, Yang Q. 5,10-Methylenetetrahydrofolate reductase gene
A rare variant of the leptin gene has large effects on blood pressure and
carotid intima-media thickness: a study of 1,428 individuals in 248
17. Copas J, Shi JQ. Meta-analysis, funnel plots and sensitivity analysis. Bio-
18. Poole C, Greenland S. Random-effects meta-analyses are not always con-
et al; MTHFR Studies Collaborative Group. Homocysteine and coronary
heart disease: meta-analysis of MTHFR case-control studies, avoiding
20. van Beynum IM, den Heijer M, Blom HJ, Kapusta L. The MTHFR 677C>
T polymorphism and the risk of congenital heart defects: a literature re-
Steegers-Theunissen R. Hyperhomocysteinemia and MTHFR polymor-
phisms in association with orofacial clefts and congenital heart defects: a
22. Nie Y, Gu H, Gong J, Wang J, Gong D, Cong X, et al. Methylenetetrahy-
drofolate reductase C677T polymorphism and congenital heart disease: a
23. Xia M, Dong L, Zhang J, Lian J, Xu Z. Meta-analysis of the asso-
ciation between MTHFR C677T polymorphism and the risk of congenital

CLINICAL PERSPECTIVE

The relationship between plasma folate and congenital heart disease (CHD) is contentious. Folate supplementation in the periconceptual period markedly reduces the incidence of neural tube defects, so randomized trials to test the effect of supplementation on CHD risk are ethically precluded. Folate fortification of cereals is the most effective mechanism to increase plasma folate in populations. Because CHD is significantly more common than neural tube defect, demonstration of an effect of folate on CHD risk would be of population health importance and could potentially inform decisions about recommended levels of folate supplementation or fortification. However, prospective observational studies to test the relationship between plasma folate and CHD would be prohibitively expensive (given CHD affects only ~0.7% of live births) and likely to be confounded by discretionary maternal folate intake. We have, therefore, taken a genetic approach to this question. We investigated the relationship between the C677T polymorphism in the methylene tetrahydrofolate reductase (MTHFR) gene, which directly acts to reduce plasma levels of folate, and CHD risk in a large meta-analysis. We find no relationship between genotype and CHD risk in large studies from areas of the world where folate levels are low, medium, or high. Our work suggests that the prevention of neural tube defects remains the principal rationale for folate fortification because our genetic approach did not show any effect on CHD risk associated with the differences in folate level caused by MTHFR C677T genotype.
Association Between C677T Polymorphism of Methylene Tetrahydrofolate Reductase and Congenital Heart Disease: Meta-Analysis of 7697 Cases and 13 125 Controls


Circ Cardiovasc Genet. 2013;6:347-353; originally published online July 22, 2013; doi: 10.1161/CIRCGENETICS.113.000191

Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1942-325X. Online ISSN: 1942-3268

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circgenetics.ahajournals.org/content/6/4/347

Data Supplement (unedited) at:
http://circgenetics.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Cardiovascular Genetics can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Cardiovascular Genetics is online at:
http://circgenetics.ahajournals.org//subscriptions/
SUPPLEMENTAL METHODS

Literature-based Meta-Analysis:

We defined the inclusion criteria for studies as follows: evaluation of the MTHFR C677T polymorphism and congenital heart disease; case-control study design; and sufficient data available either from publication or subsequent to contact with authors to calculate odds ratios and 95% confidence intervals. We searched two scientific databases, PubMed (National Library of Medicine) and HuGE Navigator (v.1.4), and Google Scholar (scholar.google.com) using the search terms: “methylene tetra-hydro-folate reductase (MTHFR)”; “heart defects, congenital”; “C677T”; “rs1801133”; “homocysteine”, “folate”, “folic acid”, alone or in combination, without restriction on language, with a cut-off date for publication of December 2011. Where studies not in English were encountered, these were translated. When eligible studies were identified, their bibliographies were hand-searched for additional references. Where genotype numbers could not be calculated from presented data, we made efforts to contact the authors for further information. We restricted inclusion in the meta-analysis to published studies, and where the same dataset had been used in two or more publications, only the original paper was included. The few previous studies that had used a family-based design in trios had used some variant of the transmission disequilibrium test (TDT), which tests for allelic rather than genotypic association. The odds ratios under the recessive genetic model of principal interest to us (that is, the risk of T/T genotype relative to the other two genotypes C/T and C/C) cannot be calculated from the numbers of transmissions and non-transmissions that are typically reported when this method is used, therefore family-based studies were excluded. Data on numbers of individuals participating were, however, extracted from these excluded studies. For each study, two authors abstracted the first author’s surname, publication
year, ethnicity of subjects, and frequencies for the three C677T genotypes in cases and controls, with discrepancies resolved by discussion.

Since the effect of MTHFR genotype on plasma folate levels is dependent upon an individual’s folate status, and this could lead to heterogeneity in any effect of MTHFR C677T genotype on CHD risk in folate-replete and folate-deplete populations, we mapped the geographic origin of each study to flour fortification status and prevalence of folate deficiency using publicly available data from the Flour Fortification Initiative (http://www.sph.emory.edu/wheatflour) and World Health Organisation (http://www.who.int). We stratified studies into three groups following the approach of Clarke et al., which was based upon consideration of plasma folate levels in 81 population-based surveys including 200,000 individuals and took account of the introduction of folate fortification in the mid-1990s in many countries. Studies conducted in countries practicing mandatory folate fortification and published following the introduction of fortification (chiefly US studies) were assigned to a high folate status group. Our New York samples were from births that occurred prior mandatory fortification but were during the “ramp up phase” when fortification was voluntary. The result was that many US food manufacturers were supplementing early and our samples were therefore placed in the “high” group in these analyses. Studies conducted in those same countries pre-fortification were grouped together with those from Europe following the introduction of voluntary fortification in a mid folate status group. Those conducted in Europe pre-fortification, and in Asian countries not practicing mandatory folate fortification (chiefly China) were assigned to a low folate status group.

We identified 25 publications examining the relationship between MTHFR C677T and CHD (Supplementary Table 1). Of these, three appeared to present substantially overlapping data and accordingly the largest and most recent dataset only was used.
TDT data only was presented in four studies which had enrolled families; \textsuperscript{17 10, 21} as discussed above, these studies could not be analysed for the model of interest based on the summary data available and were excluded. One study included both TDT and case-control data; the case-control data was used in the pooled analyses. One study \textsuperscript{24} was concerned with the contribution of C677T to risk of CHD only in the setting of Down’s syndrome, and was therefore excluded. Despite attempts to communicate with authors, it was not possible to obtain case/control genotype numbers from one study, published in Chinese, which included 115 disease cases. Of the studies remaining after the above exclusions, there were 14 in which comparison had been made between genotypes in cases of CHD and healthy controls, and there were 8 in which comparison had been made between genotypes in mothers of cases of CHD and healthy controls. The flow chart summarising the selection process for the meta-analysis is presented in Supplementary Figure 1. Among the 14 studies comparing case and control genotypes, four were conducted in China, five in Europe, three in North America, one in Brazil, and one in Taiwan. These studies included a total of 1,883 cases and 3,103 controls. Among the 8 studies comparing genotypes between mothers of cases and controls, five were conducted in Europe, two in North America, and one in China. These studies included a total of 952 mothers of cases and 15,513 controls.

**Estimation of power of genetic instrument for “Mendelian randomisation”:**

In the absence of data on plasma folate levels in our primary samples, we adopted an approach based on extrapolation from the effect sizes for the associations between MTHFR genotype and folate levels, and between differences in folate levels and CHD risk, observed in previously published epidemiological studies. Among 6793 participants in the NHANES cohort, Yang et al. showed that the T/T genotype was associated with an approximately 25% lower plasma folate level than the C/C genotype. \textsuperscript{27} A recent analysis by Clarke et al. of 200,000 people in 81 population surveys of plasma folate levels,
spanning the introduction of fortification in many countries in the mid-1990s, showed an approximately 50% increase in plasma folate following supplementation both in European and US/Australasian populations. ¹ Finally, the Canadian time trend analysis of incident CHD conducted by Ionescu-Ittu et al. showed a 36% fall in CHD during the six years following the introduction of folate fortification. ²⁸ Assuming a linear relationship between folate levels and a putative effect on CHD risk, from these three pieces of information we can calculate that the T/T genotype might be anticipated to confer about an 18% increase in CHD risk compared to the other two genotypes, through its effect on plasma folate levels, if the relationship between plasma folate and CHD were causal. Power calculations using this effect size, together with the observed allele frequencies at C677T in our cohort and the appropriate population prevalence of CHD, were performed using Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/). ²⁹ These indicated the study had >95% power to detect the anticipated effect under the recessive model (T/T versus C/T and C/C), and 80% power under the allelic association model.
SUPPLEMENTAL REFERENCES


14. van Beynum IM, Kapusta L, den Heijer M, Vermeulen SH, Kouwenberg M, Daniels O, Blom HJ. Maternal mthfr 677c>t is a risk factor for congenital heart defects:


29. Purcell S, Cherny SS, Sham PC. Genetic power calculator: Design of linkage and association genetic mapping studies of complex traits. *Bioinformatics*. 2003;19:149-150.


<table>
<thead>
<tr>
<th>First Author</th>
<th>Country</th>
<th>Year</th>
<th>Year of enrolment</th>
<th>Types of CHD</th>
<th>Exclusion criteria</th>
<th>Ethnicity of cases</th>
<th>Ethnicity of controls</th>
<th>CC</th>
<th>CC- Mat</th>
<th>TDT</th>
<th>Folate status</th>
<th>N cases/ N controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wenstrom 2</td>
<td>USA</td>
<td>2001</td>
<td>1988–1998</td>
<td>All types</td>
<td>Syndromes, Teratogens, DM</td>
<td>Black 27% White 69% Other 4%</td>
<td>Black 20% White 78% Other 2%</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>High</td>
<td>26/116</td>
</tr>
<tr>
<td>Junker 3</td>
<td>Germany</td>
<td>2001</td>
<td>1995–2000</td>
<td>All types except PFO</td>
<td>Chromosomal, Teratogens</td>
<td>Caucasian</td>
<td>Caucasian</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>Mid</td>
<td>114/228</td>
</tr>
<tr>
<td>Storti 4</td>
<td>Italy</td>
<td>2003</td>
<td>2000–2001</td>
<td>Conotruncal (11 cases 22q11 del)</td>
<td>Not described</td>
<td>Caucasian</td>
<td>Caucasian</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>Mid</td>
<td>103/200</td>
</tr>
<tr>
<td>Ying 5</td>
<td>China</td>
<td>2003</td>
<td>Unknown</td>
<td>PDA, TOF, ASD, VSD, PS</td>
<td>Not described</td>
<td>Chinese</td>
<td>Chinese</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Nurk 6</td>
<td>Norway</td>
<td>2004</td>
<td>1950–1952, 1967–1996</td>
<td>Unknown</td>
<td>Multiple other malformation syndromes</td>
<td>Caucasian 65% Hispanic 29% Black 5% Asian 1%</td>
<td>Not described</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
<td>High</td>
<td>-</td>
</tr>
<tr>
<td>McBride 7</td>
<td>USA</td>
<td>2004</td>
<td>1998–2003</td>
<td>Left-sided CHD</td>
<td>Multiple other malformation syndromes</td>
<td>Caucasian</td>
<td>Caucasian</td>
<td>Unknown</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Shaw 8</td>
<td>USA</td>
<td>2005</td>
<td>1987–1988</td>
<td>Conotruncal</td>
<td>Aneuromies, single-gene disorders</td>
<td>White 67% Hispanic 23% Other 10%</td>
<td>White 58% Hispanic 29% Other 13%</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>High</td>
<td>151/428</td>
</tr>
<tr>
<td>Lee 9</td>
<td>Taiwan</td>
<td>2005</td>
<td>2002–2003</td>
<td>All types</td>
<td>Not described</td>
<td>Asian</td>
<td>Asian</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>High</td>
<td>213/195</td>
</tr>
<tr>
<td>Pereira 10</td>
<td>Brazil</td>
<td>2005</td>
<td>Unknown</td>
<td>All types</td>
<td>Not described</td>
<td>Unknown</td>
<td>Unknown</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liu 11</td>
<td>China</td>
<td>2005</td>
<td>Unknown</td>
<td>Conotruncal</td>
<td>Not described</td>
<td>Chinese</td>
<td>Chinese</td>
<td>Yes</td>
<td>-</td>
<td>Low</td>
<td>97/118</td>
<td>-</td>
</tr>
<tr>
<td>Li Y 12</td>
<td>China</td>
<td>2005</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Not described</td>
<td>Chinese</td>
<td>Chinese</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>Qiu XQ 13</td>
<td>China</td>
<td>2006</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Not described</td>
<td>Chinese</td>
<td>Chinese</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>Van Beynum 14</td>
<td>Netherlands</td>
<td>2006</td>
<td>2002–2003</td>
<td>All types</td>
<td>NTD, Clefts, Syndromes</td>
<td>Caucasian</td>
<td>Caucasian, same area</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Mid</td>
<td>165/220</td>
</tr>
<tr>
<td>Zhu 15</td>
<td>China</td>
<td>2006</td>
<td>Unknown</td>
<td>ASD, PDA</td>
<td>DM, PKU, Teratogens X-ray</td>
<td>Asian, province in China</td>
<td>Asian, same area</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>Low</td>
<td>56/103</td>
</tr>
<tr>
<td>Hobbs 16</td>
<td>USA</td>
<td>2006</td>
<td>1998–2004</td>
<td>Septal, Conotruncal, right-left sided CHD</td>
<td>Syndromes Chromosomal</td>
<td>White</td>
<td>White</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>First Author</td>
<td>Country</td>
<td>Year</td>
<td>Year of inclusion</td>
<td>Types of CHD</td>
<td>Exclusion criteria</td>
<td>Ethnicity of cases</td>
<td>Ethnicity of controls</td>
<td>CC</td>
<td>CC- Mat</td>
<td>TDT</td>
<td>Folate status</td>
<td>N cases/ N controls</td>
</tr>
<tr>
<td>--------------</td>
<td>---------</td>
<td>------</td>
<td>-------------------</td>
<td>--------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>---------------------</td>
<td>----</td>
<td>---------</td>
<td>-----</td>
<td>-------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Hobbs 17</td>
<td>USA</td>
<td>2006</td>
<td>1998–2004</td>
<td>Septal Conotruncal, right-left sided CHD</td>
<td>Syndromes, Chromosomal</td>
<td>Not described</td>
<td>Not described</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Galdieri 18</td>
<td>Brazil</td>
<td>2007</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Syndromes, Multiple malformations</td>
<td>White 22% Non-White 78%</td>
<td>White 53% Non-White 47%</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
<td>High</td>
<td>58/38</td>
</tr>
<tr>
<td>Wintner 19</td>
<td>Austria</td>
<td>2007</td>
<td>1993-2004</td>
<td>All types</td>
<td>Aneuploidy Syndromes Maternal DM Teratogens</td>
<td>Caucasian</td>
<td>Caucasian</td>
<td>-</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Van Driel 20</td>
<td>Netherlands</td>
<td>2008</td>
<td>Unknown</td>
<td>Multiple types</td>
<td>Not described</td>
<td>Dutch natives 89% European others 11%</td>
<td>Dutch natives 89% European others 11%</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>Mid</td>
<td>229/251</td>
</tr>
<tr>
<td>Goldmuntz 21</td>
<td>USA</td>
<td>2008</td>
<td>1997-2007</td>
<td>Conotruncal</td>
<td>Syndromes Chromosomal</td>
<td>Any racial/ethnic group</td>
<td>Any racial/ethnic group</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Marinho 22</td>
<td>Portugal</td>
<td>2009</td>
<td>Unknown</td>
<td>TOF</td>
<td>Not described</td>
<td>White</td>
<td>Unknown</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>Mid</td>
<td>38/251</td>
</tr>
<tr>
<td>Li D 23</td>
<td>China</td>
<td>2009</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Not described</td>
<td>Chinese</td>
<td>Chinese</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>Low</td>
<td>104/208</td>
</tr>
<tr>
<td>Brandalize 24</td>
<td>Brazil</td>
<td>2009</td>
<td>Unknown</td>
<td>CHD in trisomy 21</td>
<td>Other syndrome Other offspring with another syndrome</td>
<td>90% European descent 6.4% African descent 3.4% other</td>
<td>93.8% European descent 4.8% African descent 1.4% other</td>
<td>-</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Garcia- Fragoso 25</td>
<td>Puerto-Rico</td>
<td>2010</td>
<td>Unknown</td>
<td>Multiple</td>
<td>Chromosomal, Syndromes, PDA associated with prematurity, Antiepileptics, conditions associated with food intolerance, malabsorption, or wasting syndromes, maternal DM</td>
<td>White 76% Black 7% Other 17%</td>
<td>White 76% Black 7% Other 17%</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>High</td>
<td>27/220</td>
</tr>
</tbody>
</table>
CC: case-control study involving genotype frequency comparison in affected people and controls
CC-Mat: case-control study involving genotype frequency comparison in mothers of affected people and controls
TDT: study involving the examination of genotype transmission in families, typically using a variant of the “transmission disequilibrium test”
22q11del: Chromosome 22q11 deletion syndrome (also known as DiGeorge/velocardiofacial/CATCH-22 syndromes)
ASD: atrial septal defect
DM: diabetes mellitus
NA: not applicable
PDA: persistent ductus arteriosus
PFO: patent foramen ovale
PKU: phenylketonuria
PS: pulmonary stenosis
TOF: tetralogy of Fallot
VSD, Ventricular septal defect
Supplementary Table 2: Previous meta-analyses of MTHFR C677T association with CHD

<table>
<thead>
<tr>
<th>Meta-analysis, by first author name and year</th>
<th>Number of studies</th>
<th>OR [95% CI]</th>
<th>Number of cases/controls</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van Beynum, 2007(^{30})</td>
<td>8</td>
<td>1.3 [0.97–1.73]</td>
<td>882/1511</td>
<td>TT vs. CC</td>
</tr>
<tr>
<td>Verkleij-Hagoort, 2007(^{31})</td>
<td>6</td>
<td>1.14 [0.86–1.53]</td>
<td>774/1393</td>
<td>TT+CT vs. CC</td>
</tr>
<tr>
<td>Nie et al, 2011(^{32})</td>
<td>13</td>
<td>1.27 [0.98-1.66] ALL 1.45 [1.08-1.95] Caucasian</td>
<td>1898/3003</td>
<td>TT vs. CC+CT</td>
</tr>
<tr>
<td>Yin et al, 2012(^{33})</td>
<td>13</td>
<td>1.55 [1.25, 1.93]</td>
<td>1655/2327</td>
<td>TT vs. CC</td>
</tr>
</tbody>
</table>
Supplemental Figure 1: PRISMA flow diagram for case-control meta-analysis

- Records identified through database searching (n = 25)
- Additional records identified through other sources (n = 0)

Records after duplicates removed (n = 22)

Records screened (n = 22)

Records excluded (n = 0)

Full-text articles assessed for eligibility (n = 14)

Full-text articles excluded (n = 8)

Studies included in qualitative synthesis (n = 14)

Studies included in quantitative synthesis (meta-analysis) (n = 14 + primary data from 2 studies)
Supplemental Figure 2: Copas’ selection model plot. The upper panel shows the p-value for residual selection bias (on y-axis) at diminishing probability of publication, indicating that this crosses a threshold of 0.1 at a probability of publication of ~0.7. The right panel shows the corresponding odds ratios and 95% CIs for the association of T/T genotype with CHD risk at diminishing probability of publication.