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Association between C677T Polymorphism of Methylene Tetrahydrofolate Reductase and Congenital Heart Disease: Meta-Analysis of 7,697 Cases and 13,125 Controls

Running title: *Mamasoula: MTHFR C677T polymorphism and congenital heart disease*

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Abstract

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 5,814 cases with primary genotype data and 10,056 controls, there was no evidence of association between *MTHFR* C677T genotype and CHD risk (OR 0.96 [95% CI 0.87-1.07]). A random-effects meta-analysis of all studies (involving 7,697 cases and 13,125 controls) suggested the presence of association (OR 1.25 [95% CI 1.03-1.51]; p=0.022), but with substantial heterogeneity among contributing studies (I^2=64.4%), and evidence of publication bias. Meta-analysis of large studies only (defined by a variance of the log OR less than 0.05), which together contributed 83% of all cases, yielded no evidence of association (OR 0.97 [95% CI 0.91-1.03]), without significant heterogeneity (I^2=0). Moreover, meta-analysis of 1,781 mothers of CHD cases (829 of whom were genotyped in this study) and 19,861 controls revealed no evidence of association between maternal C677T genotype and risk of CHD in offspring (OR 1.13 [95% CI 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, MTHFR, genetic association, folate, Mendelian randomization

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Congenital heart disease (CHD) is the commonest birth defect. It affects around 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 one previous randomized trial of preconceptual folate supplementation and CHD, conducted in Hungary, which suggested a potentially substantial effect on CHD risk of a multivitamin supplement containing folic acid. However, statistical significance was borderline (OR=0.48 [95% CI 0.23-1.03]; p=0.055), in an analysis involving only thirty cases of CHD. [7] Given the proven protective effect of folate on neural tube defect (NTD), further placebo-controlled trials are ethically precluded. A recent Canadian study used the introduction of mandatory folate fortification of grain products in 1998 as an opportunity to conduct a time trend analysis based on medical reimbursement data in which CHD incidence rates prior and subsequent to fortification were compared. In the six years following fortification, CHD incidence rates fell by 36%. [8] However, that study was potentially vulnerable to confounding by unmeasured coincident secular trends (for example the introduction of fetal echocardiography leading to a higher termination rate for fetuses determined to have severe CHD).

Homozygosity for the thymidine allele (T/T genotype) at the C677T polymorphism (dbSNP ID: rs1801133) of the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene, which is observed in about 11% of Caucasians and 1-2% of those of African origin, directly causes lower levels of plasma folate (and higher levels of plasma homocysteine). The nucleotide

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substitution of thymidine (T) for cytosine (C) leads to the replacement of the amino acid alanine with valine at position 222 of the protein and a reduction in the activity of the *MTHFR* enzyme (by about one third per copy of the T allele). [9] The effect of genotype is non-additive, with small and inconsistent differences in plasma folate between C/C and C/T individuals, but ~25% lower plasma folate in T/T than C/C individuals. [10] Determining the presence or absence of association between *MTHFR* C677T genotypes and CHD risk could confirm or refute a causal association between folate and CHD risk, through "Mendelian randomization", the background of which has been extensively reviewed. [11] In keeping with this notion, the T/T genotype has been shown to be a risk factor for NTD when present either in mothers of affected offspring or the offspring themselves. [12-14] A number of previous studies have investigated the association between C677T genotype and CHD risk, but all were small, and the number of genotyped cases in the literature has precluded robust conclusions even when these studies were combined in meta-analyses. Here we report findings in 5,814 newly genotyped CHD cases and 10,056 controls, set in context of a meta-analysis including a total of 7,698 cases and 13,159 controls.

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Methods

Ethics Statement:

Collection of the European and Australian populations, and conduct of the genetic investigation, was approved by the appropriate ethical committees in the participating institutions. Informed consent was obtained from all participants (or from parents, if the patients were children too young to themselves consent). The specimens and associated data from the New York State newborn screening program were made anonymous prior to testing. The use of these samples was approved by the Institutional Review Board of the New York State Department of Health and reviewed by the Office for Human Research Protections at the National Institutes of Health.

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

Populations studied:

EU-Caucasian Cohort: Cases of CHD were collected from UK congenital heart disease units in Bristol, Leeds, Liverpool, Leicester, Newcastle, Oxford and London, and from centres in Amsterdam (Netherlands), Leuven (Belgium), Erlangen (Germany), and Sydney (Australia). All cases were of European Caucasian ancestry. Patients with known genetic causes of CHD (for example, Down's syndrome, 22q11 deletion syndrome, Noonan's 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. Since 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 3,800 healthy Caucasian 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 Caucasian controls free of CHD ascertained as previously described who were genotyped on both platforms employed in this cohort (see below). [15]

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

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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 EU-Caucasian 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 660W-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 NY 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, USA). In both cohorts, at least 10% of samples were randomly plated a second time and re-genotyped. The concordance rate between these replicates was >95%.

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Literature search:

The methods for the literature search are described in the Supplementary Information online.

Statistical analysis:

In the principal analyses, we estimated odds ratios (ORs) for CHD risk with T/T genotype compared to (C/T + C/C) genotypes, and their 95% confidence intervals, using logistic

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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 OR's and 95% CIs using the DerSimonian and Laird random-effect model, as we anticipated substantial heterogeneity between the studies, possibly related to inter-population 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's Q, and also quantified heterogeneity using the I^2 statistic, which describes the percentage of variation across studies that is due to heterogeneity rather than chance. [16] Values of I^2 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's and Begg's 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 three diagnostic subgroups: septal defects (ASD, VSD and AVSD); conotruncal lesions (chiefly tetralogy of Fallot, pulmonary stenosis with VSD, pulmonary atresia, and transposition of the great arteries); and left-sided lesions (chiefly 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 one of these three groups were designated "Other" – examples

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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%) confidence intervals for the odds ratios 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 two 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; and 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, using meta-regression; we also calculated the heterogeneity between studies within each of the three groups. All p-values were two-tailed, and other than in the subgroup analyses $p<0.05$ was accepted as the threshold for significance. Analyses were performed using STATA (Version 10, Stata Corporation, College Station, TX, USA).

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Results

Primary genotyping data:

MTHFR genotypes were successfully assigned in over 95% of participants. A total of 5,814 CHD cases and 10,056 controls in the combined EU-Caucasian and NY cohorts had *MTHFR* C677T genotype available. Of these, 4,495 cases (77%) were of Caucasian ethnicity. The allele frequencies corresponded closely with those observed in previous large studies of this

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polymorphism. Previously reported differences in the allele frequencies were observed between those of African ancestry and other groups. Genotypes were in Hardy-Weinberg equilibrium at the p>0.05 level. In the total cohort, there was no significant association between genotype and CHD risk under a recessive model examining the risk of T/T genotype relative to the combined C/T and C/C groups (OR=0.96 [95% CI 0.87-1.07]; Table 1). Nor was there any evidence of association under an additive model (OR=1.00 [95% CI 0.96-1.05]; Table 1). There was no evidence of heterogeneity in the odds ratios between ethnic groups. Genotypes were available on 829 European Caucasian mothers of CHD cases (336 C/C, 396 C/T, and 97 T/T); there was no difference in genotype frequency between mothers of CHD cases and healthy controls (OR for TT versus CT+CC =1.05 [95% CI 0.83-1.33]).

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Meta-analysis:

The selection procedure resulting in the inclusion of 14 published case-control studies and 8 published studies of mothers of CHD cases and controls in the final meta-analyses is described in Supplementary Table 1 online. The meta-analysis of case-control data incorporating the primary genotyping data from the present study included 7,697 cases and 13,159 controls, and the corresponding meta-analysis of data in mothers of cases and controls included 1,781 mothers of cases and 19,861 controls.

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The random-effects meta-analysis of offspring genotypes suggested association between T/T genotype and CHD risk (summary OR=1.25 [95% CI 1.03-1.51], p=0.022; Figure 1) but moderate to high heterogeneity was present (I^2=64.4%; p<0.0001 by Cochran's Q). The random-effects meta-analysis of maternal genotypes yielded no significant association of maternal T/T genotype with CHD risk (summary OR 1.13 [95% CI 0.87-1.47]; Figure 2) and low heterogeneity between these studies (I^2=30.7%; p=0.163 by Cochran's Q). We considered

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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, [18] 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 panel), 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 panel). We used two 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; Supplementary Figure 2). Finally, we followed the approach of previous investigators by designating studies in which the variance of the log OR was less than 0.05 as large. [19] Using these criteria identified three studies (the present EU-Caucasian and NY studies and the previous study of Xu et al 2010; these were also the only studies which included greater than 500 cases). Among these studies, which included 83% of all CHD cases in the meta-analysis (6,416 of 7,697 cases), the summary OR was 0.97 (95% CI 0.91-1.03), with no evidence of heterogeneity (I^2=0%; p-value by Cochran's Q=0.6); while among the 13 smaller studies the OR was 1.62 (95% CI 1.19-2.21), further reinforcing the role of publication bias.

We conducted analyses comparing the risk of CHD by C677T genotype in four diagnostic subgroups: septal defects (2723 cases and 5022 controls); conotruncal defects (1718

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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 odds ratio for TT genotype was 0.68 (95% CI 0.49-0.95; p=0.021). However, the 99% CIs we pre-specified 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 four subgroup analyses likewise rendered that result non-significant (corrected p=0.084). Moreover, a test for interaction was non-significant (χ^2_1=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 towards 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%), while there was no significant heterogeneity (I^2=0%) in the high folate group of studies. The low folate group included three small studies with extreme odds ratios (between 2.10 and 3.44) and one 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 three large studies identified as above, which by chance represented each of the three folate status groups. This analysis yielded no relationship between folate status and CHD risk associated with *MTHFR* genotype

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(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 three 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 analysed primary genotyping data on 5,814 CHD cases and 10,056 controls, together with meta-analysis of a further 1,883 cases and 3,103 controls, and we found no significant effect of *MTHFR* C677T genotype on CHD risk. Among the three largest studies, which contributed 83% of the genotyped cases, the confidence intervals 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 4,348 healthy controls, together with meta-analysis of a further 952 mothers of cases and 15,513 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. Since *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

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early meta-analyses suggesting no effect of *MTHFR* genotype and more recent meta-analyses suggesting the presence of an effect, possibly more marked in Caucasian populations (Supplementary Table 2). [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 employed. 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 (Supplementary Information). 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);[12, 14] 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 (for example, Noonan's syndrome, the second most common syndromic cause of CHD after Down's 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 towards the null. However, it is unlikely that our sample contains significant numbers of undiagnosed Down's syndrome patients, and the prevalence of other syndromes (e.g. Noonan's: 1/1000-1/2500) is

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insufficiently 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 analysed individually in our sample (eg Ebstein's anomaly). Since 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 (BAV). Further studies focused on BAV, the commonest 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 NTD. 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 adds to the results of previous investigations showing the substantial degree to which publication bias may influence the results of genetic meta-analyses.

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analysis and interpretation of data; manuscript writing; or decision to submit the paper for publication.

Conflict of Interest Disclosures: None.

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Table 1: *MTHFR* C677T polymorphism and risk of CHD in cases and controls genotyped in this study

Population	OR(95% CI) T vs. C	OR(95% CI) TT vs. CC+CT	CC Cases	CT Cases	TT Cases	CC Controls	CT Controls	TT Controls	Allele Frequencies Cases		Allele Frequencies Controls	
									C	T	C	T
EU-Caucasian	1.06 (0.98, 1.13)	1.00 (0.86, 1.16)	1244	1338	325	1885	1816	467	0.66	0.34	0.67	0.33
NY-Caucasian	0.94 (0.86, 1.03)	0.91 (0.76, 1.10)	676	716	196	1306	1474	428	0.65	0.35	0.64	0.36
NY-Black	0.90 (0.74, 1.10)	0.97 (0.51, 1.85)	508	132	14	992	293	29	0.88	0.12	0.87	0.13
NY-Hispanic	0.93 (0.79, 1.10)	0.97 (0.72, 1.31)	200	172	76	385	375	160	0.64	0.36	0.62	0.38
NY-other	0.91 (0.70, 1.20)	0.89 (0.47, 1.71)	131	72	14	258	156	32	0.77	0.23	0.75	0.25
Total	1.00 (0.96, 1.05)	0.96 (0.87, 1.07)	2759	2430	625	4826	4114	1116	0.68	0.32	0.69	0.31

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Figure Legends:

Figure 1: Meta-analysis of offspring genotypes, random effects model. The size of the grey square around the point estimate is proportional to the study size, and 95% confidence intervals for each study are shown. The point estimate of the summary odds ratio is denoted by the broken red line, with 95% confidence interval denoted by a diamond.

Figure 2: Meta-analysis of maternal genotypes, random effects model. Conventions as in Figure 1.

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Figure 3: Assessment of publication bias by funnel plots. The left panel shows the funnel plot for the offspring genotype meta-analysis, and the right panel shows the funnel plot for the maternal genotype meta-analysis. Each study is represented by a dot, with the 95% pseudo-confidence intervals shown as broken lines on either side of the summary odds ratio from the respective meta-analysis.

Figure 4: Meta-analysis of CHD diagnostic subgroups. Patients from both the NY and EU-Caucasian cohorts are included, grouped by diagnostic category of their dominant defect. Conventions as in Figure 1, other than 99%, rather than 95% confidence intervals are shown.

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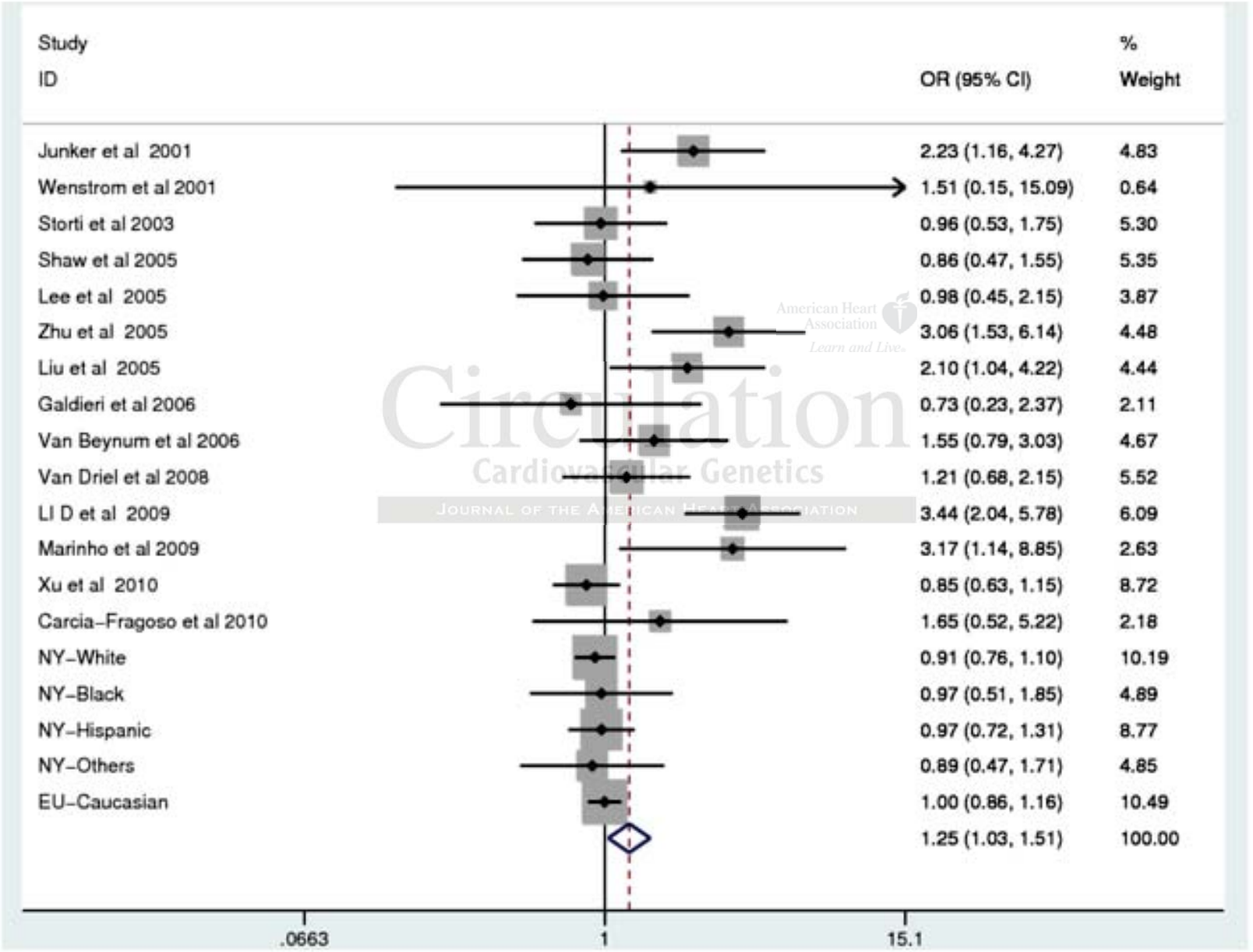

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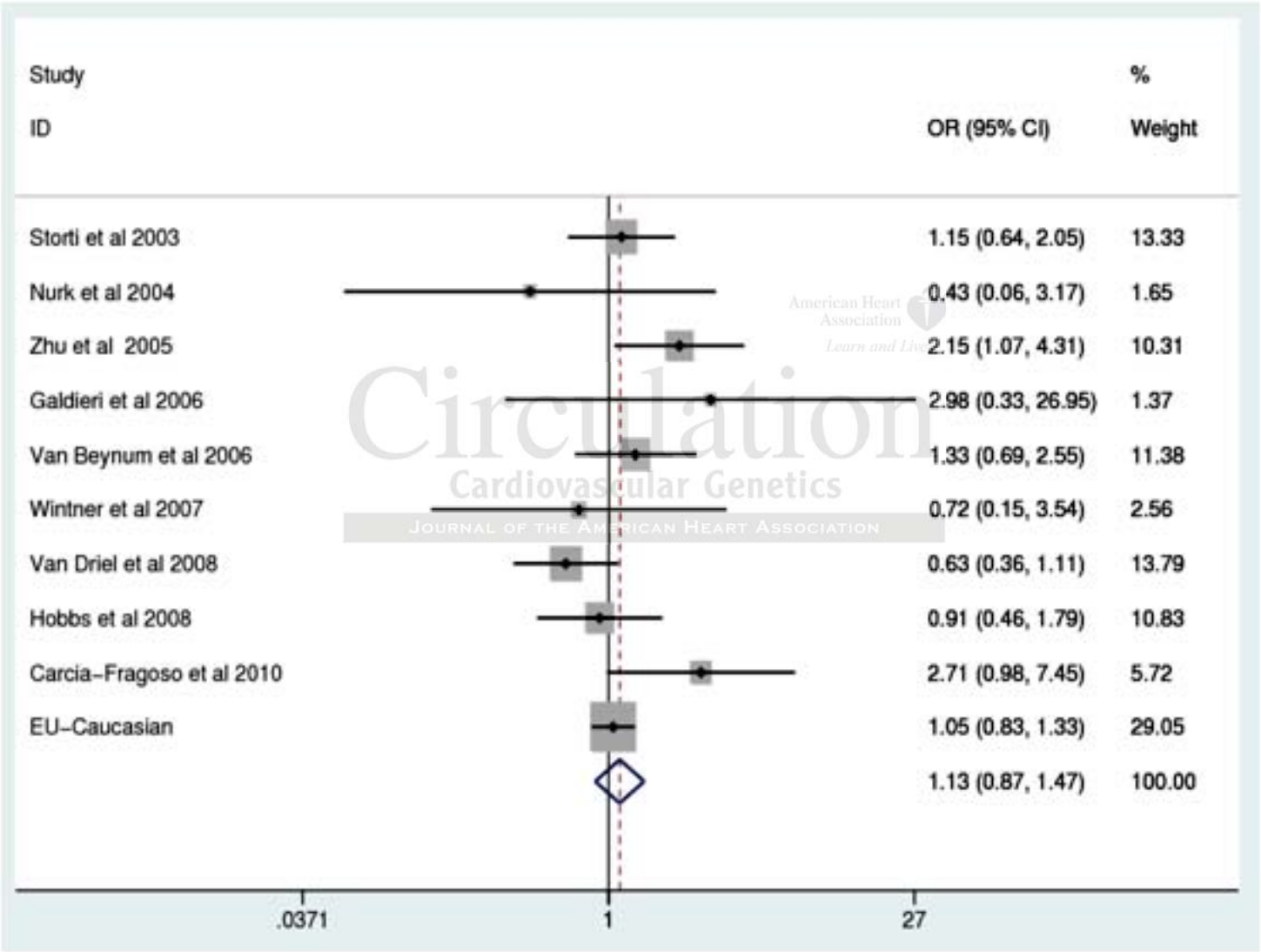

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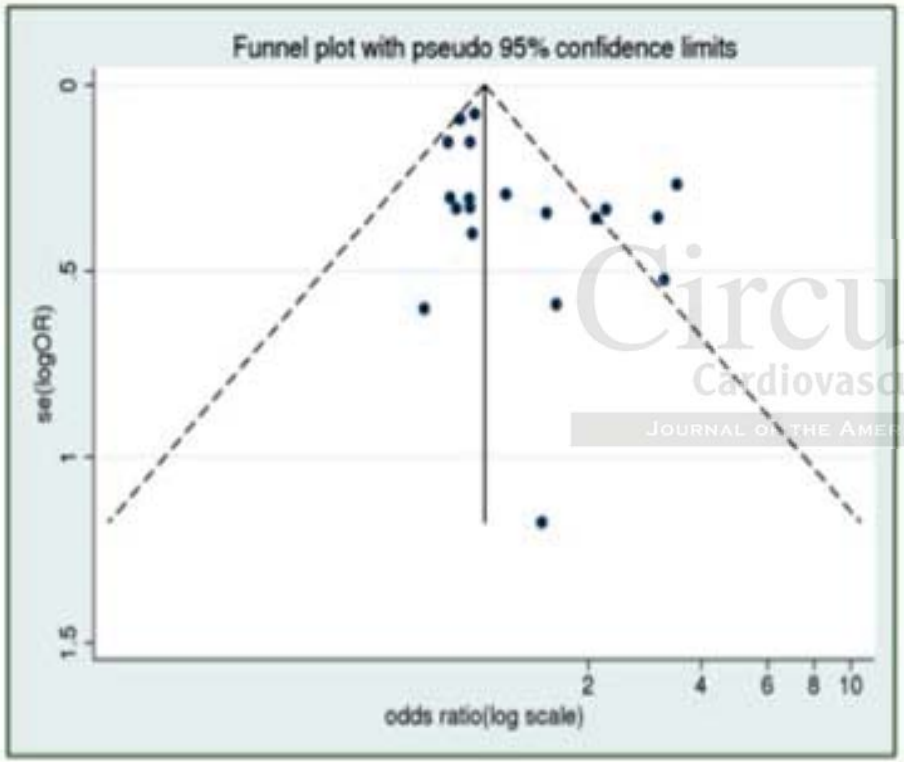

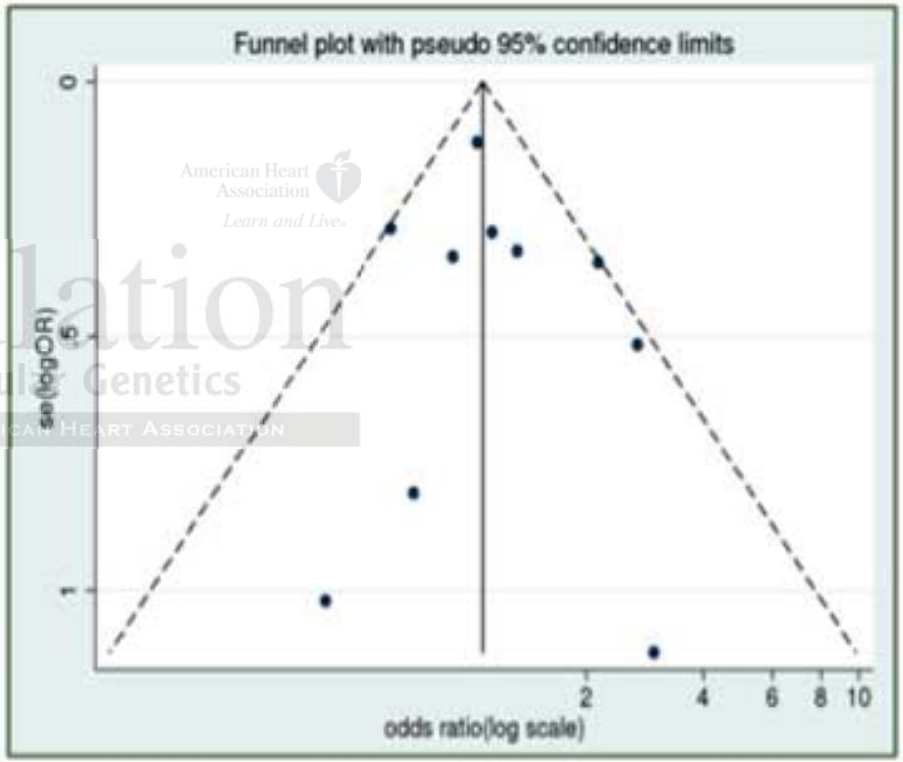

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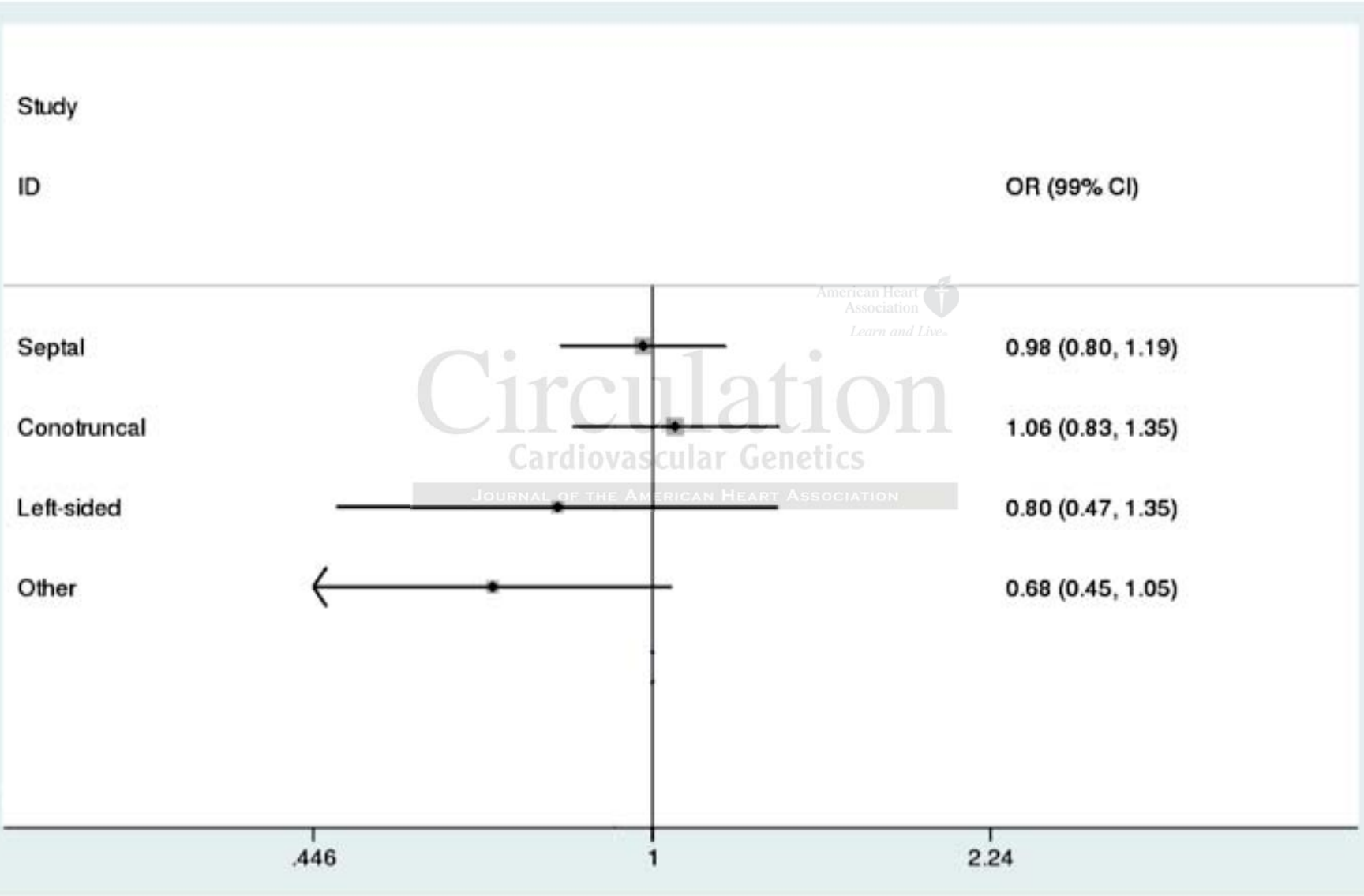

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SUPPLEMENTAL MATERIAL

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

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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. [1] 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). [2-26] Of these, three [11 5, 15] appeared to present substantially overlapping data and accordingly the largest and most recent dataset only [15] was used.

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TDT data only was presented in four studies which had enrolled families; [17 7 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 [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. [27] A recent analysis by Clarke et al. of 200,000 people in 81 population surveys of plasma folate levels,

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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.

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Supplemental Table 1 : Studies investigating association between MTHFR C677T and CHD identified by literature search

First Author	Country	Year	Year of enrolment	Types of CHD	Exclusion criteria	Ethnicity of cases	Ethnicity of controls	CC	CC- Mat	TDT	Folate status	N cases/ N controls
Wenstrom [2]	USA	2001	1988–1998	All types	Syndromes Teratogens DM	Black 27% White 69% Other 4%	Black 20% White 78% Other 2%	Yes	-	-	High	26/116
Junker [3]	Germany	2001	1995–2000	All types except PFO	Chromosomal	Caucasian	Caucasian	Yes	-	-	Mid	114/228
Storti [4]	Italy	2003	2000–2001	Conotruncal (11 cases 22q11 del)	Not described	Caucasian	Caucasian	Yes	Yes	-	Mid	103/200
Ying [5]	China	2003	Unknown	PDA, TOF, ASD,VSD, PS	Not described	Chinese	Chinese	Yes	-	-	-	N/A
Nurk [6]	Norway	2004	1950–1952, 1967–1996	Unknown	Not described	Unknown	Unknown	-	Yes	-	-	-
McBride [7]	USA	2004	1998–2003	Left-sided CHD	Multiple other malformation syndromes	Caucasian 65% Hispanic 29% Black 5% Asian 1%	Not described	-	-	Yes	-	-
Shaw [8]	USA	2005	1987–1988	Conotruncal	Aneusomies, single-gene disorders	White 67% Hispanic 23% Other 10%	White 58% Hispanic 29% Other 13%	Yes	-	-	High	151/428
Lee [9]	Taiwan	2005	2002–2003	All types	Not described	Asian	Asian	Yes	-	-	High	213/195
Pereira [10]	Brazil	2005	Unknown	All types	Not described	Unknown	Unknown	-	-	Yes		-
Liu [11]	China	2005	Unknown	Conotruncal	Not described	Chinese	Chinese	Yes	-	-	Low	97/118
Li Y [12]	China	2005	Unknown	Unknown	Not described	Chinese	Chinese	Yes	Yes	-	-	N/A
Qiu XQ [13]	China	2006	Unknown	Unknown	Not described	Chinese	Chinese	Yes	Yes	-	-	N/A
Van Beynum [14]	Netherlands	2006	2002–2003	All types	NTD, Clefts, Syndromes	Caucasian	Caucasian, same area	Yes	Yes	Yes	Mid	165/220
Zhu [15]	China	2006	Unknown	ASD, PDA	DM, PKU, Teratogens X-ray	Asian, province in China	Asian, same area	Yes	Yes	-	Low	56/103
Hobbs [16]	USA	2006	1998–2004	Septal, Conotruncal, right-left sided CHD	Syndromes Chromosomal	White	White	-	Yes	-	-	-

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First Author	Country	Year	Year of inclusion	Types of CHD	Exclusion criteria	Ethnicity of cases	Ethnicity of controls	CC	CC- Mat	TDT	Folate status	N cases/ N controls
Hobbs [17]	USA	2006	1998–2004	Septal Conotruncal, right-left sided CHD	Syndromes, Chromosomal	Not described	Not described	-	–	Yes	-	-
Galdieri[18]	Brazil	2007	Unknown	Unknown	Syndromes, Multiple malf.	White 22% Non-White 78%	White 53% Non-White 47%	Yes	Yes	–	High	58/38
Wintner [19]	Austria	2007	1993-2004	All types	Aneuploidies Syndromes Maternal DM Teratogens	Caucasian	Caucasian	-	Yes	-	-	-
Van Driel [20]	Netherlands	2008	Unknown	Multiple types	Not described	Dutch natives 89% European others 11%	Dutch natives 89% European others 11%	Yes	Yes	-	Mid	229/251
Goldmuntz [21]	USA	2008	1997-2007	Conotruncal	Syndromes Chromosomal	Any racial/ethnic group	Any racial/ethnic group	-	-	Yes	-	-
Marinho [22]	Portugal	2009	Unknown	TOF	Not described	White	Unknown	Yes	-	-	Mid	38/251
Li D [23]	China	2009	Unknown	Unknown	Not described	Chinese	Chinese	Yes	-	-	Low	104/208
Brandalize [24]	Brazil	2009	Unknown	CHD in trisomy 21	Other syndrome Other offspring with another syndrome	90% European descent 6.4% African descent 3.4% other	93.8% European descent 4.8% African descent 1.4% other	-	Yes	-	-	-
Garcia-Fragoso [25]	Puerto-Rico	2010	Unknown	Multiple	Chromosomal, Syndromes, PDA associated with prematurity, Antiepileptics, conditions associated with food intolerance, malabsorption, or wasting syndromes, maternal DM	White 76% Black 7% Other 17%	White 76% Black 7% Other 17%	Yes	Yes	-	High	27/220
Xu J [26]	China	2010	2006-2008	Left-sided CHD, Septal, PDA, Cyanotic	Chromosomal, Family history Maternal DM, PKU Teratogens Maternal drugs	Chinese	Chinese	Yes	-	-	Low	502/527

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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

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Supplementary Table 2: Previous meta-analyses of MTHFR C677T association with CHD

Meta-analysis, by first author name and year	Number of studies	OR [95% CI]	Number of cases/controls	Model
Van Beynum, 2007[30]	8	1.3 [0.97–1.73]	882/1511	TT vs. CC
Verkleij-Hagoort, 2007[31]	6	1.14 [0.86–1.53]	774/1393	TT+CT vs. CC
Nie et al, 2011 [32]	13	1.27 [0.98-1.66] ALL 1.45 [1.08-1.95] Caucasian	1898/3003	TT vs. CC+CT
Yin et al, 2012 [33]	13	1.55 [1.25, 1.93]	1655/2327	TT vs. CC

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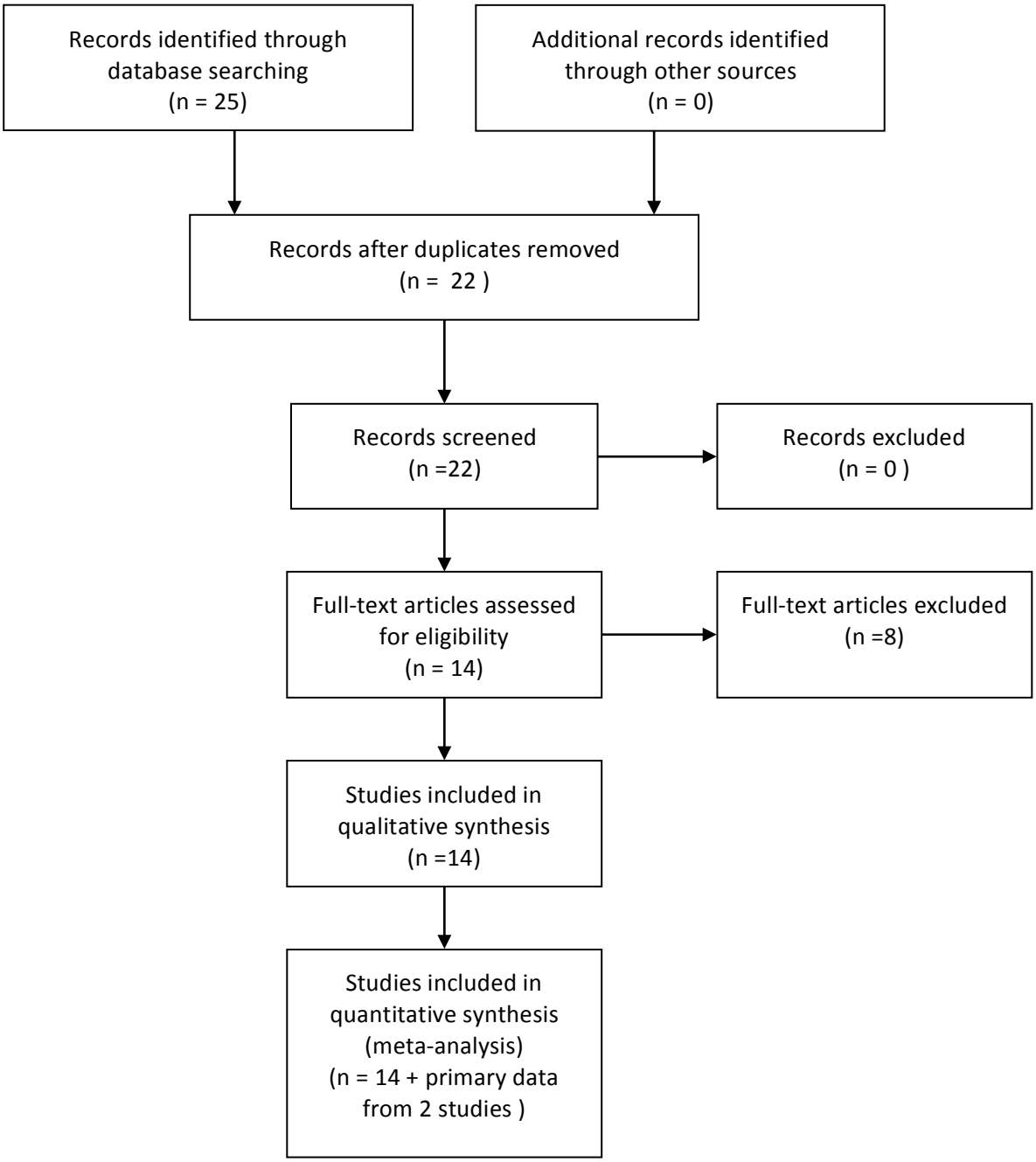
Supplemental Figure 1: PRISMA flow diagram for case-control meta-analysis



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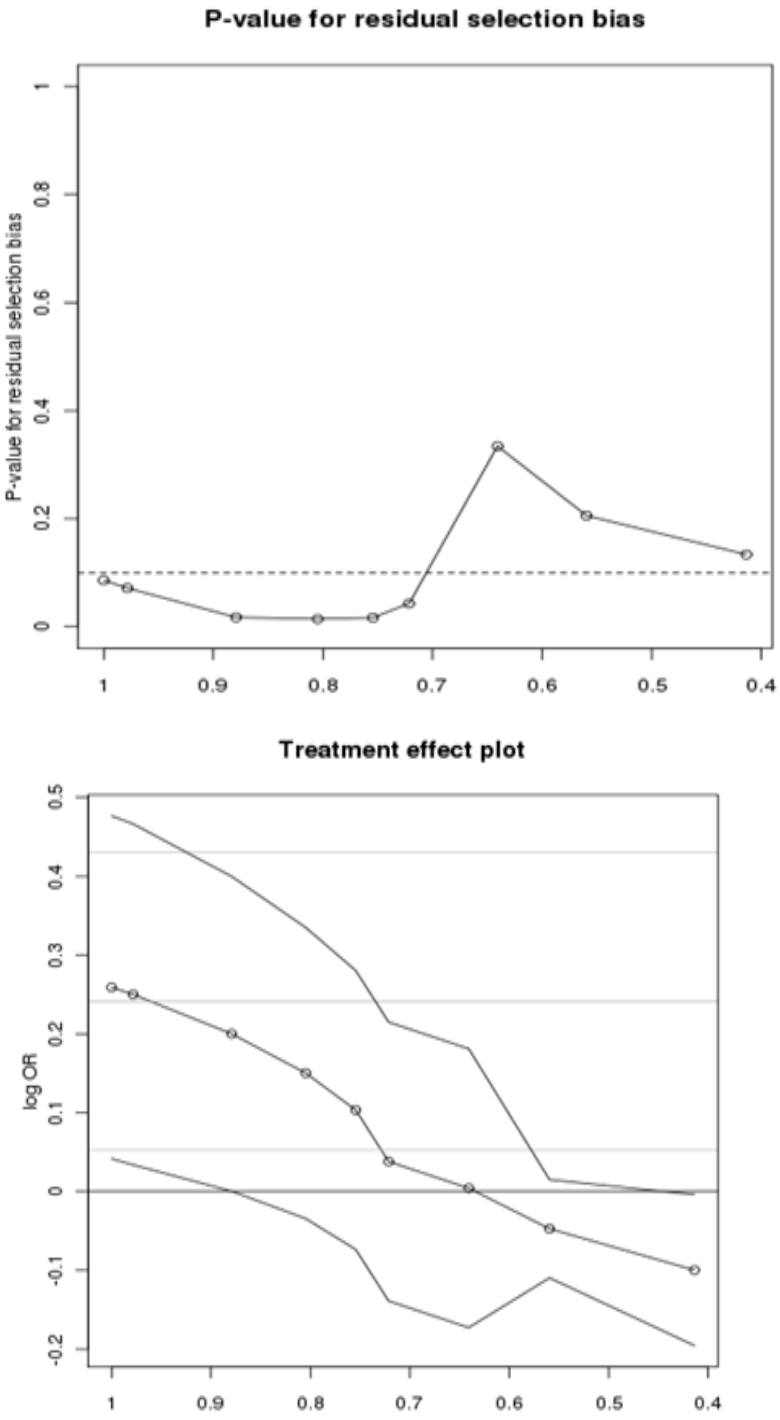
Supplemental Figure 2: Copas' selection model plot. The upper panel shows the p-value for residual selection bias (on y-axis) at diminishing probability of publication, indicating that this crosses a threshold of 0.1 at a probability of publication of ~0.7. The right panel shows the corresponding odds ratios and 95% CIs for the association of T/T genotype with CHD risk at diminishing probability of publication.



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