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# Papers

## Meta-analysis of MTHFR 677C→T polymorphism and coronary heart disease: does totality of evidence support causal role for homocysteine and preventive potential of folate?

Sarah J Lewis, Shah Ebrahim, George Davey Smith

### Abstract

**Objectives** To investigate the association between the MTHFR 677C→T polymorphism and coronary heart disease, assessing small study bias and heterogeneity between studies.

**Data sources** Medline and Embase citation searches between January 2001 and August 2004; no language restrictions.

**Study selection** Case-control and prospective studies of association between MTHFR 677C→T variant and myocardial infarction, coronary artery occlusion, or both; 80 studies were included.

**Data extraction** Data on genotype frequency and mean homocysteine concentrations by genotype were extracted. Odds ratios were calculated for TT genotype versus CC genotype. Heterogeneity was explored, with stratification by geographical region of the study samples, and meta-regression by difference in mean serum homocysteine concentrations (CC minus TT genotypes) was carried out.

**Results** 26 000 cases and 31 183 controls were included. An overall random effects odds ratio of 1.14 (95% confidence intervals 1.05 to 1.24) was found for TT versus CC genotype. There was strong evidence of heterogeneity ( $P < 0.001$ ,  $I^2 = 38.4\%$ ), which largely disappeared after stratification by geographical region. Odds ratios in Europe, Australia, and North America attenuated towards the null, unlike those in the Middle East and Asia.

**Conclusions** No strong evidence exists to support an association of the MTHFR 677 C→T polymorphism and coronary heart disease in Europe, North America, or Australia. Geographical variability may be due to higher folate intake in North America and Europe or to publication bias. The conclusion drawn from previous meta-analyses that folic acid, through lowering homocysteine, has a role in prevention of cardiovascular disease is in some doubt.

### Introduction

Observational studies have consistently shown that higher plasma homocysteine concentrations are associated with an greater risk of coronary heart disease.<sup>1</sup> However homocysteine-coronary heart disease associations may be confounded (for example, by smoking and socioeconomic position) and existing atherosclerosis could itself increase homocysteine concentrations.<sup>2,3</sup> This last explanation (reverse causality) is supported by evidence that the homocysteine-coronary heart disease associations derived from cohort studies are weaker than those from case-control studies, which are, inevitably, more prone to being

biased by existing disease leading to higher homocysteine concentrations.<sup>4</sup>

Associations between the genetic variant MTHFR 677C→T and coronary heart disease have been reported.<sup>5</sup> The genetic variant is associated with elevated homocysteine concentrations but, following the principles of mendelian randomisation,<sup>6</sup> is not subject to reverse causation or the confounding that exists in observational studies of coronary heart disease risk in relation to directly measured homocysteine concentrations. Indeed, inclusion of folate—which lowers homocysteine concentrations<sup>7</sup>—in the proposed Polypill (a combination of low dose aspirin, a statin, three blood pressure lowering drugs, and folic acid, intended for prevention of coronary heart disease) is supported by reference to two meta-analyses of such genetic studies, and the evidence of causation is said to be “compelling.”<sup>8</sup> However, the sample sizes needed for studies to accurately estimate the causal influence of intermediate phenotypes such as homocysteine concentration on disease outcomes by using common genetic variants are necessarily very large,<sup>9</sup> and publication bias (selective publication of positive findings) is likely to be a major concern with genetic association studies.<sup>10</sup>

In their meta-analysis, Wald et al tested for small study bias, reporting an Egger test of  $P = 0.55$ .<sup>5</sup> Another meta-analysis of the association between MTHFR 677C→T and coronary heart disease showed potential publication bias, in which non-publication of small negative studies would lead to overestimation of the strength of association between the MTHFR 677C→T variant and coronary heart disease.<sup>11</sup> Since the publication of these two meta-analyses several new studies have appeared, including one larger than any previous study.<sup>12</sup> Adding these and studies already published but presumably missed by the search strategy used by previous investigators more than doubles the number of cases available for analysis compared with the two previous comprehensive meta-analyses.<sup>5,11</sup> We have therefore carried out an updated meta-analysis using a comprehensive literature search and fully investigated potential publication bias. This work is important, as it is essential to define carefully the evidence for inclusion of folic acid in the Polypill, which has been strongly promoted, has been patented, and is in the process of being manufactured.



References to included studies are on [bmj.com](http://bmj.com)

**Table 1** Summary findings by geographical region: odds ratios for coronary heart disease comparing TT with CC genotypes, and difference in geometric mean serum homocysteine concentrations

Region	Odds ratio (95% CI)	No of cases:controls	No of studies	Difference in serum homocysteine CC-TT (95% CI)	Odds ratio (95% CI) for 5 $\mu\text{mol/l}$ increase in homocysteine
Europe	1.08 (0.99 to 1.18)	17 275:21 313	41	2.03 (1.15 to 2.90)	1.21 (0.98 to 1.50)
North America	0.93 (0.80 to 1.10)	3714:3969	15	1.35 (0.32 to 2.38)	0.76 (0.44 to 1.42)
Middle East	2.61 (1.81 to 3.75)	971:1316	5	8.80 (1.23 to 16.4)	1.72 (1.12 to 4.90)
Asia	1.23 (0.94 to 1.62)	2755:4735	16	3.76 (2.29 to 5.23)	1.32 (0.92 to 1.90)
Australia	1.04 (0.73 to 1.49)	1285:480	3	–	–
Overall	1.14 (1.05 to 1.24)	26 000:31 813	80	2.24 (1.55 to 2.94)	1.34 (1.12 to 1.62)

## Methods

We identified eligible studies by searching Medline and Embase for all publications between January 2001 and August 2004, updating the search used in earlier meta-analyses.<sup>5</sup> We used the search terms “mthfr,” “methylenetetrahydrofolate reductase,” “5,10 methylenetetrahydrofolate reductase,” “677C,” and “C677T” in combination with “cardiovascular disease,” “ischemic heart disease,” “coronary,” “heart disease,” “myocardial infarction,” and “angina.” We did not exclude any studies on the basis of language. We used the outcome as defined by previous investigators of myocardial infarction or coronary artery occlusion (> 50% of the luminal diameter).

We based unadjusted odds ratios on published genotype frequencies or extracted them directly from publications where raw data were not available. We used random effects models.

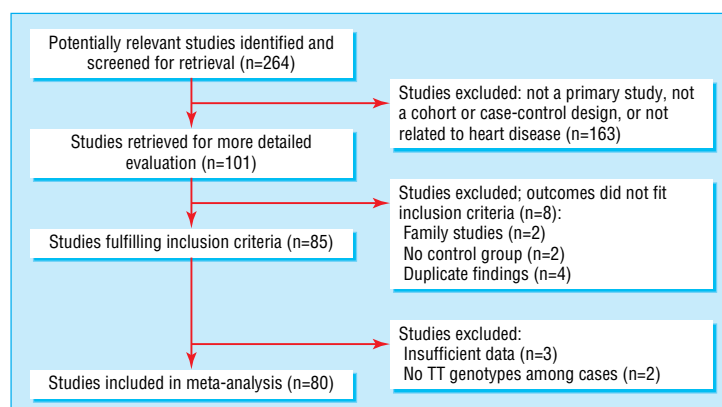
Where possible, we assessed whether the frequencies of CC, CT, and TT genotypes among controls in individual studies were consistent with the expected distribution (that is, in Hardy-Weinberg equilibrium) by using the Pearson  $\chi^2$  test. In line with previous work, we compared the TT genotype with the CC genotype. In our meta-analysis of all studies, we stratified by the geographical region in which the studies were done—Asia, Australia, the Middle East, North America, and Europe—and gave odds ratios by region. We quantified the extent of heterogeneity by using  $I^2$ , which represents the percentage of total variation across studies that is attributable to heterogeneity rather than chance.<sup>13</sup> Where mean serum homocysteine concentrations were given by genotype, we extracted these for all samples or for controls only where values for all samples were not provided or could not be calculated. We estimated the difference in serum homocysteine concentrations between CC and TT genotypes by using a random effects meta-analysis. We used meta-regression analysis to assess whether differences in serum homocysteine concentration affected MTHFR-disease associations. We used Stata version 8 for all statistical analyses.

We calculated the predicted odds ratio for coronary heart disease risk for a 5  $\mu\text{mol/l}$  increase and a 3  $\mu\text{mol/l}$  decrease in homocysteine concentrations, for comparison with the Wald et al meta-analysis,<sup>5</sup> by raising the odds ratio and confidence intervals to the power of 5/2.24 (the overall difference in mean homocysteine between CC and TT genotypes) and to the power of  $-3/2.24$ .

## Results

In all, we included 81 data points from 80 studies in this meta-analysis (table 1; fig 1), with a total of 26 000 cases and 31 813 controls. We identified 24 studies that we believe fitted the earlier reviews' inclusion criteria,<sup>5 11</sup> but which were not included in either of the original meta-analyses either because they were not identified by the original search strategies or because they have been published since this meta-analysis. We found an overall random effect odds ratio for coronary artery disease comparing TT with CC genotypes of 1.14 (95% confidence interval 1.05 to 1.24). Five studies showed evidence to suggest ( $P < 0.05$ ) that genotypes were not in Hardy-Weinberg equilibrium in controls. However, exclusion of these studies made very little difference to the overall result (odds ratio 1.15, 1.06 to 1.26). We also found evidence that effect estimates were related to study size, suggesting that small study bias, such as publication bias, may have distorted the findings (Egger test  $P = 0.03$ ).

We found strong evidence of between study heterogeneity ( $P < 0.001$ ,  $I^2 = 38.4\%$ ). Stratified analysis by geographical region removed much of the heterogeneity within Europe, North America, Australia, and the Middle East; the random effect odds ratio were 1.08 (0.99 to 1.18;  $P_{\text{heterogeneity}} = 0.19$ ,  $I^2 = 16.1\%$ ) among the 42 European studies, 0.93 (0.80 to 1.10;  $P_{\text{heterogeneity}} = 0.64$ ,  $I^2 = 0\%$ ) among the 15 North American studies, 1.04 (0.73 to 1.49;  $P_{\text{heterogeneity}} = 0.97$ ,  $I^2 = 0\%$ ) among the three Australian studies, and 2.61 (1.81 to 3.75;  $P_{\text{heterogeneity}} = 0.57$ ,  $I^2 = 0\%$ ) among

**Fig 1** Results of search strategy

the five Middle Eastern studies (fig 2). Evidence of heterogeneity remained between Asian studies, but this was only apparent between Japanese studies and others and within studies done in Japan. The random effects odds ratio for Japanese studies was 1.71 (1.23 to 2.37;  $P_{\text{heterogeneity}} = 0.01$ ,  $I^2 = 62.5\%$ ); studies done in China, Taiwan, and Korea produced homogeneous results, with an overall odds ratio of 0.81 (0.60 to 1.10;  $P_{\text{heterogeneity}} = 0.41$ ,  $I^2 = 3.0\%$ ).

To minimise the effect of inter-laboratory variation in measurement of homocysteine concentrations, we used differences in homocysteine concentrations between TT and CC genotypes rather than absolute homocysteine concentrations as a measure of geographical differences in dietary folate, as differences in homocysteine concentrations by MTHFR C677T genotype have been shown to be greater at lower levels of folate intake and are reduced after folate supplementation.<sup>14</sup> We examined whether these differences were associated with the size of effect of the MTHFR genotype on coronary heart disease risk. Of the 20 studies that gave mean homocysteine concentrations and standard deviations by genotype, the differences ranged from  $-0.8$  to  $11 \mu\text{mol/l}$  (table 2). A random effects meta-analysis of mean difference in homocysteine concentrations between CC and TT genotypes found an overall difference of  $2.24 \mu\text{mol/l}$  (95% confidence interval 1.55 to 2.94). In a meta-regression analysis we found evidence to suggest that differences in serum homocysteine concentrations by genotype were associated with the effect of genotype on coronary artery disease risk ( $\beta = 0.103$ ,  $P = 0.02$ ), although this does not take into account the uncertainty in homocysteine differences by genotype. This equates to an increase in the odds ratio of exponential 0.103 (0.019 to 0.186) or 1.11 (1.01 to 1.20) per  $1 \mu\text{mol/l}$  increase in the difference in homocysteine concentrations between CC and TT genotypes.

We calculated the predicted odd ratios for coronary heart disease risk for given changes in homocysteine concentrations by region (table 1) and compared them with the Wald et al<sup>5</sup> meta-analysis (table 3). Findings were broadly similar for increased odds of coronary heart disease associated with the CC genotype and the effect of a  $5 \mu\text{mol/l}$  increase or a  $3 \mu\text{mol/l}$  decrease in homocysteine concentration between our meta-analysis and Wald's.

## Discussion

### Do high homocysteine concentrations cause heart disease?

Our meta-analysis of 80 studies gives an estimate of a 14% (95% confidence interval 5% to 24%) greater risk of coronary heart disease associated with the MTHFR CC genotype. However, because of the marked heterogeneity between studies, causal inferences about the association between homocysteine and coronary heart disease must be guarded. Because of the large amount of data in our meta-analysis, an examination of the geographical variation was possible; this showed wide variation between regions, and in European, north American, and Australian studies no strong evidence existed to support any effect of homocysteine on coronary heart disease risk. Only in studies carried out in the Middle East and in Asia did any evidence exist to support the hypothesis, and in Asia the effect was confined to Japanese studies only.

### Is regional heterogeneity due to differences in folate intake?

It has been suggested that the observed geographical differences relate to nutritional habits, widespread use of vitamin supplements, or fortification of breakfast cereals with folate in North America and Europe in contrast with more unfavourable

intakes of folic acid in other regions.<sup>11</sup> Differences in homocysteine concentrations by MTHFR C677T genotype have been shown to be greater at lower levels of folate intake and are reduced after folate supplementation.<sup>14</sup> In our meta-analysis we found relatively modest differences in homocysteine concentrations by genotype in the United States, where fortification of foods with folate is mandatory, but larger differences in other regions. A recent meta-analysis that used mendelian randomisation to examine the association of homocysteine with stroke confirmed the lower mean difference between TT and CC MTHFR C677T genotypes in homocysteine concentration for North American but not European studies, but the numbers of participants studied were much smaller.<sup>15</sup> If increased folate intake in some parts of the world does explain the heterogeneity of the associations, folic acid as a preventive intervention would be unlikely to have any major role to play in the regions where there is no MTHFR-coronary heart disease association.

### Regional heterogeneity and publication bias

We found that differences in homocysteine concentrations by genotype were positively associated with the size of the effect of genotype on risk of coronary heart disease. However, this does not necessarily provide convincing evidence of a causal relation between homocysteine concentrations and risk of coronary heart disease, because the correlation of larger mean differences and larger effects in certain regions could equally represent publication bias. The strongly positive studies reported from the middle East and Japan could also represent publication bias, especially given the high levels of scientific activity around MTHFR variants. In support of this, although our meta-analysis included too few Japanese and Middle Eastern studies to provide sufficient power to test for small study bias, the largest Japanese study included gave a null result (odds ratio 0.99, 0.74 to 1.32),<sup>16</sup> and a further large Japanese study (445 cases) that could not be included in our meta-analysis owing to insufficient data also found a null result ( $P > 0.1$ ).<sup>17</sup> The largest Middle Eastern study included in our meta-analysis, which contributed more than half of the total cases for this region, gave a result that was not incompatible with the null hypothesis.<sup>18</sup> A similar meta-analysis of MTHFR and ischaemic stroke also found a greater increase in risk among TT homozygotes in Japan compared with other countries and regions.<sup>19</sup> The inference that the MTHFR-disease association is greatest in Japan because folate intake is low seems to be incongruous with the low incidence of folic acid deficiency and neural tube defects in Japan relative to other countries.<sup>20 21</sup>

### Evidence from clinical trials

Recent trials of folate supplementation in North America, Europe, and Australia that have examined clinical outcomes and surrogates have been negative.<sup>22-26</sup> These trials have largely been done in populations with high background folate consumption and are unlikely to resolve the question of whether folate supplementation or folate fortified food could be an important intervention in populations with "insufficient" folate consumption. We await more randomised trial findings that, we hope, will clarify the role of homocysteine in coronary heart disease and the relevance of folate supplementation for prevention.

### Limitations

Any meta-analysis has limitations, and although we invested considerable effort in searching for published studies of genetic associations and found considerably more than previous meta-analyses, our estimates of effect are still likely to represent overestimation through publication bias. Unlike randomised controlled trials, which are becoming easier to locate, aided by

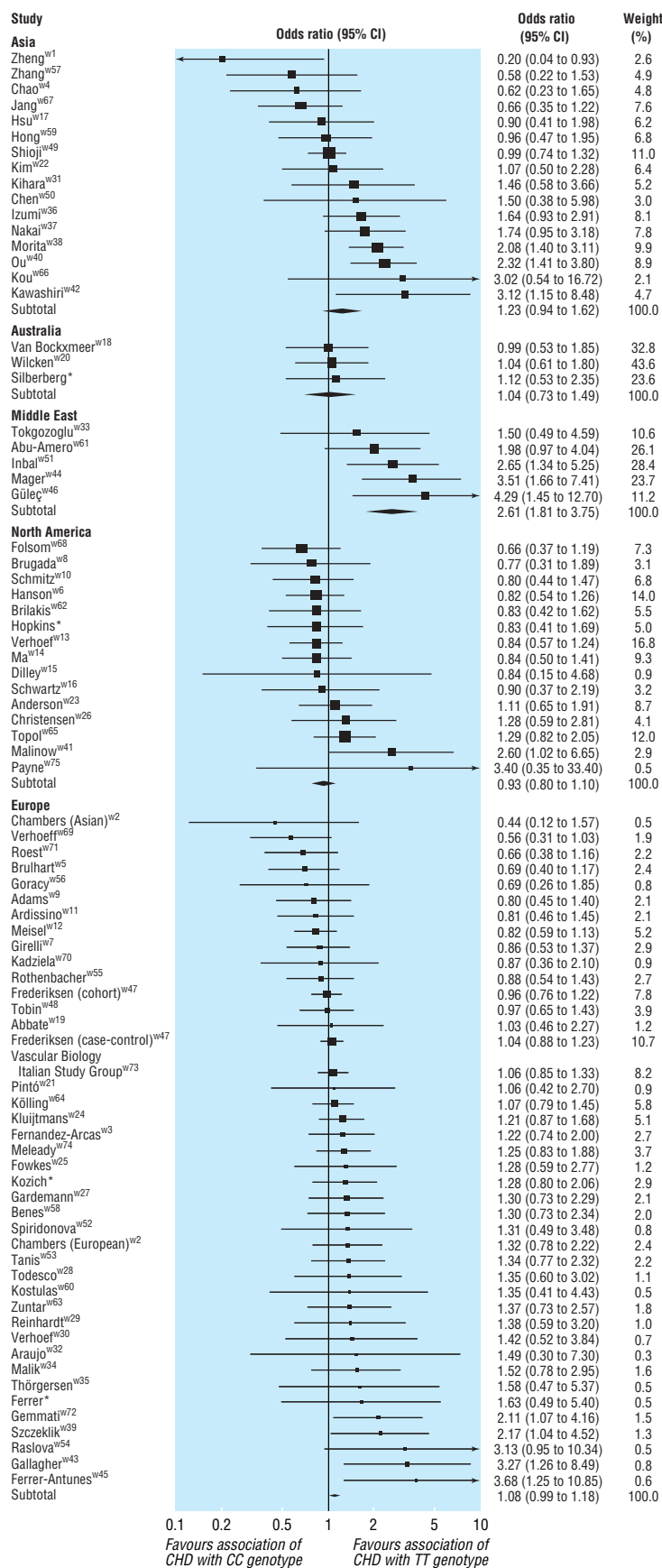


Fig 2 Association between MTHFR C677T polymorphism and coronary heart disease (TT versus CC genotype). \*Unpublished data taken from Klerk et al, 2002 meta-analysis

**Table 2** Studies reporting serum homocysteine concentrations ( $\mu\text{mol/l}$ ) according to MTHFR genotype: homozygotes for the mutant allele (TT), heterozygotes (CT), and wild type homozygotes (CC)

Study: first author	TT		CT		CC		Difference between TT and CC
	Mean (SD)	No	Mean (SD)	No	Mean (SD)	No	
Chambers (Asian) <sup>w2</sup>	11.6 (12.2)	15	11.3 (4.5)	151	11.2 (4.2)	439	0.4
Chao <sup>w4</sup>	12.6 (6.6)	19	10.8 (4.2)	72	9.7 (4.0)	101	2.9
Tsai <sup>w6b</sup>	10.4 (4.3)	52	9.4 (4.1)	212	9.1 (4.3)	194	1.3
Girelli <sup>w7b</sup>	20.7	112	15.5	322	14.6	221	6.1
Schmitz <sup>w10</sup>	9.1 (2.3)	14	10.6 (3.8)	46	9.9 (2.7)	67	-0.8
Meisel <sup>w12</sup>	10.3 (4.5)	177	9.7 (3.6)	421	9.4 (4.5)	265	0.9
Ma <sup>w14</sup>	12.6 (4.1)	72	10.9 (5.9)	240	10.6 (5.9)	271	2
Schwartz <sup>w16</sup>	13.5 (7.0)	43	10.8 (3.9)	141	10.9 (3.8)	154	2.6
Kim <sup>w22</sup>	13.5 (6.0)	16	12.6 (3.10)	41	12.2 (3.5)	30	1.3
Kluijtmans <sup>w24</sup>	15.4 (8.2)	51	13.4 (6.3)	233	12.6 (5.7)	231	2.8
Christensen <sup>w26</sup>	12.8 (4.7)	22	11.0 (3.9)	98	10.3 (3.5)	87	2.5
Chambers (European) <sup>w2</sup>	11.7 (5.6)	72	10.5 (4.1)	286	10.3 (3.0)	296	1.4
Verhoeff <sup>w30</sup>	16.9*	20	12.4*	107	11.8*	104	5.1
Tokgozoglul <sup>w33</sup>	23.3 (15)	16	16.9 (9)	57	14.5 (7)	57	8.8
Nakai <sup>w37</sup>	11.6 (5.6)	63	8.9 (4.1)	191	8.6 (3.3)	174	3
Ou <sup>w40</sup>	15.1 (6.0)	27	11.2 (1.9)	19	10.5 (3.3)	39	4.6
Malinow <sup>w41</sup>	10.3 (3.8)	25	9.4 (3.7)	128	9.1 (3.5)	89	1.2
Kawashiri <sup>w42</sup>	21.0 (11.0)	12	11.0 (3.5)	36	10.0 (4.6)	34	11
Frederiksen <sup>w47</sup>	14.7 (9.25)	342	11.7 (4.17)	1738	11.8 (4.42)	1955	2.9
Tanis <sup>w53</sup>	14.9 (5.3)	59	12.4 (3.2)	262	11.6 (2.7)	280	3.3
Raslova† <sup>w54</sup>	18.5*		14.4*		13.5*		5
Rothenbacher <sup>w55</sup>	10.2	81	8.7	336	8.4	374	1.8
Kölling <sup>w64</sup>	13.2 (5.7)	52	11.9 (4.1)	284	11.7 (4.5)	281	1.5
Jang <sup>w67</sup>	13.5 (6.79)	48	8.95 (3.65)	115	8.87 (3.77)	67	4.6
Verhoeff <sup>w69</sup>	17.6*		13.2*		12.7*		4.9
Meleady <sup>w74</sup>	12.2*	81	9.7*	314	9.3*	352	2.9

\*Geometric mean.

†Estimated from figure.

the work of the Cochrane Collaboration, observational genetic epidemiology has yet to develop mechanisms for producing robust and systematic reviews of available evidence. Mechanisms are clearly needed for sharing of data, archiving of all relevant findings whether positive or negative, and avoiding duplication of effort. Guidelines could be developed by building on experience gained over the past five years in the conduct of reviews of human genome epidemiology (HuGE).<sup>27</sup> A further limitation of our analysis is that we did not have individual level data and were not able to take account of the uncertainty in differences in mean homocysteine concentrations between people with CC and TT genotypes in our meta-regression analysis. To allow comparison with Wald et al,<sup>5</sup> we did not limit the estimation of our MTHFR-homocysteine effect to healthy people, and the inclusion of some people with disease in this analysis may have clouded the true effect of genotype.<sup>28</sup>

### Conclusions

The results of this meta-analysis cast some doubt on the implications drawn from previous reviews that support a role for folic acid in preventing cardiovascular disease. Ironically, enthusiasm

for such intervention comes from high income countries where the evidence would suggest there would be little, if anything, to gain with respect to coronary heart disease risk from increasing folic acid intake. The growing observational evidence has important implications for the inclusion of folic acid in any Polypill strategy.

### What is already known on this topic

The MTHFR 677C→T polymorphism is associated with homocysteine concentration; people with TT genotype have higher concentration than those with the CC genotype

Two recent meta-analyses reported associations between MTHFR C677T genotype and coronary heart disease, concluding that high homocysteine concentrations are causally related to coronary heart disease risk

### What this study adds

Since the publication of the previous meta-analyses several more studies have been published, and the total number of cases and controls has doubled

This meta-analysis provides no evidence of a causal relation between homocysteine and coronary heart disease risk in European, North American, and Australian populations

This study casts doubt on the beneficial role of lowering homocysteine concentrations, through supplementation with folic acid, to prevent heart disease

**Table 3** Summary results from three meta-analyses of MTHFR C677T and coronary heart disease

Study	Overall odds ratio (95% CI)	5 $\mu\text{mol/l}$ increase in homocysteine	3 $\mu\text{mol/l}$ decrease in homocysteine
Wald et al <sup>5</sup>	1.21 (1.06 to 1.39)	1.33 (1.22 to 1.46)	0.84 (0.80 to 0.89)
Klerk et al <sup>11</sup>	1.11 (1.05 to 1.28)	Not available	Not available
Our meta-analysis	1.14 (1.05 to 1.24)	1.34 (1.12 to 1.62)	0.84 (0.75 to 0.94)*

\*Our confidence intervals are larger because we included only studies with information on coronary heart disease in the analysis of difference in homocysteine concentration by genotype, whereas Wald et al also included studies of deep vein thrombosis and stroke.

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Ethical approval: Not needed.

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