The M235T Polymorphism in the AGT Gene and CHD Risk: Evidence of a Hardy-Weinberg Equilibrium Violation and Publication Bias in a Meta-Analysis

Mohammad Hadi Zafarmand¹,², Yvonne T. van der Schouw², Diederick E. Grobbee², Peter W. de Leeuw³, Michiel L. Bots²*¹

¹Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands, ²Persian Gulf Health Research Center, Bushehr University of Medical Sciences and Health Services, Bushehr, Iran, ³Department of Internal Medicine, University Hospital Maastricht, Maastricht, The Netherlands

Abstract

Background: The M235T polymorphism in the AGT gene has been related to an increased risk of hypertension. This finding may also suggest an increased risk of coronary heart disease (CHD).

Methodology/Principal Findings: A case-cohort study was conducted in 1,732 unrelated middle-age women (210 CHD cases and 1,522 controls) from a prospective cohort of 15,236 initially healthy Dutch women. We applied a Cox proportional hazards model to study the association of the polymorphism with acute myocardial infarction (AMI) (n = 71) and CHD. In the case-cohort study, no increased risk for CHD was found under the additive genetic model (hazard ratio [HR] = 1.20; 95% confidence interval [CI], 0.86 to 1.68; P = 0.28). This result was not changed by adjustment (HR = 1.17; 95% CI, 0.83 to 1.64; P = 0.38) nor by using dominant, recessive and pairwise genetic models. Analyses for AMI risk under the additive genetic model also did not show any statistically significant association (crude HR = 1.14; 95% CI, 0.93 to 1.39; P = 0.20). To evaluate the association, a comprehensive systematic review and meta-analysis were undertaken of all studies published up to February 2007 (searched through PubMed/MEDLINE, Web of Science and EMBASE). The meta-analysis (38 studies with 13284 cases and 18722 controls) showed a per-allele odds ratio (OR) of 1.08 (95% CI, 1.01 to 1.15; P = 0.02). Moderate to large levels of heterogeneity were identified between studies. Hardy-Weinberg equilibrium (HWE) violation and the mean age of cases were statistically significant sources of the observed variation. In a stratum of non-HWE violation studies, there was no effect. An asymmetric funnel plot, the Egger’s test (P = 0.066), and the Begg-Mazumdar test (P = 0.074) were all suggestive of the presence of publication bias.

Conclusions/Significance: The pooled OR of the present meta-analysis, including our own data, presented evidence that there is an increase in the risk of CHD conferred by the M235T variant of the AGT gene. However, the relevance of this weakly positive overall association remains uncertain because it may be due to various residual biases, including HWE-violation and publication biases.

Introduction

Angiotensinogen (AGT) is a liver protein that interacts with renin to produce angiotensin I, the pro-hormone of angiotensin II. Angiotensin II is the major effector molecule of the renin-angiotensin-aldosterone system (RAAS) and plays a key role in the regulation of blood pressure (BP) by increasing vascular tone and promoting sodium retention. Genetic variants in the angiotensinogen gene modify the plasma concentration of angiotensinogen, which has been directly related to arterial blood pressure [1]. The molecular variant (M235T) of the AGT gene, encoding a threonine instead of a methionine at residue 235 of the mature protein, has been associated with a higher plasma AGT level and higher BP in patients homozygous for the T allele and occurs among various ethnic populations [1–3]. In a meta-analysis, the TT genotype was associated with a 32% increase in the risk of hypertension in white people but not in non-white people, when compared with the MM genotype [4].

Given the importance of hypertension in the occurrence of coronary heart disease [5], this finding suggests that this polymorphism may be related to increased risk of CHD. A few studies [6–8], including recent publications, [9,10] have found that there is an association of the M235T AGT variant with increased CHD risk; however, this relationship was not confirmed in several other studies [11–13] as well as in a meta-analysis [14]. Marked ethnic differences in the frequency of the T allele, small sample
sizes and genotyping or phenotyping errors could partly account for discrepancies among these gene-disease association studies. Therefore, we investigated the association of the M235T polymorphism in the AGT gene (National Center for Biotechnology Information single nucleotide polymorphism cluster ID rs699) with acute myocardial infarction (AMI) and CHD in a large population-based cohort of middle-aged Dutch women and conducted an updated meta-analysis of the available studies to clarify the role of the M235T polymorphism in CHD risk.

Methods

Case-cohort study

Study design, general questionnaire, anthropometric and laboratory measurements have been described in detail elsewhere [15–16]. Briefly, the study population consisted of participants of the Prospect-EPIC cohort. Participants were recruited between 1993 and 1997 among women living in Utrecht and the vicinity who attended the regional population-based breast cancer-screening program. A total of 17,357 women, aged 49–70, were included. At baseline, a general and a dietary questionnaire were administered, a limited physical examination was performed and a non-fasting blood sample was taken. Follow-up event information was obtained from the Dutch Centre for Health Care Information, which holds a standardized computerized register of hospital discharge diagnoses. Using the International Classification of Diseases, ninth Revision (ICD-9) codes for the main discharge reason, we categorized cardiovascular disease (codes 390–459) as CHD (codes 410–414), including AMI (code 410), and other cardiovascular diseases. Whenever multiple events (AMI and CHD) occurred, the first occurrence of that endpoint was taken as the endpoint of interest in endpoint-specific analyses. All women signed an informed consent form prior to study inclusion. The study was approved by the Institutional Review Board of the University Medical Center Utrecht.

We applied the case-cohort design introduced by Prentice [17]. From the 17,357 women in the total cohort, we randomly selected a sample of 10% as the sub-cohort (n = 1736). Women who did not consent to linkage with vital status registries or who were not traceable (cases n = 3/sub-cohort n = 38) were not included. Women who reported a diagnosis of cardiovascular disease (ICD-9: 390–459) at baseline or who had missing questionnaires, blood, or DNA samples were excluded. This resulted in 15,236 women in the total cohort and 1522 women in the sub-cohort (as the control group) at baseline. All individuals with first fatal and non-fatal CHD and ischemic stroke events that arose during follow-up until January 1st 2000 were selected as cases. These were 211 CHD cases, including 71 AMIs. For all case subjects, follow-up ended at the date of diagnosis or at the date of death due to cardiovascular disease.

Genetic analysis. Genetic analysis was performed at the Cardiovascular Genotyping (CAGT) laboratory of the Department of Internal Medicine of the University Hospital Maastricht. Genomic DNA was extracted fromuffy coats using the QIAamp® Blood Kit (Qiagen Inc., Valencia, California, USA). Genotyping of the polymorphisms was performed using a multilocus genotyping assay for candidate markers of cardiovascular disease risk (Roche Molecular Systems Inc., Pleasanton, CA, USA) [18]. Briefly, each DNA sample was amplified using two multiplex polymerase chain reactions, and the alleles were genotyped simultaneously using an array of immobilized sequence-specific oligonucleotide probes. This array of probes was blotted on plastic strips, and, after staining, genotypes were scored based on the intensity of each band and checked manually. Genotyping was performed blinded to the case-control status. A random double-check was performed to detect potential genotyping errors in a subset of 100 samples. The check confirmed the previous genotyping results by 100%.

Data analysis. Hardy-Weinberg equilibrium (HWE) was tested with the χ² test among the controls. Allele frequencies were estimated by gene counting. We used the ANOVA F-test to estimate differences among the M235T genotypes and continuous variables, while we tested the significance of any difference in proportions by applying the χ² statistic. A p-value <0.05 (2-sided) was considered statistically significant.

To assess the relationship of the M235T polymorphism in the AGT gene with the outcome, we used a Cox proportional hazards model with an estimation procedure adapted for case-cohort designs. We used the unweighted method by Prentice [17,19], which is incorporated in a SAS macro at http://lib.stat.cmu.edu/
general/robphreg. A previous meta-analysis [14] showed that the effect of the AGT M235T variant on its intermediate phenotype (plasma angiotensinogen level) follows an additive model according to the number of T alleles [5% (95% CI: 2 to 8%) increase for the MT and 11% (95% CI: 7 to 15%) increase for the TT genotype versus the MM genotype]. Therefore, our priori hypothesis was that the association between the M235T polymorphism in the AGT gene and CHD follows an additive model according to the number of T alleles. However, other genetic models were evaluated as well. We considered different modes of inheritance as follows: the additive ‘‘per-allele’’ model, the T allele was compared between cases and controls by assigning scores of 0, 1, and 2 to homozygotes for the M allele, heterozygotes, and homozygotes for the T allele, respectively; the recessive model, the TT genotype versus the MT and MM combined genotypes; and the dominant model, the MT and TT genotypes combined versus the MM genotype. We also performed separate pairwise comparisons of the MT and TT genotypes versus the MM genotype.

Meta-analysis

Searching. We searched PubMed/MEDLINE, Web of Science, and EMBASE up to February 2007 for observational studies evaluating an association between the M235T polymorphism in the AGT gene and CHD. Terms used for the search contained both medical subject heading terms and text words: (Met235Thr OR M235T OR T704C) AND (angiotsensinogen OR AGT) AND (polymorphism OR mutation OR genetic OR genotype) AND (“coronary disease” OR “coronary heart disease” OR CHD OR “myocardial infarction” OR MI OR “myocardial infract” OR “coronary artery disease” OR CAD OR “ischemic heart disease” OR IHD OR “cardiovascular disease” OR “heart disease” OR angina). We also retrieved additional studies by hand searching the bibliographies of original research reports and review articles and through the MEDLINE option ‘‘related articles’’. Search results were limited to articles published in English and studies on human subjects.

Selection. All studies were considered potentially eligible if they aimed to investigate the relationship between the M235T genotypes and risk of CHD or MI. Any observational study, regardless of sample size, which fulfilled the following criteria, was included: (i) AGT M235T genotype frequencies were provided by case-control status (studies without controls were excluded); (ii) risk of CHD or MI was evaluated (studies on recurrent coronary events
were excluded); (iii) relevant data were presented to calculate the effect size and its 95\% CI; (iv) non-overlapping data were contained. For duplicate publications, the study with the smaller data set was excluded.

Data abstraction. The following information was extracted from each study that we included: the first author’s name; country; year of publication; the population evaluated; study design; mean age or age range for case-patients and controls; definition and number of cases and controls; allele frequencies and genotype distribution in case-patients and controls (where data were not given, they were calculated from the corresponding genotyping frequencies of the case and control groups); consistency of genotype frequencies with HWE (calculated); gender in the evaluated population and male percentage; matching variables, use of blinding of genotyping staff, performing re-genotyping of a genotype frequencies with HWE (calculated); gender in the evaluated population and male percentage, matching variables, use of blinding of genotyping staff, performing re-genotyping of a genotype frequencies with HWE (calculated); gender in the evaluated population and male percentage, matching variables, use of blinding of genotyping staff, performing re-genotyping of a genotype frequencies with HWE (calculated); gender in the evaluated population and male percentage, matching variables, use of blinding of genotyping staff, performing re-genotyping of a genotype frequencies with HWE (calculated); gender in the evaluated population and male percentage, matching variables, use of blinding of genotyping staff, performing re-genotyping of a genotype frequencies with HWE (calculated); gender in the evaluated population and male percentage, matching variables, use of blinding of genotyping staff, performing re-genotyping of a genotype frequencies with HWE (calculated); gender in the evaluated population and male percentage, matching variables, use of blinding of genotyping staff, performing re-genotyping of a genotype frequencies with HWE (calculated); gender in the evaluated population and male percentage, matching variables, use of blinding of genotyping staff, performing re-genotyping of a genotype. For evaluating the impact of HWE-violated studies on effect estimates (at the 0.05 significance level) under different genetic models, odds ratios, and variances were corrected by using the HWE-predicted genotype counts in the control instead of the observed counts as previously suggested [20]. Thereafter, they were included in the sensitivity analysis.

Results

Prospect-EPIC study results

The general characteristics of the randomly sampled participants of the cohort (N = 1522) are given in Table 1. The genotype distribution was in Hardy-Weinberg equilibrium (\( \chi^2 = 0.020; \ P = 0.89 \)). General and clinical characteristics of CHD cases and controls are shown in Table 1. The median follow up time for the random sample was 4.3 years, with a total of 6,523 person years. The actual follow-up in the baseline cohort of 15,236 women was 64,768 person years. Due to the case-cohort design, 23 women in the sub-cohort eventually were CHD cases (among which there were nine AMI cases).

Due to the association of the M235T genotypes with some risk factors of CHD, we presented crude models and models adjusted for hypertension, total cholesterol and waist to hip ratio as potential confounding factors. Table 2 presents hazard ratios of AMI and CHD under different genetic models. Under the additive model of inheritance, no increased risk for CHD was found (HR = 1.20; 95\% CI, 0.86 to 1.68; \( P = 0.28 \)), which did not alter after adjustment (HR = 1.17; 95\% CI, 0.83 to 1.64; \( P = 0.38 \)). The same was true for other comparisons (Table 2). Analyses for AMI risk did not show any statistically significant associations (Table 2).

Meta-Analysis results

Flow of included studies

A total of 44 gene-disease association studies, including the present study, evaluating the AGT M235T gene variant and CHD risk were identified. Seven articles were excluded, three of which were duplicate publications [12,23,24], three of which did not provide relevant data [25–27], and one of which studied the risk of recurrent coronary events [28]. Finally, 37 studies met the selection criteria. In one paper, the provided results were based on two different studies [6], so both were included in the meta-analysis. Therefore, 38 studies with 13,284 cases and 18,722 controls were included in the final meta-analysis (Figure 1).

Study characteristics

Characteristics of the studies are shown in Table 3 [6–8,10,11,13,29–58]. There were 25 studies in Caucasians, eight studies in East Asians, and five studies in other populations (West Asian, South Asian, African, African-American, and South American). The last was collapsed into a miscellaneous group. The design of the studies was case-control, except for three studies that were prospective cohort [56], case-cohort [present study], and cross-sectional [40]. The T allele frequency varied from 26 to 54 percent in Caucasians, 65 to 91 percent in East Asians, and 34 to 83 percent in the miscellaneous group.

All studies used polymerase chain reaction methods for genotyping, and most used a restriction fragment length method for polymorphism analysis. Blinding of investigators involved in genotyping with respect to the case/control status of the participants was reported in six studies [8,32,30,51,56]. A random double-check to detect potential genotyping errors was mentioned in five studies [37,50,33,56]. In most of the studies, the genotype frequencies were consistent with HWE. However, statistically significant deviations from HWE were found in five studies.
Table 1. Baseline characteristics of the sub-cohort according to genotype, and clinical characteristics of CHD cases and controls in the Prospect –Epic cohort.

| Characteristics | sub-cohort (N = 1522) | CHD cases | Sub-cohort | P-value |
|-----------------|-----------------------|-----------|------------|---------|
| N total (%)     | M235M                 | M235T     | T235T      | P-value |
| Age at intake (yr) | 57.1±5.8             | 57.1±6.2  | 57.4±6.3  | 0.83    | 60.5±5.9 | 57.1±6.1 | <0.01  |
| Body mass index (kg/m²) | 26.0±4.1           | 25.6±3.8  | 25.8±4.1  | 0.19    | 26.8±3.9 | 25.8±4.0 | <0.01  |
| Weight (kg)     | 70.1±11               | 69.±11    | 69.±11    | 0.17    | 71.±11   | 69.±11   | 0.07   |
| Height (cm)     | 164.4±5.9             | 164.2±6.0 | 164.0±6.1 | 0.66    | 162.8±6.0 | 164.3±6.0 | <0.01  |
| Waist to hip ratio | 0.79±0.057           | 0.78±0.058 | 0.78±0.055 | 0.03    | 0.81±0.060 | 0.78±0.057 | <0.01  |
| Hypertension (%)| 39.4                  | 41.2      | 48.4      | 0.06    | 60.5      | 41.8      | <0.01  |
| Systolic blood pressure (mm Hg) | 131±19              | 133±21    | 135±20    | 0.07    | 143±22    | 133±20    | <0.01  |
| Diastolic blood pressure (mm Hg) | 79±10               | 79±11     | 80±11     | 0.14    | 82±11     | 79±11     | <0.01  |
| Presence of diabetes (%) | 2.2                 | 2.0       | 2.8       | 0.78    | 5.7       | 2.2       | <0.01  |
| Presence of hypercholesterolemia (%) | 3.6                 | 4.6       | 2.8       | 0.38    | 11.4      | 3.9       | <0.01  |
| Current alcohol consumption (%) | 88.7                | 87.1      | 89.2      | 0.60    | 80.7      | 88.0      | <0.01  |
| Smoking status (%) | Past                | 35.1      | 33.8      | 0.73    | 26.2      | 34.7      | 0.02   |
| Current         | 23.2                  | 22.4      | 23.6      | 0.90    | 33.8      | 22.9      | <0.01  |
| Pack- years *   | 6.8±9.5               | 6.5±9.5   | 6.7±9.3   | 0.87    | 9.7±11.4  | 6.7±9.5   | <0.01  |
| Total cholesterol (mmol/L) | 5.9±1.0             | 5.8±0.9   | 5.9±1.1   | 0.05    | 6.4±1.0   | 5.9±1.0   | <0.01  |
| HDL cholesterol (mmol/L) | 1.6±0.4             | 1.6±0.4   | 1.6±0.4   | 0.33    | 1.4±0.3   | 1.6±0.4   | <0.01  |
| LDL cholesterol (mmol/L) | 4.0±1.0             | 3.9±0.9   | 3.9±0.9   | 0.25    | 4.4±1.0   | 3.9±0.9   | <0.01  |
| Serum glucose (mmol/L) | 4.6±1.5             | 4.5±1.3   | 4.5±1.2   | 0.52    | 5.1±2.5   | 4.5±1.4   | <0.01  |

HDL, high-density lipoprotein; LDL, low-density lipoprotein; CHD, coronary heart disease (ICD 410–414).

Table 2. Association of the AGT M235T polymorphism and AMI and CHD under different genetic models.

| Mode of Inheritance | Crude: model 1 | Adjusted: model 2 a |
|---------------------|----------------|---------------------|
|                     | Hazard ratio  | 95% CI   | P-value | Hazard ratio  | 95% CI   | P-value |
| AMI                 |                |          |         |                |          |         |
| Additive a          | 1.20           | 0.86–1.68 | 0.28    | 1.17           | 0.83–1.64 | 0.38    |
| Recessive (TT vs. M-carriers) | 0.77             | 0.43–1.41 | 0.40    | 0.87           | 0.46–1.58 | 0.62    |
| Dominant (T-carriers vs. MM) | 0.79             | 0.47–1.32 | 0.36    | 0.79           | 0.46–1.33 | 0.37    |
| MT vs. MM           | 1.09           | 0.84–1.41 | 0.53    | 1.11           | 0.85–1.45 | 0.45    |
| TT vs. MM           | 1.21           | 0.86–1.70 | 0.28    | 1.17           | 0.83–1.63 | 0.38    |
| CHD                 |                |          |         |                |          |         |
| Additive a          | 1.14           | 0.93–1.39 | 0.20    | 1.11           | 0.90–1.38 | 0.33    |
| Recessive (TT vs. M-carriers) | 0.87             | 0.60–1.26 | 0.45    | 0.98           | 0.66–1.47 | 0.93    |
| Dominant (T-carriers vs. MM) | 0.82             | 0.60–1.12 | 0.21    | 0.80           | 0.58–1.10 | 0.18    |
| MT vs. MM           | 1.09           | 0.93–1.27 | 0.31    | 1.13           | 0.95–1.34 | 0.16    |
| TT vs. MM           | 1.14           | 0.93–1.40 | 0.20    | 1.11           | 0.90–1.37 | 0.33    |

AMI = acute myocardial infarction (ICD 410); CHD = coronary heart disease (ICD 410–414).

aThe additive genetic model assumes that there is a linear gradient in risk between the MM, MT and TT genotypes (MM genotype baseline). This is equivalent to a comparison of the T allele versus the M allele (baseline).

bWe used a cox proportional hazards model with an estimation procedure adapted for case-cohort designs; adjusted for waist to hip ratio, hypertension, total cholesterol.

doi:10.1371/journal.pone.0002533.t001
doi:10.1371/journal.pone.0002533.t002
CHD cases were defined in 16 studies as a >50% stenosis of at least one coronary vessel [7,8,10,11,34,40,41,43,46,48,50,51,54–57], while, in four studies, a >70% stenosis was considered [36,42,52,58]. In 14 studies [13,29–32,35,37–39,44,47,49,53], the WHO criteria were used, and, in four studies, CHD was diagnosed based on a clinical diagnosis [6,33,45]. Controls arose from the source population of the cases in 21 studies [6,8,13,29,31–33,35–38,45,47,49–53,55], while hospital-based/not population-based controls were used in 17 studies [7,10,11,30,34,39–44,46,48,54,56–58].

Quantitative data synthesis

The overall OR under a random-effects model using an additive model for CHD risk was 1.08 (95% CI, 1.01 to 1.15; \( P = 0.025 \); Figure 2). However, there was evidence of substantial between-study heterogeneity (\( \tau^2 = 0.025 \)). Table 4 shows the association of the AGT T235M polymorphism with CHD risk under different genetic contrasts. When a recessive model was evaluated, a significant association was found between individuals homozygous for the T allele (T235T genotype) and CHD risk, when compared to carriers of the M allele (OR = 1.11; 95% CI, 1.02 to 1.22; \( P = 0.016 \). Under the dominant model, the association was not significant. Under pairwise comparisons, there was a significant modest association between the T235T genotype and CHD risk, as compared to the M235M genotype (OR = 1.15; 95% CI, 1.00 to 1.32; \( P = 0.045 \). There was evidence for moderate to large between-study heterogeneity under all models (Table 4). Sub-group analysis, by study characteristics under the additive model, showed that matching, blinded genotyping staff, and regenotyping of a random sub-sample explained little of the heterogeneity. However, stratification showed an attenuated effect estimates in the large studies, in studies that CHD was defined based on angiography or WHO criteria, and in particular in studies that were in HWE (Table 5). Further evaluation of potential sources of the heterogeneity was performed using a meta-regression analysis.

Meta-regression

First, an empty regression was run with only the log of the effect estimate of pooled studies under the additive model to determine the baseline value for \( \tau^2 \), an estimate of between-study variation (baseline \( \tau^2 = 0.025 \)). Next, single covariates were added in a series of univariate models. We performed the regression analysis for ten pre-defined potential sources of heterogeneity, including ethnicity, sex, mean age of cases, study size, case definition, source of controls, HWE-violation, blinding in genotyping, performing a sub-sample regenotyping, and matching (we hypothesized that studies that used matching might produce more conservative estimates of association). Univariate regression analyses showed that violation of HWE (\( \beta \) coefficient = 0.27 (0.06 to 0.48); \( P_{\text{het}} = 0.015 \), \( \tau^2 = 0.019 \)), the mean age of cases (\( \beta = -0.01 \) (-0.02 to 0.0008); \( P_{\text{het}} = 0.066 \), \( \tau^2 = 0.024 \)), and the method of case definition, clinically diagnosed CHD versus WHO criteria adjusted for other definitions (\( \beta = 0.50 \) (0.02 to 0.50); \( P_{\text{het}} = 0.038 \), \( \tau^2 = 0.020 \)), were significant sources of heterogeneity among studies. The study size (\( P_{\text{het}} = 0.241 \), \( \tau^2 = 0.024 \)), the ethnicity (\( P_{\text{het}} = 0.591 \), \( \tau^2 = 0.025 \)), the male percentage in the study (\( P_{\text{het}} = 0.701 \), \( \tau^2 = 0.029 \)), blinded genotyping (\( P_{\text{het}} = 0.890 \),...
Table 3. Characteristics of published studies of the association between the M235T polymorphism in AGT gene and CHD included in the meta-analysis.

| Author                      | Year | Country   | Ethnicity | Total cases | Total controls | Study size based on average weight | Cases MM | Cases MT | Cases TT | Controls MM | Controls MT | Controls TT |
|-----------------------------|------|-----------|-----------|-------------|----------------|------------------------------------|----------|----------|----------|--------------|--------------|--------------|
| Katsuya et al. [45]         | 1995 | New Zealand | Caucasian | 422         | 406            | Large                              | 144      | 186      | 92       | 156          | 191          | 59           |
| Tiret et al. [13]           | 1995 | France and UK | Caucasian | 630         | 741            | Large                              | 229      | 301      | 100      | 258          | 372          | 111          |
| Ludwig et al. [6] (Framingham study) | 1997 | USA | Caucasian | 58          | 55             | Small                              | 17       | 30       | 11       | 20           | 23           | 12           |
| Ludwig et al. [6] (ARIC study) | 1997 | USA | Caucasian | 235         | 245            | Large                              | 79       | 117      | 59       | 85           | 118          | 42           |
| Wenzel et al. [55]          | 1997 | Germany | Caucasian | 111         | 102            | Small                              | 25       | 59       | 27       | 39           | 46           | 17           |
| Ludwig et al. [57]          | 1999 | Germany | Caucasian | 329         | 92             | Small                              | 103      | 148      | 78       | 28           | 53           | 11           |
| Fernandez-Arcas et al. [37] | 1999 | Spain | Caucasian | 272         | 182            | Small                              | 84       | 132      | 56       | 36           | 96           | 50           |
| Gardemann et al. [40]       | 1999 | Germany | Caucasian | 1739        | 511            | Large                              | 536      | 920      | 283      | 168          | 247          | 96           |
| Fatini et al. [36]          | 2000 | Italy    | Caucasian | 205         | 209            | Large                              | 61       | 91       | 53       | 84           | 86           | 39           |
| Fomicheva et al. [38]       | 2000 | Russia  | Caucasian | 198         | 152            | Small                              | 63       | 85       | 50       | 43           | 75           | 34           |
| Reinhardt et al. [50]       | 2000 | Germany | Caucasian | 184         | 155            | Small                              | 56       | 101      | 27       | 38           | 91           | 26           |
| Bataille et al. [31]        | 2000 | Spain   | Caucasian | 220         | 200            | Small                              | 69       | 99       | 52       | 64           | 96           | 40           |
| Wierzbicki et al. [56]      | 2000 | UK      | Caucasian | 48          | 108            | Small                              | 23       | 21       | 4        | 58           | 44           | 6            |
| Rodriguez-Perez et al. [8]  | 2001 | Spain   | Caucasian | 299         | 315            | Large                              | 67       | 145      | 87       | 97           | 158          | 60           |
| Olivieri et al. [7]         | 2001 | Italy   | Caucasian | 454         | 245            | Large                              | 148      | 205      | 101      | 74           | 114          | 57           |
| Sethi et al. [29]           | 2001 | Denmark | Caucasian | 943         | 7973           | Large                              | 335      | 460      | 148      | 2779         | 3886         | 1310         |
| Ortlepp et al. [48]         | 2002 | Germany | Caucasian | 100         | 100            | Small                              | 25       | 58       | 17       | 29           | 55           | 16           |
| Emis et al. [35]            | 2002 | Turkey  | Caucasian | 102         | 114            | Small                              | 32       | 48       | 22       | 39           | 59           | 16           |
| Bis et al. [32]             | 2003 | USA     | Caucasian | 208         | 717            | Large                              | 71       | 98       | 39       | 215          | 349          | 153          |
| Buraczynska et al. [33]     | 2003 | Poland  | Caucasian | 200         | 200            | Small                              | 28       | 122      | 50       | 72           | 80           | 48           |
| Tobin et al. [53]           | 2004 | UK      | Caucasian | 547         | 505            | Large                              | 212      | 252      | 83       | 197          | 226          | 82           |
| Sekuri et al. [10]          | 2005 | Turkey  | Caucasian | 115         | 128            | Small                              | 46       | 42       | 27       | 33           | 71           | 24           |
| Mhetoth et al. [47]         | 2005 | Canada  | Caucasian | 198         | 149            | Small                              | 65       | 93       | 40       | 60           | 70           | 19           |
| Renner et al. [51]          | 2005 | Austria | Caucasian | 2582        | 732            | Large                              | 841      | 1205     | 536      | 237          | 357          | 138          |
| Zafarmand et al. (present study) | 2008 | Netherlands | Caucasian | 210        | 1522           | Large                              | 64       | 108      | 38       | 535          | 737          | 250          |
| Kamitani et al. [44]        | 1995 | Japan   | East Asian | 103        | 103            | Small                              | 6        | 31       | 66       | 10           | 41           | 52           |
| Ishigami et al. [43]        | 1995 | Japan   | East Asian | 82          | 160            | Small                              | 6        | 22       | 54       | 30           | 51           | 79           |
| Yamakawa-Kobayashi et al. [58] | 1995 | Japan | East Asian | 315         | 380            | Small                              | 15       | 91       | 209      | 9            | 131          | 240          |
| Ko et al. [46]              | 1997 | China   | East Asian | 267         | 337            | Small                              | 6        | 36       | 225      | 4            | 54           | 279          |
| Ichihara et al. [42]        | 1997 | Japan   | East Asian | 327         | 352            | Small                              | 15       | 103      | 209      | 13           | 112          | 227          |
| Cong et al. [34]            | 1998 | Japan   | East Asian | 104         | 170            | Small                              | 2        | 31       | 71       | 16           | 43           | 111          |
| Sheu et al. [52]            | 1998 | China   | East Asian | 102         | 145            | Small                              | 1        | 26       | 75       | 1            | 37           | 107          |
| Tsai et al. [54]            | 2006 | Taiwan  | East Asian | 735         | 519            | Large                              | 15       | 195      | 525      | 5            | 111          | 403          |
| Author                  | Year | Country      | Ethnicity       | Total cases | Total controls | Study size based on average weight | Cases MM | Cases MT | Cases TT | Controls MM | Controls MT | Controls TT |
|------------------------|------|--------------|-----------------|-------------|----------------|------------------------------------|----------|----------|----------|--------------|--------------|--------------|
| Frossard et al. [39]   | 1998 | UAE          | Arab            | 74          | 61             | Small                              | 21       | 32       | 21       | 16           | 26           | 19           |
| Hooper et al. [41]     | 2002 | USA          | African-American| 100         | 100            | Small                              | 4        | 29       | 67       | 2            | 67           | 31           |
| Nair et al. [11]       | 2003 | India        | South Asian     | 141         | 131            | Small                              | 9        | 36       | 96       | 11           | 40           | 80           |
| Araujo et al. [30]     | 2004 | Brazil       | South American  | 110         | 104            | Small                              | 46       | 52       | 12       | 43           | 51           | 10           |
| Ranjith et al. [49]    | 2004 | South Africa | African         | 195         | 300            | Small                              | 24       | 80       | 91       | 29           | 127          | 144          |

Table 3. Cont.
| Author                      | Study design | Mean age ± SD (years) in Cases | Mean age ± SD (years) in Controls | Male percent | Matching variable (s)                                                                 | Allele frequency 235T (%) | P (HWE) | Blinding of genotyping staff | Regenotyping of random subsample |
|-----------------------------|--------------|--------------------------------|-----------------------------------|--------------|--------------------------------------------------------------------------------------|---------------------------|----------|-------------------------------|----------------------------------|
| Kamitani et al. [44]       | Case-control | 52±1                           | 54±1                              | M            | Age, sex, BMI, blood pressure, total cholesterol, smoking and history of diabetes     | 70                        | 0.65     | NR                           | NR                               |
| Ishigami et al. [43]       | Case-control | 62±1                           | 60±1                              | M/F          | None                                                                                 | 65                        | 0.0002   | NR                           | NR                               |
| Yamakawa-Kobayashi et al. [58]| Case-control | 57±8                           | 51±8                              | M/F          | None                                                                                 | 80                        | 0.07     | NR                           | NR                               |
| Ko et al. [46]             | Case-control | 62±1                           | 56±1                              | M/F          | None                                                                                 | 77                        | 0.51     | NR                           | NR                               |
| Ichihara et al. [42]       | Case-control | 53±6                           | 53±5                              | M            | Age, sex, BMI and some CHD risk factors (history of smoking, hypertension, diabetes, hypercholesterolemia) | 80                        | 0.86     | NR                           | NR                               |
| Cong et al. [34]           | Case-control | 65±1                           | NR                                | M/F          | None                                                                                 | 76                        | 0.0006   | NR                           | NR                               |
| Sheu et al. [52]           | Case-control | 63±1                           | 58±1                              | M            | None                                                                                 | 87                        | 0.47     | NR                           | NR                               |
| Tsai et al. [54]           | Case-control | 64±11                          | 59±13                             | M/F          | None                                                                                 | 88                        | 0.38     | NR                           | NR                               |
| Frossard et al. [39]       | Case-control | 57±12                          | 54±14                             | M/F          | None                                                                                 | 52                        | 0.26     | NR                           | NR                               |
| Hooper et al. [41]         | Case-control | NR                             | NR                                | M/F          | None                                                                                 | 83                        | 0.73     | NR                           | NR                               |
| Nair et al. [11]           | Case-control | 56±5                           | 48±6                              | M/F          | Age and sex                                                                          | 82.3                      | 0.08     | NR                           | NR                               |
| Araujo et al. [30]         | Case-control | NR                             | NR                                | M/F          | None                                                                                 | 66.6                      | 0.36     | NR                           | NR                               |
| Ranjith et al. [49]        | Case-control | NR                             | NR                                | M/F          | Age                                                                                  | 69                        | 0.90     | NR                           | NR                               |

| Author                      | End point | Case definition                                                                 | Source of controls                                                                 |
|-----------------------------|-----------|---------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Katsuya et al. [45]         | CHD       | Admission for treatment of myocardial infarction or unstable angina, PTCA, or CABG, or stable angina with angiographic evidence of CHD or a positive exercise test result | Controls without a history of CHD and symptoms suggesting angina from two previous studies |
| Tiret et al. [13]           | MI        | WHO MONICA category I                                                            | Electoral rolls in France and the list of general practitioners in N. Ireland     |
| Ludwig et al. [6]           | CHD       | Diagnosed MI by a physician, a PTCA, a CABG, prior MI in ECG, fatal CHD          | Healthy controls without the conditions, no lipid-lowering medications and no family history |
| Ludwig et al. [6]           | ARIC study| Diagnosed MI by a physician, a percutaneous coronary angioplasty, a coronary artery bypass, prior MI, fatal CHD | Healthy controls without the conditions, no lipid-lowering medications and no family history |
| Wenzel et al. [55]          | CHD       | >50% stenosis of at least one major coronary vessel, defined as MI, PTCA, CABG   | Healthy young persons without any symptoms for CVD                              |
| Winkelmann et al. [57]      | CHD, MI   | At least one coronary stenosis ≥ 50%                                             | Controls without coronary artery disease in coronary angiography                  |
| Fernandez-Arcas et al. [37] | MI        | Typical prolonged chest pain or atypical symptoms, acute congestive heart failure, syncope, and serial cardiac enzymes elevation exceeding twice the upper limit of reference range and dynamic ECG changes typical of MI | Healthy controls with no CVD using health service identity card                   |
| Gademenmann et al. [40]     | CHD, MI   | CHD: coronary stenosis ≥ 30% MI; Using the WHO criteria                           | No vessel disease in the coronary angiography                                      |
| Fatini et al. [36]          | CHD       | History of CHD (previous MI or angina pectoris) with coronary stenosis ≥75% by angiography | Random healthy controls from the staff of the University                          |
| Author                      | End point | Case definition                                                                 | Source of controls                                                                 |
|---------------------------|-----------|----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| Fomicheva et al. [38]     | MI        | Using the WHO criteria                                                          | From secondary schools                                                               |
| Reinhardt et al. [50]     | CHD       | At least one coronary stenosis $\geq 50\%$ of a major coronary artery with or without prior MI | Random healthy controls from the local registry office                                |
| Batalla et al. [31]       | MI        | WHO MONICA protocol                                                             | Healthy controls from residents of the region                                        |
| Wierzbicki et al. [56]    | CHD       | Confirmed cardiac event, angioplasty, coronary bypass surgery, or significant lesions on angiography | No CHD                                                                              |
| Rodriguez-Perez et al. [8] | CHD       | Hospital-admitted with a diagnosis of MI or unstable angina and documented evidence of coronary artery disease by angiography | Random controls without CVD                                                          |
| Olivieri et al. [7]       | CHD, MI   | CHD: Candidate patients for CABG, having $>50\%$ stenosis of at least one major coronary vessel MI: By medical records showing diagnostic electrocardiogram and enzyme changes, and/or the typical sequelae of myocardial infarction on ventricular angiography | CHD-free group documented by angiography who were examined for other reasons in the institute |
| Sethi et al. [29]         | CHD, MI   | CHD: ICD, 8th edition, codes 410-414 MI: ICD, 8th edition, code 410               | Random healthy controls without CHD, MI or CVA from the city of Copenhagen           |
| Ortlepp et al. [48]       | CHD       | $>50\%$ stenosis of at least one coronary vessel                                 | Patients without any signs of atherosclerosis in angiography                          |
| Ermis et al. [35]         | MI        | WHO criteria                                                                    | Healthy subjects without a history of CHD, hypertension or diabetes                  |
| Bis et al. [32]           | MI        | Criteria were adapted from the Cardiovascular Health Study                       | Randomly selected subjects from the members of a health maintenance organization     |
| Buraczynska et al. [33]   | CHD       | Hospitalized patients with unstable angina, stable angina or acute MI           | Healthy subjects without family history of CHD                                        |
| Tobin et al. [53]         | MI        | Using the WHO criteria                                                          | Healthy visitors to patients                                                         |
| Sekuri et al. [10]        | CHD       | At least one stenosis $\geq 50\%$ in a major coronary artery or one of their branches | Healthy subjects without history of CVD                                              |
| Methot et al. [47]        | CHD       | Acute coronary syndrome: AMI or unstable angina defined according to standard criteria | Postmenopausal women without signs or symptoms of acute or previous acute coronary syndrome |
| Renner et al. [51]        | CHD, MI   | CHD: At least one stenosis $\geq 50\%$ in one of 15 coronary segments MI: positive history of MI or patients presented with ST elevation or non-ST elevation | Subjects without CHD (with stenoses $<20\%$) from a cohort study                     |
| Zafarmand et al. (present study) | CHD, MI | CHD: ICD, 9th edition, codes 410-414 MI: ICD, 9th edition, code 410 | Members of a 10% random sample from the whole cohort at the baseline without CVD |
| Kamitani et al. [44]      | MI        | Having MI by coronary angiography, ECG criteria, and measurements of heart-specific serum enzymes | Randomly selected subjects attending the same hospital with no CVD                   |
| Ishigami et al. [43]      | CHD       | At least one coronary artery with $>25\%$ luminal obstruction on average according to multiple coronary angiographic views | Hospital-admitted patients for other diseases with no CHD                            |
| Yamakawa-Kobayashi et al. [58] | CHD | At least one 75% stenosis in coronary arteries                                    | Healthy controls                                                                     |
| Ko et al. [46]            | CHD       | $>50\%$ stenosis of at least one major coronary vessel                           | Healthy subjects and patients without angiographic evidence of CHD                   |
| Ichihara et al. [42]      | CHD       | MI was based on typical ECG changes and increased serum enzymes and by the presence of wall motion abnormality on left ventriculography, Angina pectoris by typical ECG changes and stenosis of $>70\%$ in any major coronary artery or of $>50\%$ in the left main trunk, without wall motion abnormality on left ventriculography | Random healthy controls with no history or sign of CHD from attendants of the hospitals |
| Cong et al. [34]          | CHD       | $\geq 50\%$ stenosis in at least one major coronary artery                        | Subjects with no history of CHD or abnormal resting electrocardiogram                |
| Sheu et al. [52]          | CHD       | A postnitroglycerin stenosis of major vessels $\geq 50\%$ or a $>70\%$ reduction of luminal diameter of a first-order branch | Healthy subjects in their annual physical checkups                                  |
| Author                  | End point | Case definition                                                                 |
|------------------------|-----------|----------------------------------------------------------------------------------|
| Tsai et al. [54]       | CHD       | 50% stenosis of at least one coronary vessel CHD-free group documented by angiography |
| Frossard et al. [39]   | CHD, MI   | Exertional angina, unstable angina or MI; ECG changes; presence of regional wall motion abnormalities on trans-thoracic echocardiography; and serial enzyme elevations |
| Hooper et al. [41]     | MI        | Prior MI confirmed by ECG and/or cardiac enzymes or cardiac thallium scanning or catheterization |
| Nair et al. [11]       | CHD       | At least one coronary artery with 50% stenosis Healthy controls with BP, Araujo et al. [30] | MI | Using the WHO criteria confirmed by stenosis 50% in an angiography |
| Ranjith et al. [49]    | MI        | Using the WHO criteria Healthy normotensive subjects with no CVD or other associated risk factors |

**Table 3.**

- **Source of controls:**
  - CHD-free group documented by angiography
  - Healthy controls
  - Outpatients with no history of heart attack, stroke, or thrombosis
  - Hospital-admitted patients for other diseases with a normal coronary angiography and ventricular damage in a ventriculography
  - Healthy normotensive subjects with no CVD or other associated risk factors

- **Source of controls (PHet)**
  - $\phi^2 = 0.026$, sub-sample regenotyping ($\phi_{HT} = 0.131, \phi^2 = 0.023$), the source of controls ($\phi_{HT} = 0.640, \phi^2 = 0.025$), and matching ($\phi_{HT} = 0.942, \phi^2 = 0.026$) were not significant sources of heterogeneity among studies. Violation of HWE in multivariable regression analysis remained a statistically significant source of heterogeneity after adjustment for the effect of study size ($\phi_{HT} = 0.031, \phi^2 = 0.020$). Adding the mean age of cases and method of case definition to the model with violation of HWE decreased the $\phi^2$ value to 0.017 ($\phi_{HT} = 0.073$ for violation of HWE, $\phi_{HT} = 0.057$ for the mean age of cases, and $\phi_{HT} = 0.162$ for clinically diagnosed CHD). It also showed that the effect of method of case definition on the variation among the studies was through the effect of the mean age on the heterogeneity and not as an independent factor. A model that included only violation of HWE and the mean age of cases reduced the $\phi^2$ value to 0.018 ($\phi_{HT} = 0.019$, and 0.052, respectively).

**Sensitivity Analysis**

First, the influence of deviation from the HWE on effect estimates was examined by using HWE-deviated adjusted ORs. Table 6 presents the genotype-based contrasts with corrected ORs, as well as the allele-based contrast. After adjustment, a smaller overall effect was seen under the additive, dominant, and pairwise comparisons. Moreover, after adjustment, the previously significant association under the additive model, as well as the TT vs. MM comparison, was no longer statistically significant. The association under the recessive model still remained significant.

Figure 3 shows a funnel plot in which the log of the OR of CHD risk under the additive genetic model was plotted against the standard error of the log of the OR in each study. The funnel plot for the overall results was substantially asymmetric for small negative studies. Moreover, tests for potential publication bias (The Egger’s test and the Begg-Mazumdar test; $P$ values equal to 0.066 and 0.074, respectively) suggested the presence of a publication bias. By using the trim and fill method, we showed that, if the publication bias was the only source of the funnel plot asymmetry, it needed seven more studies to be symmetrical (Figure 4).

**Discussion**

**Prospect-EPIC study**

In this prospective study of healthy women aged 49 to 70 years, we investigated the relationship between the M235T polymorphism in the AGT gene and risk of AMI and CHD later in life. Under the additive genetic model, increased risks, albeit not statistically significant, were found for the incidence of AMI and CHD, which did not alter after adjustment. Likewise, we did not find a clear association between the variant and risk of CHD or AMI using different genetic models. This may be explained by: (i) the absence of a biological effect, (ii) the presence of real genetic heterogeneity according to ethnic background, or (iii) failure to detect a small effect because the epidemiologic risk for an individual genetic variant is likely to be small and a large sample size is needed for adequate statistical power. It has been commonly proposed that, as well as a need for much larger and more rigorous studies those that are currently used, there is a greater need for international collaborations, particularly for a complex disease like CHD [59].

**Strengths and limitations.** In our study, the data collection was prospective, before the diagnosis of AMI or CHD and equal for all participants. This ensures that the cases and the randomly selected controls are comparable [17]. For a multifactorial trait, like CHD, this provides a valid approach to evaluate the...
relationship between genetic factors and the risk of AMI and CHD, while taking into account co-existing and risk-modifying factors. In this study, prevalent cases of CHD were excluded from the analyses to prevent introducing bias due to potentially selective survival. The Prospect study was a population-based cohort, which makes it less susceptible to selection bias. Additional strengths were the comprehensiveness of our data and sample collection, as well as the morbidity and mortality follow-up for the entire cohort.
through linkage with nation-wide registries. The case-cohort design of the study combined the advantages of cohort studies (multiple outcomes and time-dependent covariates) with those of case-control analyses (fewer subjects); thus, it was more efficient than cohort studies. Classical case-control studies might be affected by selection bias since only non-fatal cases can be included, which was not the case in this study because of our endpoint definition. Moreover, we did not have misclassification of exposure (genotypes), which, when present, generally lead to a bias toward the null because we used standard laboratory protocols.

Table 4. ORs and 95% CI for coronary heart disease and the M235T polymorphism in AGT gene under different genetic models.

| Genetic model       | Random effects OR (95% CI) | P-value | $I^2$ (%) (95% CI) | $\chi^2$ statistic for heterogeneity (df = 37) | P-value for heterogeneity | Egger’s test P-value | Begg’s test P-value |
|---------------------|---------------------------|---------|-------------------|-----------------------------------------------|--------------------------|---------------------|---------------------|
| Additive            | 1.08 (1.01–1.15)          | 0.025   | 55.5 (36–69)      | 83.21                                         | <0.001                   | 0.066               | 0.074               |
| Recessive (TT vs. M-carriers) | 1.11 (1.02–1.22)          | 0.016   | 37.5 (7–58)       | 59.23                                         | 0.012                    | 0.011               | 0.070               |
| Dominant (T-carriers vs. MM) | 1.07 (0.96–1.19)          | 0.253   | 56.0 (37–69)      | 84.02                                         | <0.001                   | 0.549               | 0.706               |
| MT vs. MM           | 1.02 (0.91–1.14)          | 0.724   | 51.3 (29–66)      | 75.99                                         | <0.001                   | 0.895               | 0.960               |
| TT vs. MM           | 1.15 (1.00–1.32)          | 0.045   | 53.3 (33–68)      | 79.30                                         | <0.001                   | 0.286               | 0.615               |

*The additive genetic model assumes that there is a linear gradient in risk between the MM, MT and TT genotypes (MM genotype baseline). This is equivalent to a comparison of the T allele versus the M allele (baseline).

doi:10.1371/journal.pone.0002533.t004

Table 5. Studies of the M235T polymorphism in AGT gene and risk of coronary heart disease under additive model grouped by study characteristics.

| Study characteristics | Number of studies | Per-allele OR (95%CI) | P-value | $I^2$ (%) (95%CI) | $\chi^2$ statistic for heterogeneity | P-value for heterogeneity | Egger’s test P-value | Begg’s test P-value |
|-----------------------|-----------------|---------------------|---------|-------------------|-----------------------------------|--------------------------|---------------------|---------------------|
| Overall               | 38              | 1.08 (1.01–1.15)    | 0.025   | 55.5 (36–69)      | 83.21                             | <0.001                   | 0.066               | 0.074               |
| Study size            |                 |                     |         |                   |                                   |                          |                     |                     |
| Small                 | 26              | 1.12 (1.02–1.24)    | 0.021   | 50.2 (35–73)      | 50.24                             | 0.002                    |                     |                     |
| Large                 | 12              | 1.03 (0.95–1.12)    | 0.502   | 62.0 (29–80)      | 28.92                             | 0.002                    |                     |                     |
| Ethnicity             |                 |                     |         |                   |                                   |                          |                     |                     |
| Caucasians            | 25              | 1.08 (1.01–1.17)    | 0.028   | 58.2 (35–73)      | 57.43                             | <0.001                   | 0.002               |                     |
| Eastern Asians        | 8               | 1.12 (0.89–1.40)    | 0.325   | 69.5 (36–85)      | 22.96                             | 0.002                    |                     |                     |
| Others                | 5               | 0.99 (0.84–1.18)    | 0.944   | 0.00 (0–79)       | 2.31                              | 0.679                    |                     |                     |
| Matching              |                 |                     |         |                   |                                   |                          |                     |                     |
| Matched               | 11              | 1.07 (0.96–1.18)    | 0.211   | 26.2 (0–63)       | 13.56                             | 0.194                    |                     |                     |
| Unmatched             | 27              | 1.08 (0.99–1.17)    | 0.072   | 62.7 (44–75)      | 69.65                             | <0.001                   | 0.002               |                     |
| Violating HWE         |                 |                     |         |                   |                                   |                          |                     |                     |
| Violated              | 5               | 1.38 (1.05–1.63)    | 0.022   | 70.7 (26–88)      | 13.65                             | 0.009                    |                     |                     |
| Confirmed             | 33              | 1.04 (0.98–1.11)    | 0.188   | 43.5 (5–63)       | 56.66                             | 0.005                    |                     |                     |
| Blinding of genotyping staff |       |                     |         |                   |                                   |                          |                     |                     |
| Blinded               | 6               | 1.07 (0.92–1.24)    | 0.391   | 62.6 (9–85)       | 13.36                             | 0.020                    |                     |                     |
| Not reported          | 32              | 1.08 (1.00–1.16)    | 0.040   | 55.5 (34–70)      | 69.88                             | <0.001                   | 0.001               |                     |
| Regenotyping of a random subsample |       |                     |         |                   |                                   |                          |                     |                     |
| Performed             | 5               | 0.94 (0.79–1.14)    | 0.544   | 58.9 (0–85)       | 9.74                              | 0.045                    |                     |                     |
| Not reported          | 33              | 1.10 (1.03–1.18)    | 0.007   | 54.7 (33–69)      | 70.64                             | <0.001                   | 0.001               |                     |
| Case definition       |                 |                     |         |                   |                                   |                          |                     |                     |
| >50% stenosis of ≥1 major vessels | 16         | 1.09 (0.97–1.23)    | 0.135   | 62.4 (35–78)      | 39.9                              | <0.001                   | 0.001               |                     |
| >70% stenosis of ≥1 major vessels | 4           | 1.10 (0.90–1.34)    | 0.358   | 40.7 (0–80)       | 5.1                               | 0.167                    |                     |                     |
| WHO criteria          | 14              | 1.00 (0.93–1.09)    | 0.942   | 36.9 (0–67)       | 20.6                              | 0.081                    |                     |                     |
| Clinical diagnosis    | 4               | 1.31 (1.15–1.49)    | <0.001  | 0.00 (0–85)       | 2.7                               | 0.439                    |                     |                     |
| Source of controls    |                 |                     |         |                   |                                   |                          |                     |                     |
| Population-based      | 21              | 1.09 (1.01–1.19)    | 0.036   | 62.6 (40–77)      | 53.5                              | <0.001                   |                     |                     |
| Hospital-based        | 17              | 1.05 (0.95–1.17)    | 0.354   | 44.6 (2–69)       | 28.9                              | 0.025                    |                     |                     |

doi:10.1371/journal.pone.0002533.t005
performed a random double-check to detect potential genotyping errors, and had our AGT genotypes in Hardy-Weinberg equilibrium. The limitations of this study were the relatively short period of follow-up and the small number of cases. Moreover, because this cohort was exclusively composed of Dutch women, these results cannot be generalized to men or other ethnic groups, for whom the rates of the events or the allele frequency are known to differ.

Meta-Analysis

The current meta-analysis, which includes new data from a prospective study in a large population-based cohort of Dutch women, represents a comprehensive evaluation of the M235T variant of the AGT gene in CHD risk. Although a pooled per-allele OR was suggestive of a modest increase in the risk of CHD of 1.08 (95% CI, 1.01 to 1.15), the robustness of this summary estimate is uncertain. First, in the pre-specified sub-groups analyses in the meta-analysis, larger studies, those with validated genotyping quality controls, and studies that used standardized criteria for case definition did not provide strong evidence for a positive statistically significant association between the M235T variant of the AGT gene and CHD risk. Second, the meta-regression analysis revealed that the HWE violation was a significant source of the moderate to large heterogeneity in the meta-analysis. Taking violation of HWE into account in the meta-analysis decreased the overall effect (Figure 5). Third, the previous result was confirmed by using HWE-deviation adjusted ORs in the meta-analysis.

Meta-Analysis

The current meta-analysis, which includes new data from a prospective study in a large population-based cohort of Dutch women, represents a comprehensive evaluation of the M235T variant of the AGT gene in CHD risk. Although a pooled per-allele OR was suggestive of a modest increase in the risk of CHD of 1.08 (95% CI, 1.01 to 1.15), the robustness of this summary estimate is uncertain. First, in the pre-specified sub-groups analyses in the meta-analysis, larger studies, those with validated genotyping quality controls, and studies that used standardized criteria for case definition did not provide strong evidence for a positive statistically significant association between the M235T variant of the AGT gene and CHD risk. Second, the meta-regression analysis revealed that the HWE violation was a significant source of the moderate to large heterogeneity in the meta-analysis. Taking violation of HWE into account in the meta-analysis decreased the overall effect (Table 5). Third, the previous result was confirmed by using HWE-deviation adjusted ORs in the meta-analysis.

Table 6. ORs and 95% CI after adjustment for HWE-deviation under different genetic models.

| Genotype contrasts | Population | Number of studies | Random effects model | I² (%) (95%CI) | Q statistic for heterogeneity | P-value for heterogeneity |
|--------------------|------------|-------------------|----------------------|---------------|-------------------------------|---------------------------|
|                    |            |                   | Odds ratio 95%CI P-value |                |                              |                           |
| Additive           | All        | 38                | 1.11 0.81–1.53 0.522 0 (0–37) 2.04 1.000 |               |                              |                           |
|                    | Caucasians | 25                | 1.11 0.75–1.64 0.616 0 (0–44) 1.04 1.000 |               |                              |                           |
|                    | East Asians| 8                 | 1.19 0.60–2.36 0.626 0 (0–68) 0.82 0.997 |               |                              |                           |
| Recessive          | All        | 38                | 1.14 1.04–1.26 0.007 56 (37–70) 84.66 <0.001 |               |                              |                           |
|                    | Caucasians | 25                | 1.15 1.03–1.29 0.014 56 (32–72) 55.02 <0.001 |               |                              |                           |
|                    | East Asians| 8                 | 1.18 0.90–1.55 0.242 73 (45–87) 26.15 <0.001 |               |                              |                           |
| Dominant           | All        | 38                | 1.05 0.96–1.15 0.330 49 (26–65) 72.52 <0.001 |               |                              |                           |
|                    | Caucasians | 25                | 1.08 0.98–1.20 0.121 58 (35–73) 57.82 <0.001 |               |                              |                           |
|                    | East Asians| 8                 | 0.92 0.64–1.33 0.656 33 (0–70) 10.41 0.166 |               |                              |                           |
| MT vs MM            | All        | 38                | 1.00 0.92–1.09 0.996 15 (0–43) 43.41 0.217 |               |                              |                           |
|                    | Caucasians | 25                | 1.03 0.94–1.14 0.497 25 (0–54) 31.99 0.127 |               |                              |                           |
|                    | East Asians| 8                 | 0.82 0.60–1.11 0.204 0 (0–68) 6.53 0.480 |               |                              |                           |
| TT vs MM            | All        | 38                | 1.13 0.99–1.28 0.080 52 (31–67) 77.88 <0.001 |               |                              |                           |
|                    | Caucasians | 25                | 1.19 1.02–1.38 0.023 60 (38–74) 60.11 <0.001 |               |                              |                           |
|                    | East Asians| 8                 | 1.01 0.65–1.59 0.952 50 (0–77) 13.87 0.054 |               |                              |                           |

doI:10.1371/journal.pone.0002533.t006

doi:10.1371/journal.pone.0002533.g003
Moreover, there was evidence for publication bias in the meta-analysis. Taken together, these findings point to a violation of HWE and publication biases as the potential explanations for the results observed in the meta-analysis.

Some aspects of the current meta-analysis need to be considered to appreciate the findings. First, it might not be very practical to adjust for violation of HWE in the studies that mentioned that the violation is not due to genotyping errors. However, in the current meta-analysis, the HWE-violated studies that were included in the pooled estimate did not provide any reason for the violation. Therefore, we performed sensitivity analyses by using HWE-adjusted ORs and corresponding variances. Thereafter, a smaller overall effect was seen under most of the genetic models. Second, the power of tests for HWE and the power to detect genotyping errors are low. Therefore, the inability to detect a deviation from the HWE does not mean that there is no deviation, nor does it rule out the presence of genotyping errors, especially for small sample sizes. Third, our meta-analysis was based on published studies and we did not have access to the original data. However, it could be possible that an association between the genotype and disease exists in certain contexts rather than in all people studied. For example, a case-control study showed that the TT genotype was associated with an increased risk of CHD and MI only in smokers [33]. Finally, in all meta-analyses of gene-disease association studies, the inclusion criteria of cases and controls can be a potentially confounding factor. In this meta-analysis, cases were well defined and the source of controls was not a significant source of variation. However, the advantages of this study were the large sample size of the meta-analysis of 38 studies with 13284 cases and 18722 controls, which was twice the number of studies and sample sizes that had been reported in the previous meta-analysis [14]. Thus, the exploration of potential sources of heterogeneity in the meta-analysis, and the evaluation of the association under different modes of inheritance.

Approximately 10% of gene-disease association studies are affected by statistically significant deviation from HWE, which could result from genotyping error, chance, inbreeding, non-random mating, differential survival of marker carriers, genetic drift, population stratification, or a combination of these reasons [20,60]. Of these, genotyping error could be avoided by using standard genotyping methods and performing quality assessment. It has been recommended that authors specify the quality measures for the genotyping analysis, such as the blinding of laboratory staff to the donor subjects and hypotheses being investigated, procedures for establishing duplicates, degree of reproducibility between quality control replicates, and the inspection for conformity to HWE [61]. However, violation of HWE, which tends to inflate the chance of a false positive association, may be the strongest indicator of genotyping error [62].

Violation of HWE cannot solely explain the observed between-study variation in gene-disease association studies. The large between-study heterogeneity presented in most meta-analyses could be due to true heterogeneity (i.e., racial differences or differences in gene-environment interactions among various populations) or bias [63]. Bias, which could invalidate the results of the studies, should, therefore, be explored in detail. Biological plausibility, publication bias, selection bias, biased definition of cases, biased selection of controls, and population stratification should be assessed [63]. In this meta-analysis, we found strong evidence for publication bias. This is said to occur when the chance of the publication of a smaller study increases when it shows a stronger effect. Further exploration for sources of biases among studies showed that the selection of controls was not biased. However, using different case definitions resulted in a significant difference in the risk of CHD between those studies using WHO criteria and those using clinically diagnoses of CHD. Studies using definition of cases based on coronary angiography or based on WHO criteria had the same results. Considering a multivariate model in the meta-regression results, case definition was not a significant source of bias in the meta-analysis, while the different
mean age of cases and violation of HWE were significant sources of heterogeneity. Since increasing age is a risk factor for CHD and the mean age of cases in the included studies ranged from 42 to 67 years, it is more likely that the studies with older individuals would show a stronger effect and produce heterogeneity. As case-parental history of myocardial infarction and stroke: the PEGASE study. Projet d’Etude des Genes de l’Hypertension Arterielle Severe a moderee Essentielle.

In conclusion, the present meta-analysis, including our own experiments: Pd Yv. Analyzed the data: MZ MB. Contributed reagents/analytical tools: Yv DG. Performed the experiments: MZ MB Yv. Planned the experiments: MZ MB Yv DG. The authors declare that they have no competing interests.

References

1. Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, et al. (1995) Molecular basis of human hypertension: role of angiotensinogen. Cell 71: 169–180.
2. Caulfield M, Lavender P, Newell-Price J, Farrall M, Kamdar S, et al. (1995) Linkage of the angiotensin gene locus to human essential hypertension in African Caribbeans. J Clin Invest 96: 667–669.
3. Paillard F, Chansel D, Brand E, Benetos A, Thomas F, et al. (1999) Genotype-phenotype relationships for the renin-angiotensin-aldosterone system in a normal population. Hypertension 34: 423–429.
4. Staessen JA, Kuznetsova T, Wang JG, Emelianov D, Vlacies R, et al. (1999) M235T angiotensin gene polymorphism and cardiovascular renal risk. J Hypertens 17: 9–17.
5. Sattar N, Greer LA (2002) Pregnancy complications and maternal cardiovascular risk: opportunities for intervention and screening? BMJ 325: 157–160.
6. Ludwig EH, Borecki IB, Ellison RC, Follom AR, Heiss G, et al. (1997) Associations between candidate loci angiotensin-converting enzyme and angiotensinogen with coronary heart disease and myocardial infarction: the NHLBI Family Heart Study. Ann Epidemiol 7: 3–12.
7. Olivieri O, Stranieri C, Girelli D, Pizzolo F, Grazioli S, et al. (2001) Homozygosity for angiotensin 235T variant increases the risk of myocardial infarction in patients with multivessel coronary artery disease. J Hypertens 19: 879–884.
8. Rodriguez-Perez JC, Rodriguez-Esparragon F, Hernandez-Perez O, Anabitarte A, Losada A, et al. (2001) Association of angiotensin M235T and A-6G gene polymorphisms with coronary heart disease with independence of maternal history of myocardial infarction and stroke: the PROCAGENE study. Prospective Cardiac Gene J Am Coll Cardiol 35: 1536–1542.
9. Lanz JR, Pereira AG, Lemos PA, Martinez E, Krieger JE (2005) Angiotensin M235T polymorphism is associated with coronary artery disease severity. Am J Cardiol 96: 176–181.
10. Sekeri C, Cam FS, Ercan CE, Ercan A, Sagan A, et al. (2005) Renin-angiotensin system gene polymorphisms and premature coronary heart disease. J Renin Angiotensin Aldosterone Syst 6: 38–42.
11. Nishida K, Shih HH, Asahara T, Dalal JJ (2001) Coronary heart disease, hypertension, and angiotensin gene variants in Indian population. J Clin Lab Anal 17: 141–146.
12. Sethi AA, Nordestgaard BG, Gronholt ML, Steffensen R, Jensen G, et al. (2003) Angiotensinogen single nucleotide polymorphisms, plasma angiotensinogen, and risk of hypertension and ischemic heart disease: a meta-analysis. Arterioscler Thromb Vasc Biol 23: 1269–1275.
13. Boker LS, van Noord PA, van der Schouw YT, Koot NV, Bueno de Mesquita HB, et al. (2001) Prospective-Epic Utrecht: study design and characteristics of the cohort population. European Prospective Investigation into Cancer and Nutrition. Eur J Epidemiol 17: 1047–1053.
14. Zafarmand MH, van der Schouw YT, Grobbee DE, de Leeuw PW, Bot ML (2000) T allele polymorphism in beta-adrenoreceptor gene (ADRB2) and coronary heart disease: a case-cohort study and meta-analysis. J Intern Med 26: 79–89.
15. Prentice RL (1986) A case-cohort design for epidemiologic cohort studies and disease prevalence trials. Biometrika 73: 1–11.
16. Cheng S, Geow MA, Pallaud C, Kiltz W, Erlich HA, et al. (1999) A multifocus genotyping assay for candidate markers of cardiovascular disease risk. Genome Res 9: 936–949.
17. Onland-Moret NC, van der AD, van der Schouw YT, Buschers W, Elias SG, et al. (2007) Analysis of case-cohort data: a comparison of different methods. J Clin Epidemiol 60: 350–355.
18. Trikalinos TA, Salanti G, Khoury MJ, Ioannidis JP (2006) Impact of violations and deviations in Hardy-Weinberg equilibrium on postulated gene-disease associations. Am J Epidemiol 163: 300–309.
19. Higgins JP, Thompson SG, Deeks JJ, Altman DG (2003) Measuring inconsistency in meta-analyses. BMJ 327: 557–560.
20. Aggerholm-Larsen B, Nordestgaard BG, Tybjaerg-Hansen A (2000) ACE gene polymorphism in cardiovascular disease: meta-analyses of small and large studies in whites. Arterioscler Thromb Vasc Biol 20: 484–492.
21. Berdiel A, Sekeri C, Sirri CE, Ercan E, Sagan A, et al. (2005) Association between the ENOS (Glu298Asp) and the RAS genes polymorphisms and premature coronary artery disease in a Turkish population. Clin Chim Acta 331: 87–94.
22. Fernandez-Arcas N, eguez-Lacena JL, Munoz-Moran E, Ruiz-Galdon M, Espinoza-Caldua S, et al. (2001) Both alleles of the M235T polymorphism of the angiotensinogen gene can be a risk factor for myocardial infarction. Clin Genet 60: 52–57.
23. Zee RV, Cook NR, Cheng S, Erlich HA, Lindpaintner K, et al. (2006) Multifocus candidate gene polymorphisms and risk of myocardial infarction: a population-based, prospective genetic analysis. J Thromb Haemost 4: 341–348.
24. Petrovic D, Zorc M, Kanic V, Peterlin B (2001) Interaction between gene polymorphisms of renin-angiotensin system and metabolic risk factors in premature myocardial infarction. Angiology 52: 247–252.
25. Krizanova O, Obeldzalkova D, Polakova H, Jelok I, Hudecova S (1997) Molecular variants of the renin-angiotensin system components in the Slovak population. Physiol Res 46: 357–361.
26. Goldenberg I, Moss AJ, Ryan D, McNin S, Eberly SW, et al. (2006) Polymorphism in the angiotensinogen gene, hypertension, and ethnic differences in the risk of recurrent coronary events. Hypertension 48: 693–699.
27. Sethi AA, Tybjaerg-Hansen A, Gronholt ML, Steffensen R, Schnohr P, et al. (2001) Angiotensinogen mutations and risk for ischemic heart disease, myocardial infarction, and ischemic cerebrovascular disease. Six case-control studies from the Copenhagen City Heart Study. Ann Intern Med 134: 941–954.
28. Araujo MA, Goulart LR, Cordeiro ER, Gatti RR, Menezes BS, et al. (2005) Genotypic interactions of renin-angiotensin system genes in myocardial infarction. Int J Cardiol 103: 27–32.
29. Albatalla A, Alvarez R, Reguero JR, Hevia S, Iglesias-Cabero G, et al. (2000) Synergistic effect between apolipoprotein E and angiotensinogen gene polymorphisms in the risk for early myocardial infarction. Clin Chem 46: 1910–1915.
30. Bu JC, Smith NL, Patsy BM, Heckbert SR, Edwards KL, et al. (2003) Angiotensinogen Mer235Thr polymorphism, angiotensin-converting enzyme inhibitor therapy, and the risk of nonfatal stroke or myocardial infarction in hypertensive patients. J Hypertens 16: 1011–1017.
31. Buraczynska M, Pijanowski Z, Spasiewicz D, Nowicka T, Sodolski T, et al. (2003) Renin-angiotensin system gene polymorphisms: assessment of the risk of coronary heart disease. Kardiol Pol 45: 1–8.
32. Tong ND, Hamaguchi R, Sakawa T, Hara M, Sakata T (1998) A polymorphism of angiotensinogen gene codon 174 and coronary artery disease in Japanese subjects. Am J Med Sci 316: 339–344.
33. Ferris C, Tsai MY, Hamar A, Akar N, Arv T (2002) Angiotensin I converting enzyme, angiotensin II type 1 receptor and angiotensinogen polymorphisms and early myocardial infarction in Turkish population. Thromb Haemost 88: 693–694.
34. Fatini C, Abbate R, Pepe G, Battaglini B, Genini F, et al. (2008) Searching for a better assessment of the individual coronary risk profile. The role of angiotensin-converting enzyme, angiotensin II type 1 receptor and angiotensinogen gene polymorphisms. Eur Heart J 29: 633–638.
37. Fernandez-Arcas N, eguez-Lacera JL, Munoz-Moran E, Ruiz-Galardon M, Espinosa-Caliani S, et al. (1999) The genotype interactions of methylenetetrahydrofolate reductase and renin-angiotensin system genes are associated with myocardial infarction. Atherosclerosis 145: 293–300.

38. Economou EF, Gkioura SP, Lariotis-Vasina VI, Kovalovs YR, Schwartz EI (2000) Gen gene interaction in the RAS system in the predisposition to myocardial infarction in elderly population of St. Petersburg (Russia). Mol Genet Metab 69: 76–80.

39. Frossard PM, Hill SH, Eshahat YL, Obineche EN, Bokhari AM, et al. (1998) Associations of angiotensinogen gene mutations with hypertension and myocardial infarction in a gulf population. Clin Genet 54: 285–293.

40. Gardemann A, Stricker J, Humme J, Nguyen QD, Kanz N, et al. (1999) Angiotensinogen T174M and M235T gene polymorphisms are associated with the extent of coronary atherosclerosis. Atherosclerosis 145: 309–314.

41. Hooper WC, Dowling NF, Wengser NK, Dilley A, Ellingsen D, et al. (2002) Relationship of venous thromboembolism and myocardial infarction with the renin-angiotensin system in African-Americans. Am J Hematol 70: 1–8.

42. Ichihara S, Yokota M, Fujimura T, Kato S, Hirayama H, et al. (1997) Lack of association between variants of the angiotensinogen gene and the risk of coronary artery disease in middle-aged Japanese men. Am Heart J 134: 260–263.

43. Ishigami T, Umemura S, Iwamoto T, Tamura K, Hibi K, et al. (1995) Molecular variant of angiotensinogen gene is associated with coronary atherosclerosis. Circulation 91: 951–954.

44. Kamitani A, Rakugi H, Higaki J, Ohishi M, Shi SJ, et al. (1995) Enhanced predictability of myocardial infarction in Japanese by combined genotype analysis. Hypertension 25: 950–953.

45. Katsuya T, Koike G, Yee TW, Sharpe N, Jackson R, et al. (1995) Association of angiotensin gene T235 variant with increased risk of coronary heart disease. Lancet 345: 1600–1603.

46. Ko YL, Ko YS, Wang SM, Chia PH, Teng MS, et al. (1997) Angiotensinogen and angiotensin-I converting enzyme gene polymorphisms and the risk of coronary artery disease in Chinese. Hum Genet 100: 210–214.

47. Methot J, Hamelin BA, Boguty P, Assenza RL, Plante S, et al. (2003) ACE-DD genotype is associated with the occurrence of acute coronary syndrome in postmenopausal women. Int J Cardiol 105: 306–314.

48. Offe LP, Lautsch J, Janssen U, Minkenberg R, Hanrath P, et al. (2002) Analysis of several hundred genetic polymorphisms may improve assessment of the individual genetic burden for coronary artery disease. Eur J Intern Med 13: 481–492.

49. Ranjith N, Pegoraro RJ, Rom L, Lanning PA, Naidoo DP (2004) Renin-angiotensin system and associated gene polymorphisms in myocardial infarction in young South African Indians. Cardiovasc J South Afr 15: 22–26.

50. Reinhart D, Signac HH, Vogt SF, Zeiss C, Farker K, et al. (2000) A common variant of the angiotensinogen gene and the risk of coronary artery disease in a German population. Pharmazie 55: 69–71.

51. Renner W, Nauck M, Winkelmann BR, Hofmann MM, Schmagrau H, et al. (2005) Association of angiotensinogen haplotypes with angiotensinogen levels but not with blood pressure or coronary artery disease: the Ludwigshafen Risk and Cardiovascular Health Study. J Mol Med 83: 235–239.

52. Sheu WH, Lee WJ, Jeng CY, Young MS, Ding YA, et al. (1998) Angiotensinogen gene polymorphism is associated with insulin resistance in nondiabetic men with or without coronary heart disease. Am Heart J 136: 123–131.

53. Tobin MD, Braund PS, Burton PR, Thompson JR, Steeds R, et al. (2004) Genotypes and haplotypes predisposing to myocardial infarction: a multilocus case-control study. Eur Heart J 25: 459–467.

54. Tsai CT, Hwang JJ, Ritchie MD, Moore JH, Chiang FT, et al. (2007) Renin-angiotensin system gene polymorphisms and coronary artery disease in a large angiographic cohort: detection of high order gene-gene interaction. Atherosclerosis 195: 172–180.

55. Weltzel K, Blackburn A, Ernst M, Allford M, Hanke R, et al. (1997) Relationship of polymorphisms in the renin-angiotensin system and in E-selectin of patients with early severe coronary heart disease. J Mol Med 75: 57–61.

56. Wierzbicki AS, Lambert-Hammill M, Lamb PJ, Crook MA (2000) Renin-angiotensin system polymorphisms and coronary events in familial hypercholesterolemia. Hypertension 36: 808–812.

57. Winkelmann BR, Russ AP, Nauck M, Klein B, Bohm BO, et al. (1999) Angiotensinogen M235T polymorphism is associated with plasma angiotensinogen and cardiovascular disease. Am Heart J 137: 796–805.

58. Yamakawa-Kobayashi K, Arinami T, Hamaouchi H (1995) Absence of association of angiotensinogen gene T235 allele with increased risk of coronary heart disease in Japanese. Lancet 345: 515.

59. Seminara D, Khoury MJ, O’Brien TR, Manolio T, Gwinn ML, et al. (2007) The emergence of networks in human genome epidemiology: challenges and opportunities. Epidemiology 18: 1–8.

60. Sheu WH, Lee WJ, Jeng CY, Young MS, Ding YA, et al. (2005) Association of angiotensinogen haplotypes with angiotensinogen levels but not with blood pressure or coronary artery disease: the Ludwigshafen Risk and Cardiovascular Health Study. J Mol Med 83: 235–239.

61. Salanti G, Ambrozats G, Nizani EE, Ioannidis JP (2003) Hardy-Weinberg equilibrium in genetic association studies: an empirical evaluation of reporting, deviations, and power. Eur J Hum Genet 13: 840–848.

62. Little J, Bradley L, Bray MS, Clynne M, Dorman J, et al. (2002) Reporting, appraising, and integrating data on genome prevalence and gene-disease associations. Am J Epidemiol 156: 300–310.

63. Xu J, Turner A, Little J, Blecker ER, Meyers DA (2002) Positive results in association studies are associated with departure from Hardy-Weinberg equilibrium: hint for genotyping error? Hum Genet 111: 573–574.

64. Ntzani EE, Rizos EC, Ioannidis JP (2002) Counterpoint: bias from population stratification is not a major threat to the validity of conclusions from large-scale evidence. Am J Epidemiol 165: 973–984.

65. Seminara D, Khoury MJ, O’Brien TR, Manolio T, Gwinn ML, et al. (2007) The emergence of networks in human genome epidemiology: challenges and opportunities. Epidemiology 18: 1–8.

66. Sheu WH, Lee WJ, Jeng CY, Young MS, Ding YA, et al. (2005) Association of angiotensinogen haplotypes with angiotensinogen levels but not with blood pressure or coronary artery disease: the Ludwigshafen Risk and Cardiovascular Health Study. J Mol Med 83: 235–239.