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Endothelial Nitric Oxide Synthase Genotype and Ischemic Heart Disease

Meta-Analysis of 26 Studies Involving 23 028 Subjects

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Background— Polymorphisms in the endothelial nitric oxide synthase (eNOS) gene may influence the risk of ischemic heart disease (IHD), but data from published studies with individually low statistical power are conflicting. To evaluate the role of polymorphisms in the eNOS gene in IHD, we considered all available studies in a meta-analysis.

Methods and Results— Case-control studies evaluating the association between the Glu298Asp, −786T>C, and intron-4 polymorphisms and IHD were searched in MEDLINE and EMBASE up to January 2003. The principal prior hypothesis was that homozygosity for eNOS Asp298, the −786C allele in the promoter, or the intron-4 (*a* allele) would be associated with an increased risk of IHD. Data were available for 9867 cases and 13 161 controls from 26 studies. Homozygosity for the Asp298 was associated with an increased risk of IHD (OR, 1.31; 95% CI, 1.13 to 1.51). Although there was significant heterogeneity among studies of Asp298 (P_{Het}=0.0002), which was largely accounted for by a single study, the increase in risk was still significant after exclusion of that study from analysis. Homozygosity for the intron-4*a* allele was also significantly associated with higher risk of IHD (OR, 1.34; 95% CI, 1.03 to 1.75). However, no significant association was found with the −786C allele (OR, 1.06; 95% CI, 0.89, 1.25).

Conclusions—Individuals homozygous for the Asp298 and intron-4*a* alleles of eNOS are at moderately increased risk of IHD. These findings support the proposal that common genetic variations in the eNOS gene contribute to atherosclerosis susceptibility, presumably by effects on endothelial NO availability. **(*Circulation*. 2004;109:1359-1365.)**

Key Words: coronary disease ■ meta-analysis ■ myocardial infarction ■ nitric oxide synthase ■ polymorphism (genetics)

Atherosclerosis is a partly heritable disorder, although the genes involved and the associated relative risks are not known.[1,2] Nitric oxide (NO) from the endothelium is considered an important atheroprotective mediator, and acquired defects in NO generation associated with cardiovascular risk factors cause endothelial dysfunction and may contribute to the development of atherosclerosis.[3,4] There is also evidence that inherited differences in NO availability could play a role in this process. Endothelium-dependent flow-mediated dilatation of the brachial artery (an NO-dependent response) is impaired in young healthy individuals with a first-degree relative who died of ischemic heart disease (IHD) at <55 years of age compared with age-matched individuals with no family history of IHD.[5,6] Also, mice in which the endothelial NO synthase (eNOS) gene has been deleted have an increased susceptibility to develop atherosclerosis independently of blood pressure changes.[7] Because the regulation of endothelial NO availability occurs at the level of the synthetic enzyme (eNOS), the gene that encodes eNOS is a candidate atherosclerosis-susceptible gene.[8]

A number of groups have identified polymorphisms in the eNOS gene.[9,10] These include single nucleotide polymorphisms, a variable-number tandem repeat in intron-4, and a CA-repeat microsatellite marker in intron-13. Recently, several case-control studies have evaluated the association of the eNOS polymorphisms (Glu298Asp, intron-4, and −786T>C) and the risk of developing IHD,[9–13] but data from many small, individually underpowered, case-control allele-association studies are conflicting. We conducted a meta-analysis of available studies to clarify the role of eNOS genotypes in IHD risk.

Methods

Literature Search

We searched MEDLINE and EMBASE up to January 2003 for case-control studies evaluating an association between Glu298Asp, −786T>C, or intron-4 polymorphisms and IHD. Terms used for the search (which were all MeSH terms) were "nitric oxide synthase," "ischemic heart disease," "coronary heart disease," and "myocardial infarction" combined with "genetic," "polymorphism," "mutation,"

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Characteristics of Published Studies of Association Between the eNOS Genotyping (Glu298Asp, Intron-4, and −786T/C) and IHD Included in the Meta-Analysis

		Frequency of Genotype for Each Polymorphism*				
Study	Country	Asp/Asp, %	a/a, %	C/C, %	Study Design	Outcome
Non-Asian population						
Jeerooburkhan et al[35]	United Kingdom	11.3	1.4	14.5	Nested case-control	MI
Hingorani et al[9]	United Kingdom	8.7	...	...	Case-control	MI
Hingorani et al[9]	United Kingdom	10.1	...	...	Case-control	CAD
Fowkes et al[44]	United Kingdom	...	1.6	...	Case-control	CAD
Granath et al[12]	Australia	10.5	2.2	14.9	Case-control	CAD
Wang et al[10]	Australia	...	0.67	...	Case-control	CAD
Sim et al[47]	Australia	...	...	19.2	Case-control	CAD
Cai et al[32]	Australia	7.7	...	...	Case-control	MI
Cai et al[31]	Australia	13.9	...	...	Case-control	CAD
Gardemann et al[34]	Germany	9.3	2.4	...	Case-control	CAD
Sigusch et al[46]	Germany	...	2.6	...	Case-control	CAD
Aras et al[30]	Turkey	7.6	...	...	Case-control	CAD
Cine et al[43]	Turkey	...	0.65	...	Case-control	MI
Hooper et al[45]	United States	...	9.2	...	Case-control	MI
Poirier et al[36]	Ireland	16.1	...	21.9	Case-control	MI
Pulkkinen et al[37]	Finland	10	4.5	...	Case-control	CAD
Poirier et al[36]	France	12.8	...	17.8	Case-control	MI
Alvarez et al[42]	Spain	...	2	14.3	Case-control	CAD
Colombo et al[33]	Italy	6.1	...	...	Case-control	CAD
Asian population						
Shimasaki et al[27]	Japan	0.16	...	...	Case-control	MI
Nakagami et al[40]	Japan	...	2.9	...	Case-control	CAD
Ichihara et al[38]	Japan	...	1.2	...	Case-control	MI
Hibi et al[11]	Japan	...	1.4	...	Case-control	MI
Takagi et al[48]	Japan	...	...	1.1	Case-control	MI
Yoon et al[29]	Korea	...	7	...	Case-control	CAD
Lee et al[39]	Korea	...	0.46	...	Case-control	CAD
Park et al[41]	Korea	...	0.48	...	Case-control	MI
Wang et al[28]	Taiwan	1.4	...	...	Case-control	CAD

MI indicates myocardial infarction; CAD, coronary artery disease. Where no data are presented, that polymorphism was not evaluated.

*The frequency reported corresponds to the control group.

or "genes." Search results were limited to "human" and "English language." Additional studies were identified in the references lists of publications and through the MEDLINE option "related articles." The Table gives characteristics of the published studies.

Selection Criteria

For inclusion, studies had to be case-control (retrospective or nested) in design, involve unrelated subjects, and examine the associations between IHD and the presence of the eNOS polymorphisms. Studies were excluded if subjects were <18 years age or if reported only as abstracts. For duplicate publications, the study with the smaller data set was excluded. When genotype frequency was not reported, authors were contacted to obtain the relevant information.

End Points

IHD was defined as myocardial infarction (World Health Organization criteria)[14] or angiographic coronary artery occlusion (>50% of the luminal diameter). In studies in which coronary artery disease was assessed as the primary outcome, some subjects had a history of myocardial infarction. These subjects were included as cases only once to avoid double counting.

Data Extraction

The population evaluated, study design, mean age of participants, frequency of genotypes and alleles, frequency of cardiovascular risk factors, and primary outcome were extracted independently and entered into separate databases by 2 authors. Results were compared, and disagreements were resolved by consensus.

Statistical Analysis

Our principal prior hypothesis was that homozygosity for eNOS Asp298, −786C, or intron-4*a* alleles would be associated with an increased risk of IHD (recessive genetic model). In a separate analysis, the ORs for a dominant and codominant genetic model for each polymorphism were also calculated. Data were analyzed by use of Review Manager software (version 4.1) Cochrane Collaboration 2000 and Stata 8.0. We calculated fixed- and random-effect summary ORs and 95% CIs for each polymorphism using the Mantel-Haenszel[15,16] and DerSimonian and Laird[17] methods, respectively.

We used Galbraith plots[18] and the DerSimonian and Laird *Q* test[17] to evaluate the heterogeneity and funnel plots and the Egger regression asymmetry test to assess publication bias.[19] The influence of individual studies on the summary OR was evaluated by reestimating and plotting the summary OR in the absence of each study. We used meta-regression to evaluate the extent to which different variables explained heterogeneity among the individual ORs.[20] To evaluate the possible effect of ethnic background on the variability of the individual ORs, the study populations were divided into Asian (Japanese, Korean, and Taiwanese) and non-Asian (British, American, Irish, German, French, Finnish, Australian, Italian, Spanish, and Turkish).

The population-attributable risk, which reflects the proportion of IHD in the population attributed to a particular genotype, was calculated with the following formula: 100×[prevalence (OR−1)/prevalence (OR−1)+1]. When the OR was derived from the fixed method, the proportion of the population exposed to causative factor (ie, the gene variant) was calculated by use of control genotype frequencies.

Results

Study Selection

The primary search generated 37 potentially relevant articles, of which 31 met the selection criteria. From the 6 articles excluded, 4 were published in non-English journals,[21–24] 1 was a study of related case-controls,[25] and the other was a case series.[26] Of the 31 studies, only 26 (9867 cases, 13 161 controls) were included in the final analysis.[9–12,27–48] Three studies[13,49,50] were excluded because there were no homozygous subjects in the control or case group, and another 2 studies[51,52] were excluded because genotype frequency was not reported and the relevant information could not be obtained from the authors. Besides a case-control study nested within a cohort included in our analysis,[35] no other prospective studies were available.

Subject Characteristics

Glu298Asp Polymorphism

The meta-analysis of the Glu298Asp polymorphism included 14 studies with 6036 IHD cases and 6106 controls.[9,12,27,28,30–37] The control groups were either hospital inpatients or community controls, except for 1 study in which the controls were selected from subjects who underwent coronary angiography and were found to have normal coronary arteries.[33] The mean ages for cases and controls were 58 and 53 years, respectively. There was a significant difference in the percentage of individuals homozygous for Asp298 by ethnic group (0.48% for Asians versus 10.73% for non-Asians; $P=0.0006$). Likewise, significant differences in the frequency of the Asp298 allele by ethnic group were observed (7.6% for Asians versus 32.3% for non-Asians; $P<0.0001$).

Intron-4 Polymorphism

Sixteen studies (6212 cases, 6737 controls) that evaluated the association between the intron-4 polymorphism and IHD were included in the meta-analysis.[10–12,29,34,35,37–46] As for the Glu298Asp polymorphism, the control groups were selected mainly from hospital-based or general community population.

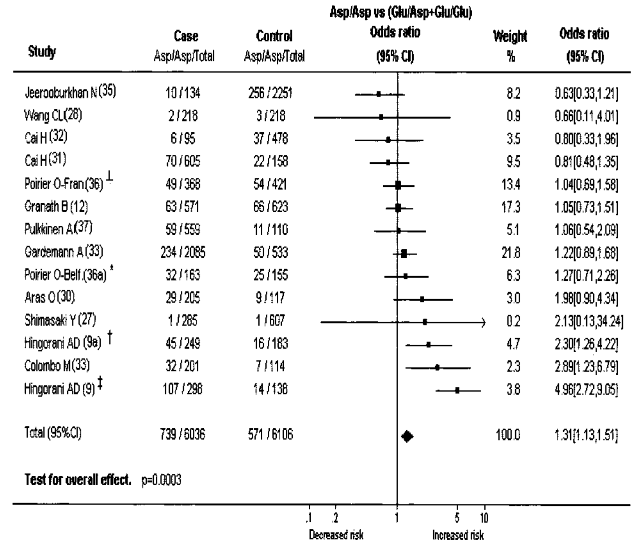

Figure 1. Results of published studies of association between Glu298Asp polymorphism and IHD. ORs for outcome compared homozygous subjects for Asp298 allele with heterozygous (Glu/Asp) plus wild type (Glu/Glu). ⊥; Poirier O-Fran. (36) indicates substudy involving French population; *Poirier O-Belf. (36a), substudy involving Irish population; †Hingorani AD (9a), study with myocardial infarction as outcome; and ‡Hingorani AD (9), study with coronary artery disease as outcome.

The mean ages for cases and controls were 58 and 52 years, respectively. The percentage of subjects homozygous for the *a* allele was similar in Asians and non-Asians (1.6% and 2.0%, respectively; $P=0.70$). The frequency of the *a* allele was also similar in Asians (12%) and non-Asians (14%).

−786T>C Polymorphism

Seven studies that evaluated the association between the −786T>C polymorphism in the gene promoter and IHD were included in the meta-analysis (2377 cases, 7702 controls).[12,35,36,42,47,48] The mean ages for cases and controls were 49 and 53 years, respectively. There were again significant differences in the proportion of homozygotes for the −786C allele by ethnic group (1.10% for Asians versus 15.36% for non-Asians; $P<0.0001$). Similarly, we observed significant differences in the frequency for the −786C allele by ethnic group (10.7% for Asians versus 39.1% for non-Asians; $P<0.0001$).

Meta-Analysis

Glu298Asp and IHD

Figure 1 shows the results of all the studies of the Glu298Asp polymorphism and IHD. Two different ORs were obtained from the study of Poirier et al,[36] 1 for the French population and 1 for the Irish population. Also, 2 ORs were obtained from the study of Hingorani et al,[9] 1 for cases of acute myocardial infarction and 1 for cases of angiographically defined coronary artery disease. Of 14 individual ORs estimates, 7 showed a lower or similar risk of IHD for individuals homozygous for the Asp298 allele compared with Glu298 allele carriers (Glu/Glu plus Glu/Asp), but none of them was statistically significant.[12,28,31,32,35,36,37] The other 7 studies showed an increase in the risk of IHD among individuals

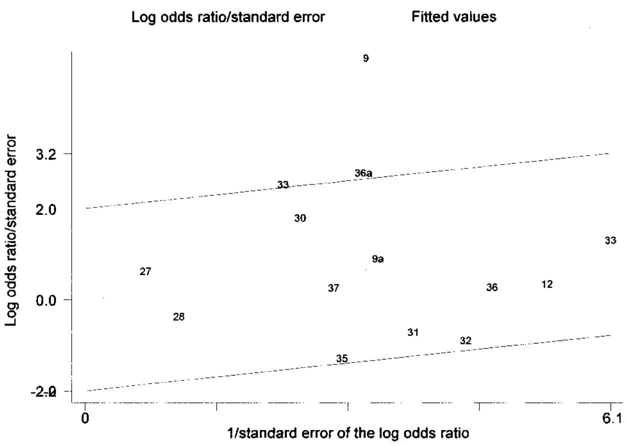

Figure 2. Galbraith plot of ORs of IHD in studies that evaluated the Glu298Asp polymorphism. Numbers inside plot correspond to reference number for each study. For reference numbers 9, 9a, 36, and 36a, see Figure 1.

homozygous for the Asp298 allele,[9,27,30,33,34,36] and 3 studies[9,33] were statistically significant.

The summary OR under a fixed-effect model showed that individuals homozygous for the Asp298 allele were 1.31 times more likely to develop IHD (95% CI, 1.13 to 1.51; $P=0.0003$). However, the individual estimates of the ORs were significantly heterogeneous (for heterogeneity, $P_{Het}=0.0002$).

The Galbraith plot (Figure 2) showed that the study on coronary artery disease conducted in the East Anglian region of the United Kingdom largely accounted for the heterogeneity.[9] This study also had the largest OR and the largest influence on the summary OR. Nevertheless, a random-effect summary OR that takes into account the intrastudy and interstudy variability resulted in a similar overall estimate (1.34; 95% CI, 1.00 to 1.79; $P=0.05$). Also, when the most influential individual OR[9] was excluded from the calculation of the summary OR, the individual ORs were no longer heterogeneous ($P_{Het}=0.11$), and the summary OR was attenuated but remained statistically significant (summary OR, 1.17; 95% CI, 1.00 to 1.36; $P=0.05$).

Despite the striking difference in the frequency of the Asp298 allele in Asian and non-Asian populations, a meta–regression analysis showed that ethnic background (Asian versus non-Asian) was not a significant source of interstudy heterogeneity ($P_{Her}=0.52$). Similarly, smoking, age, and gender were not significant sources of heterogeneity in a group of 10 studies with information in all these variables.[9,12,27,33–37]

The distribution of the ORs from individual studies in relation to their respective SD (funnel plot) was symmetric, and the Egger test suggested a low probability of publication bias ($P=0.41$). The dominant model showed a nonsignificant association between carriers of $\geq$1 Asp298 allele and the risk of IHD (OR, 1.06; 95% CI, 0.97 to 1.15; $P=0.21$). When a codominant model was evaluated, only the (Asp/Asp) versus (Glu/Glu) comparison was significant (OR, 1.26; 95% CI, 1.08 to 1.47; $P=0.003$), whereas heterozygosity (Glu/Asp versus Glu/Glu) was not associated with an increase in risk of IHD (OR, 1.0; 95% CI, 0.91 to 1.10; $P=0.93$). These results

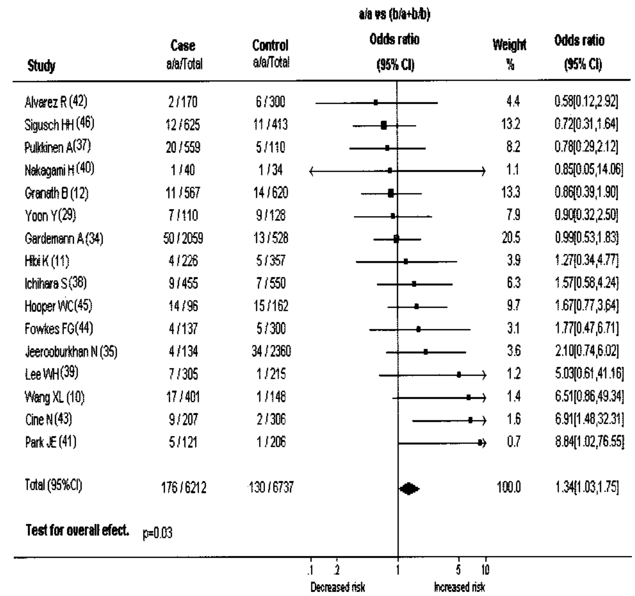

Figure 3. Results of published studies of association between intron-4 polymorphism and IHD. ORs for outcome compared homozygous subjects for intron-4*a* allele with heterozygous (*a*/*b*) plus wild type (*b*/*b*).

support a recessive model of inheritance. The population-attributable risk for the Glu298Asp polymorphism under a recessive genetic model of inheritance was 2.81% for all studies combined.

Intron-4 and IHD

Sixteen studies of the relationship between intron-4*a* allele and risk of IHD were included in the meta-analysis. Of the 16, 9 showed an increased risk in individuals homozygous for the *a* allele compared with *b* allele carriers (*b*/*b* plus *b*/*a*),[10,11,35,38,39,41,43–45] but only 1 was statistically significant[43] (Figure 3). With a fixed-effect model, the summary OR for IHD among homozygotes for the intron-4*a* allele was 1.34 (95% CI, 1.03 to 1.75; $P=0.03$) (Figure 3). No significant interstudy heterogeneity was observed ($P_{Het}=0.19$).

An asymmetric distribution of the ORs in relation to theirs SD was observed in the funnel plot, and the Egger test suggested a significant publication bias ($P=0.03$). No individual study had an undue influence on the summary OR. The dominant model indicated a nonsignificant association between carriers of at least 1 *a* allele and the risk of IHD (OR, 1.06; 95% CI, 0.97 to 1.16; $P=0.21$). A codominant model showed only a significant association for the *a*/*a* versus *b*/*b* comparison (OR, 1.38; 95% CI, 1.06 to 1.88; $P=0.02$), whereas heterozygosity for the *a* allele was not associated with an increase risk of IHD (OR, 1.02; 95% CI, 0.93 to 1.13; $P=0.62$). Again, these results are in favor of a recessive model of inheritance. The population-attributable risk for the intron-4 polymorphism under a recessive genetic model of inheritance was 0.64% for all the studies.

−786T>C and IHD

Only 7 studies of the association between the −786T>C polymorphism and risk of IHD (2377 cases, 7702 controls) were eligible for inclusion in the analysis. Three studies[36,42,48]

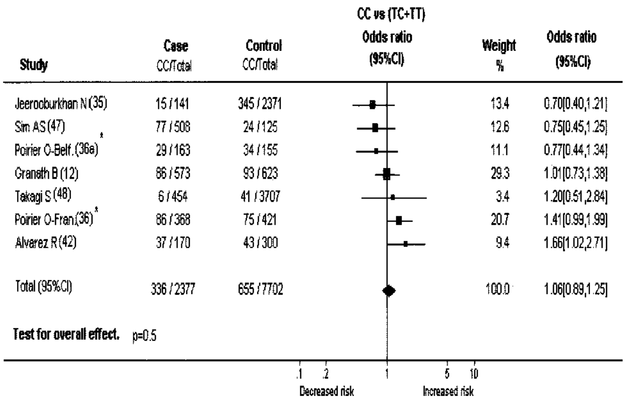

Figure 4. Results of published studies of association between the −786T>C polymorphism and ischemic heart disease. ORs for outcome compared homozygous subjects for −786C allele (C/C) with heterozygous (T/C) plus wild type (T/T). For frequency of −786T/C polymorphism in populations from Poirier O-Belf (36a) and Poirier O-Fran (36), values were inferred from the −924 genotype, which was completely associated with −786T>C variant in 26 individuals.[36]

showed an increased risk and the other 3 showed a decreased risk of IHD[35,36,47] among homozygotes for the −786C allele compared with −786T allele carriers (T/T plus T/C), but none was statistically significant (Figure 4). The summary OR obtained from a fixed-effect model indicated no significant increase in the risk of IHD among individuals homozygous for the −786C allele (summary OR, 1.06; 95% CI, 0.89 to 1.25; $P=0.50$). No significant interstudy heterogeneity was observed ($P_{Het}=0.243$). The funnel plot showed a symmetric distribution of the ORs in relation to their SD, and the Egger test did not suggest the presence of publication bias ($P=0.54$). The dominant model indicated no significant association between carriers of ≥1 −786C allele and the risk of IHD (OR, 1.10; 95% CI, 0.99 to 1.24; $P=0.09$).

Discussion

Using data from 10 399 IHD cases, we found that homozygosity for the Asp298 and intron-4*a* alleles but not the −786C allele of the eNOS gene was associated with a small but significant increase in the risk of IHD (OR, 1.31; 95% CI, 1.13 to 1.51; OR, 1.34; 95% CI, 1.03 to 1.75; and OR, 1.06; 95% CI, 0.89 to 1.25, respectively).

The ORs for Asp298 and intron-4*a* alleles are very similar in magnitude to those reported for apolipoprotein E, angiotensin-converting enzyme, and methylenetetrahydrofolate reductase polymorphisms[53–55] and suggest that the genetic contribution to IHD is through small to moderate effects of many genes. Therefore, it seems unlikely that these polymorphisms individually will make a useful contribution to risk prediction in asymptomatic individuals, but whether combined genotype analysis integrated with orthodox assessment of cardiovascular risk will enhance the prediction of IHD requires additional analysis.[56]

Whether the genotypic risks for eNOS Glu298Asp and intron-4*a*/*b* polymorphisms are independent or whether both reflect carriage of a small number of common risk haplotypes requires further study.

The Glu298Asp polymorphism is the only coding region variant identified in eNOS, and mechanistic studies indicate a functional effect of this substitution. Associations have been described between the Glu298Asp polymorphism and NO synthesis[57,58] or endothelial function,[59,60] and a mechanism by which eNOS Asp298 might reduce NO bioavailability has also been reported. eNOS Asp298 is subject to selective proteolytic cleavage in endothelial cells and vascular tissues. Because the cleaved fragments would be expected to lack NO synthase activity, this could account for reduced vascular NO generation in subjects homozygous for this variant.[57,58] Individuals homozygous for the Asp298 allele have also been shown to exhibit a reduced blood pressure fall after exercise training[61] and to have lower basal blood flow and reduced vasodilation to adenosine in their coronary arteries.[62] In addition, they have an enhanced systemic pressor response to phenylephrine[63] and a reduced flow-mediated dilatation of the brachial artery.[59] These findings suggest that subjects homozygous for the Asp298 allele generate low NO in vivo and may be more susceptible to endothelial dysfunction, which might account for the increased risk of IHD.

Conflicting associations between the intron-4 variant and NO pathway activity have also been described. Some reports indicate that carriers of this variant have lower NO plasma levels and decreased protein expression,[64,65] but this is not supported by all studies.[29,35,66] Because this variant is intronic, it is unlikely to be functional but might act as a marker in linkage disequilibrium with other functional variants in regulatory regions of the eNOS gene.[35] Nevertheless, haplotype and linkage disequilibrium analyses indicate that the degree of linkage disequilibrium observed between the intron-4 variant and Glu298Asp or −786T>C is small ($\Delta=-0.27$ and 0.36, respectively).[35]

A functional effect for the −786T>C polymorphism has also been proposed from in vitro reporter gene assays.[67] Lower eNOS mRNA and serum nitrite/nitrate levels have been found in individuals with the −786C variant in some[68] but not all studies.[35] However, this meta-analysis does not support an influence of this variant on IHD risk, although a very small effect of this variant cannot be ruled out because the analysis of available data, which included 2377 cases and 7702 controls, had only a 73% power to detect an OR of 1.2 at a significance level of 5%.

Homozygosity for the Asp298 and −786C alleles of the eNOS gene was rare (0.48% and 1.10%, respectively) in Asian population samples. Consequently, a low genotyping error rate or undetected population stratification[69] (a situation in which cases or controls are composed of substrata that differ by genetic background) would have a greater influence on the OR compared with studies in non-Asians. Studies with very large sample sizes are needed to obtain reliable estimates of the effect of these polymorphisms in Asian subjects. To estimate the effect of eNOS genetic variants in the Asian population, the sample size would have to be 19 times larger for Glu298Asp and 11 times larger for −786T>C than the sample size in the non-Asian population. Even in this meta-analysis of 2214 Asian cases, only 9 Asp298, 33 intron-4*a*, and 6 −786C allele homozygotes were identified. The results for this ethnic population must therefore be interpreted with caution.

Publication bias is an unlikely explanation of the observed association between the Glu298Asp polymorphism and IHD incidence but might have led to overestimation of association between the intron-4 variant and IHD. Although this might not affect the conclusions of this meta-analysis,[70] more studies are needed to quantify the effect size reliably.

Although confounding is generally not anticipated in analyses of an association of a genotype with disease, there may some imbalance in the distribution of cardiovascular risk factors by eNOS genotype. However, this is unlikely because no strong associations have been identified between these polymorphisms and cardiovascular risk factors.

Despite heterogeneity in the Glu298Asp meta-analysis, largely accounted for by a study in the East Anglian region of UK,[9] the random-effect estimation of the summary OR was still significant, and when this study was excluded from the analysis, the association was still preserved.

Conclusions

Homozygosity for Asp298 and intron-4*a* alleles is associated with increased risk of IHD by 31% and 34%, respectively. Common genetic variants that contribute to atherosclerosis susceptibility are likely to exert individually only a small to moderate influence on future risk of disease. Whether assessment of multiple genotypes in a single individual will enhance prediction over orthodox prediction tools based on acquired risk factors requires further examination.

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