This review examines the association of a subset of endothelial nitric oxide synthase gene (NOS3) polymorphisms (Glu298Asp, intron 4, and -786T>C) with cardiovascular disease. The Glu298Asp polymorphism within exon 7 is the only common nonsynonymous variant. The variants have been associated with low plasma nitric oxide concentrations and reduced vascular reactivity; difficulties in measuring those phenotypes means that their functional role remains unclear. A large meta-analysis of NOS3 polymorphisms in coronary heart disease revealed per-allele odds ratios of 1.17 (95% confidence interval: 1.07, 1.28) for Glu298Asp, 1.17 (95% confidence interval: 1.07, 1.28) for -786T>C, and 1.12 (95% confidence interval: 1.01, 1.24) for intron 4. However, there was evidence that small studies with more striking results could affect the associations of the Glu298Asp and -786T>C polymorphisms with coronary heart disease. Associations of NOS3 polymorphisms with hypertension, preeclampsia, stroke, and diabetes remain uncertain. To date, no reliable gene-gene or gene-environmental interactions have been described. Use of these variants in predictive testing is unlikely to be useful, although the population attributable fraction could be substantial if the modest associations are causal. The need for large-scale genetic association studies using tagging polymorphisms is warranted to confirm or refute a role of the NOS3 gene in coronary heart disease.

cardiovascular diseases; epidemiology; genotype; meta-analysis; nitric oxide synthase type III; NOS3; polymorphism, genetic; pre-eclampsia

Abbreviations: CHD, coronary heart disease; CI, confidence interval; eNOS, endothelial nitric oxide synthase; OR, odds ratio; SNP, single nucleotide polymorphism; tSNP, tagging single nucleotide polymorphism.
sequence and function (1). The NOS3 gene was cloned in 1993 and was localized to chromosome 7q35-36 (2). Spanning 4.4 kb of genomic DNA, the gene comprises 26 exons that encode a 135-kD protein containing 1,203 amino acids. Approximately 1,500 base pairs of upstream promoter sequence have also been characterized and contain transcription factor-binding sites that mediate regulation by shear stress and estrogens, among others (3). The eNOS protein synthesizes nitric oxide constitutively via a reaction including the conversion of L-arginine to L-citrulline, which involves the transfer of five electrons provided by nicotinamide adenine dinucleotide phosphate (4). The enzyme acts as a homodimer that can be divided functionally into two major domains: a C-terminal reductase domain and an N-terminal oxygenase domain (5). Catalytic activity requires the presence of heme and the cofactors tetrahydrobiop- terin, flavin adenine dinucleotide, flavin mononucleotide, and calmodulin (5). Nitric oxide is not stored but rather released upon its synthesis. Thus, nitric oxide generation is regulated through alterations in the expression or activity of the eNOS enzyme itself or through changes in the availability of activating cofactors or endogenous inhibitor molecules (6, 7).

Nitric oxide from the endothelium is considered an important atheroprotective mediator, and acquired defects in generation of nitric oxide are associated with increases in cardiovascular risk factors (8). Endothelium-dependent, flow-mediated dilatation of the brachial artery (a largely nitric oxide–dependent response) is impaired in young, healthy individuals with a first-degree relative who died from coronary heart disease (CHD) before age 55 years when compared with age-matched individuals with no family history of CHD (9, 10). In addition, mice in which the NOS3 gene has been deleted are hypertensive, and those with deletions in both the apolipoprotein E and NOS3 genes have increased susceptibility to atherosclerosis (11). Because endothelial nitric oxide availability is regulated at the level of synthesis, the gene that encodes eNOS is a candidate for cardiovascular disease (3).

GENE VARIANTS

The NOS3 gene has been extensively screened for variation. Variants detected include numerous single nucleotide polymorphisms (SNPs) (12–14), a variable-number tandem repeat in the intron 4 (15), and a CA repeat microsatellite marker in the intron 13 (12). The only common variation identified that leads to an amino acid substitution in the mature protein is the G894T or Glu298Asp (rs1799983) variant, in which a guanine → thymine substitution at exon 7 leads to a glutamate → aspartate substitution at position 298 (12). Several promoter SNPs have been identified, but there is no clear evidence that any of them lies directly within the consensus sequence for a known transcription factor of NOS3. Similarly, no variations in the 3’ untranslated region have been reported (14). Variation in this region might influence RNA stability (3).

Web appendix tables 1, 2, and 3 describe genotype frequencies in apparently healthy subjects from 64 sample populations, divided according to ethnic background. (This information is presented in the first three of six supplementary tables; each is referred to as “Web appendix table” in the text and is posted on the website of the Human Genome Epidemiology Network (http://www.cdc.gov/genomics/hugenet/reviews.htm) as well as on the Journal’s website (http://aje.oupjournals.org/). This review also includes Web Appendix text and 10 supplementary Web figures; each of these figures is referred to as “Web appendix figure” and is also posted online.) A significant difference in the frequency of Asp298 and -786C alleles by ethnic group has been reported previously (16) and was confirmed in a previous meta-analysis of NOS3 genotype and CHD, in which a lower frequency of homozygosity for the Asp298 and -786C alleles was observed among Asians (Asp/Asp—Asians: 0.48 percent vs. non-Asians: 10.73 percent; C/C—Asians: 7.6 percent vs. non-Asians: 32.3 percent) (17). The proportion of subjects homozygous for the intron 4 a allele was similar among Asians and non-Asians (1.6 percent and 2.0 percent, respectively) (17). A low frequency of subjects homozygous for the Asp298 allele has been reported among Amerindians and mixed Hispanic populations (18–20), which means that very large sample sizes would be needed to obtain reliable estimates of the effect of these polymorphisms in these populations.

Functional variation in the NOS3 gene has yet to be completely characterized. Much attention has focused on three putatively functional variants (-786T>C (rs2070744), intron 4 27-base-pair repeat, and Glu298Asp (rs1799983)), but little information has been available as to how these variants associate with one another. Focusing research efforts on the three variants examined to date limits the study of NOS3 to an isolated “candidate polymorphism” rather than a “candidate gene” approach (21). Knowledge of haplotypes and linkage disequilibrium patterning through the NOS3 gene would enable a more thorough investigation of the role of NOS3 in the development of cardiovascular disease.

We examined the association between the three commonly studied variations in a sample of 2,266 males of British descent from the Northwick Park Heart Study-II. The characteristics of this population-based prospective cohort study have been described elsewhere (22). Haplotypes generated from these three variants and corresponding linkage disequilibrium values are shown in Web appendix tables 4 and 5. The loose association between these three variants, as shown by pairwise (r²) values, justifies direct genotyping of each variant.

Investigation of NOS3 variation was then expanded by studying the International HapMap Project and the University of Washington Variation Discovery Resource (Seattle SNPs) (14, 23). The goal of the HapMap project is to provide SNP data across the entire genome with an average density of one SNP every 1 kb, a resource invaluable for haplotype-based association studies (23). The Seattle SNPs project focuses on characterizing variation across the entire length of specific genes associated with inflammation and cardiovascular disease. We used these data to characterize the pattern of linkage disequilibrium in and around the NOS3 locus in northern European populations. First, we examined linkage disequilibrium across a 110-kb region.
containing the NOS3 gene by using 21 SNPs from the HapMap database (23). We then focused specifically on the 25 kb of the NOS3 gene itself by using the University of Washington Variation Discovery Resource data (14).

The HapMap data showed the NOS3 gene to be located at the edge of a region of elevated linkage disequilibrium that extends at least 45 kb upstream of the gene, while linkage disequilibrium downstream of the NOS3 gene breaks down abruptly (Web appendix figure 1). An examination of fine-scale linkage disequilibrium across the gene itself, using genotype data from the complete resequencing of the gene by the Seattle SNPs project, confirmed the pattern of elevated linkage disequilibrium across the gene depicted in the gross-scale analysis.

We selected tagging SNPs (tSNPs) for NOS3 based on haplotypes inferred from the Seattle SNPs data by using only those variants with a minor allele frequency of greater than 5 percent. We used the haplotype $r^2$ method (24–26) and applied a minimum coefficient of determination of 0.80 in predicting the state of each tagged SNP. To combine a tagging and functional approach, we included the putatively functional variants -786T>C (rs2070744) and Glu298Asp (rs1799983) as tSNPs regardless of their coefficients. Unfortunately, the intron 4 27-base-pair repeat was not genotyped. The following set of six tSNPs satisfied these conditions: 1) tSNP1: rs2070744 (-786T>C); 2) tSNP2: rs3918167; 3) tSNP3: rs1799983 (Glu298Asp); 4) tSNP4: any one of rs3918188, rs3918181, rs3918182, or rs3918184; 5) tSNP5: any one of rs743506, rs743507, or rs2256314; and 6) tSNP6: rs11539284.

Genotyping of these six variants in a population of northwest European descent will not only directly examine the -786T>C and Glu298Asp variants but also allow assessment of all common variation across the NOS3 gene, with minimal loss of power compared with genotyping all variants directly. Haplotypes generated by these tSNPs represent the common haplotypes in populations of northwest European origin (table 1).

### FUNCTION

**Glu298Asp**

Some mechanistic studies have been published suggesting a functional effect of the Glu298Asp polymorphism. Associations have been described between the Glu298Asp polymorphism and nitric oxide synthesis (27, 28) and endothelial function (29, 30). A mechanism by which eNOS Asp298 might reduce nitric oxide bioavailability has also been reported (27, 28). In terms of enzymatic activity, studies of recombinant eNOS Asp298 and Glu298 showed no discernible difference in the Michaelis constant $K_m$, nor the $V_{max}$, of the two forms of the enzyme (3, 31, 32). Moreover, there was no difference in $K_i$ for the endogenous methylarginine inhibitors of eNOS, namely, asymmetric dimethylarginine and NG monomethyl-L-arginine (3). The Glu298Asp polymorphism lies within a loop on the external surface of the structure and does not make contact with either the active site of the enzyme or the dimerization interface (3), suggesting that, if functional, the polymorphism would have to exert its effect by a mechanism independent of nitric oxide synthase catalysis. Two studies have recently shown the eNOS protein containing Asp at position 298 to be subject to selective proteolytic cleavage in endothelial cells and vascular tissues (27, 28). If this observation is correct, the cleaved fragments would be expected to lack nitric oxide synthase activity (29, 30). However, two other reports suggest that this observation might occur in different cellular environments.

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**TABLE 1. Endothelial nitric oxide synthase gene haplotypes inferred from six tagging single nucleotide polymorphisms** in a northwest European population, based on the University of Washington Variation Discovery Resource data†

<table>
<thead>
<tr>
<th>rs2070744</th>
<th>rs3918167</th>
<th>rs1799983</th>
<th>rs3918184</th>
<th>rs743506</th>
<th>rs11539284</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>0.26</td>
</tr>
<tr>
<td>C</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>A</td>
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<tr>
<td>T</td>
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<td>C</td>
<td>A</td>
<td>G</td>
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<td>T</td>
<td>A</td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>0.11</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>0.06</td>
</tr>
<tr>
<td>T</td>
<td>A</td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>G</td>
<td>0.05</td>
</tr>
<tr>
<td>T</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>0.05</td>
</tr>
<tr>
<td>T</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>0.05</td>
</tr>
<tr>
<td>T</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>0.04</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>0.04</td>
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<td>T</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>0.03</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>G</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*Polymorphism rs2070744 corresponds to the -786T>C variant, and rs1799983 corresponds to the Glu298Asp variant.
be an artifact (32, 33), so further in vitro work is needed to resolve this issue.

Human studies suggest that individuals homozygous for Asp298 experience a reduced blood pressure fall following exercise training (34) and lower basal blood flow and reduced vasodilation to adenosine in their coronary arteries (35). In addition, in some but not all studies (29, 37, 38), subjects homozygous for the Asp298 allele have an enhanced systemic pressor response to phenylephrine (36) and a reduced flow mediated dilatation of the brachial artery. These observations require confirmation in larger studies.

**-786T>C**

Given the location of -786T>C in the promoter region of NOS3, studies examining the functionality of this polymorphism have focused on eNOS expression levels. Lower eNOS mRNA and serum nitrite/nitrate levels have been found in individuals with the -786C variant (39). Reporter gene assays support such a role (40). Recently, a nuclear protein that exhibits differential binding to the promoter containing the -786T and -786C alleles has also been described (41). Human studies suggest that subjects homozygous for the -786C allele have a decreased maximal forearm blood flow response to acetylcholine, a pharmacologic tool to evaluate nitric oxide production in vivo (42). However, these associations have yet to be reproduced in other functional and population-based studies (22, 43).

**Intron 4**

Given the intronic location of the intron 4 repeat unit, it is perhaps less likely to be functional. Conflicting associations between the intron 4 variant and nitric oxide pathway activity have been described. Some reports indicate that carriers of this variant have lower nitric oxide plasma levels and decreased protein expression (44, 45), but this finding is not supported by all studies (22, 46). It is possible that the variant is in linkage disequilibrium with other functional variants in regulatory regions of the NOS3 gene.

**DISEASES (OUTCOMES)**

Information about the epidemiology of CHD, stroke, hypertension, and preeclampsia (47–61) can be found in the online Appendix.

**GENE-DISEASE ASSOCIATIONS**

**CHD**

For genetic association studies evaluating the role of the Glu298Asp, -786T>C, or intron 4 polymorphisms of the NOS3 gene in CHD, we conducted an updated meta-analysis of studies in all languages published until February 2006. The search, selection criteria, data abstraction, and statistical methods are described in the online Appendix Methods section (62, 63). Briefly, our principal hypothesis was that an additive (per-allele) model for NOS3 Asp298, -786T>C, or intron 4 a variants would be associated with an increased risk of CHD. Secondary analyses involved recessive, dominant models and pairwise comparisons of the genotype groups generated. For all models used, the minor allele was considered the risk allele.

Of the 71 studies (69 articles) identified (12, 15, 22, 64–128), 64 (62 articles) were included in this updated meta-analysis (12, 15, 22, 64–121). Information on study design, genotype frequencies, patient characteristics, and outcomes description of studies included in the meta-analyses is outlined in Web appendix tables 1, 2, and 3. Four studies were excluded because duplication or partial overlapping was considered likely after contacting the study author (122–125), and three were omitted because relevant data were not reported and could not be obtained from study authors (126–128).

**Glu298Asp polymorphism.** The meta-analysis of the Glu298Asp polymorphism included 42 studies (40 articles) comprising 13,876 CHD cases and 13,042 controls (12, 22, 64–76, 86–89, 91–94, 96–99, 102, 103, 106–108, 111–116, 118–121). The odds ratio under an additive model for CHD was 1.17 (95 percent confidence interval (CI): 1.07, 1.28; p = 0.001; Web appendix figure 2). However, there was evidence of substantial between-study heterogeneity (I² = 67.9 percent, χ² = 124.74, phet < 0.0001). Study characteristics such as excluding participants with hypertension (129) and preeclampsia (130, 131) were associated with a diminished effect size.

**Intron 4 polymorphism.** Thirty-one studies (31 articles: 9,925 cases and 9,407 controls) evaluated the associa-
tion between the intron 4 polymorphism and CHD were included in the meta-analysis (15, 22, 64, 65, 68, 72, 74–83, 92, 94–96, 98, 100, 101, 104–106, 108–110, 113, 114). The per-allele odds ratio for the intron 4 variant was 1.12 (95 percent CI: 1.01, 1.24; p = 0.02; Web appendix figure 4). There was evidence of heterogeneity between studies (I² = 55.4 percent, χ² = 67.24, phet ≤ 0.0001); however, with the exception of outcome under evaluation for myocardial infarction, coronary stenosis, or combined outcome (χ² = 14.12, phet = 0.001), other study characteristics such as ethnicity, blinding of genotyping, study size, language of publication, and Hardy-Weinberg equilibrium did not explain much of the total heterogeneity (figure 2). Although the Egger’s and Begg’s tests suggested no evidence of publication bias (p = 0.16 and p = 0.34, respectively), the funnel plot suggested it and was due to the presence of an excess of small, positive studies conducted in Asians (Web appendix figure 5). The estimates of the effect for other genetic models of inheritance are outlined in table 2.

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-786T>C polymorphism. Twenty-two studies (20 articles) that evaluated the association between the -786T>C polymorphism in the gene promoter and CHD were included in the meta-analysis (11,236 cases and 13,562 controls) (13, 22, 65, 66, 73, 79, 84–86, 89–92, 94, 107, 111, 113, 114, 119). The per-allele odds ratio of CHD for the -786T>C variant was 1.17 (95 percent CI: 1.07, 1.28; \( p = 0.001 \); Web appendix figure 6). Substantial interstudy heterogeneity was observed (\( I^2 = 62.7 \) percent, \( \chi^2_2 = 56.36, \ p_{\text{Het}} < 0.0001 \)). From the study characteristics evaluated, the number of cases per study was the only variable that partially explained some of the observed heterogeneity (\( \chi^2_2 = 16.54, \ p_{\text{Het}} < 0.0001 \); figure 3). The funnel plot suggested evidence of a few more positive results in the smaller studies (Egger’s test = 0.052 and Begg’s test = 0.01; Web appendix figure 7). The genotypic odds ratios for other genetic models of inheritance are presented in table 2.

Summary of the association between CHD and the NOS3 gene. In these updated meta-analyses, several strategies were used to obtain unpublished data to minimize reporting and publication bias. An additional 28 studies and 7,840 cases for Glu298Asp, 15 studies and 3,713 cases for intron 4, and 14 studies and 8,859 cases for -786T>C were included in comparison with our previous report (17). Contrary to previous findings, in this updated meta-analysis, we observed statistical evidence of small-study bias in studies of the Glu298Asp and -786T>C polymorphisms. A stratified analysis of the associations of these two gene variants with CHD according to the number of cases supported these statistical findings and also suggested the presence of small-study bias for the intron 4 variant (figures 2 and 3). Interestingly, for Glu298Asp and intron 4, substantial heterogeneity was observed even within the group of studies with more than 500 cases (Glu298Asp: \( I^2 = 61.4 \) percent; intron 4: \( I^2 = 68.9 \) percent). In this updated meta-analysis, the -786T>C polymorphism was associated with an increased risk of CHD. For all three polymorphism-CHD associations, we observed substantial heterogeneity not explained by any of the study characteristics evaluated (figures 1, 2, and 3). Despite previous claims of a differential effect of ethnicity on gene-disease associations in complex diseases, in these meta-analyses, the mean estimate of the effects was highly consistent among the ethnic groups evaluated, a finding consistent with other recent results (129).

A cumulative synthesis of NOS3 polymorphisms and CHD revealed that, for Glu298Asp and intron 4, the initial positive

<table>
<thead>
<tr>
<th>Group of studies</th>
<th>Cases/controls</th>
<th>Odds ratio (95% CI)</th>
<th>( I^2 ) (%)</th>
<th>Chi-square test of heterogeneity (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study size</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;200 cases: 16 studies</td>
<td>1,773 / 4,467</td>
<td>1.42 (1.15, 1.76)</td>
<td>65.1</td>
<td>25.72 on 2 df (0.0001)</td>
</tr>
<tr>
<td>200 – 499 cases: 16 studies</td>
<td>4,782 / 5,129</td>
<td>1.18 (1.05, 1.32)</td>
<td>57.6</td>
<td></td>
</tr>
<tr>
<td>≥ 500 cases: 9 studies</td>
<td>7,321 / 3,446</td>
<td>0.97 (0.87, 1.09)</td>
<td>61.4</td>
<td></td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary stenosis: 19 studies</td>
<td>8,523 / 4,120</td>
<td>1.22 (1.06, 1.39)</td>
<td>75.6</td>
<td>4.87 on 2 df (0.08)</td>
</tr>
<tr>
<td>Myocardial infarction: 19 studies</td>
<td>4,668 / 7,885</td>
<td>1.09 (0.97, 1.23)</td>
<td>57.4</td>
<td></td>
</tr>
<tr>
<td>Combined: 3 studies</td>
<td>685 / 1,037</td>
<td>1.54 (0.95, 2.49)</td>
<td>47.6</td>
<td></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
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</tr>
<tr>
<td>Caucasian: 25 studies</td>
<td>10,377 / 9,535</td>
<td>1.13 (1.02, 1.25)</td>
<td>73.4</td>
<td></td>
</tr>
<tr>
<td>Asian: 13 studies</td>
<td>3,068 / 3,186</td>
<td>1.25 (1.02, 1.54)</td>
<td>55.7</td>
<td>4.77 on 2 df (0.09)</td>
</tr>
<tr>
<td>Other: 3 studies</td>
<td>431 / 321</td>
<td>1.36 (1.04, 1.79)</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td><strong>Blinding of genotyping staff</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blinded: 24 studies</td>
<td>9,413 / 9,192</td>
<td>1.15 (1.02, 1.28)</td>
<td>74.4</td>
<td>0.28 on 2 df (0.87)</td>
</tr>
<tr>
<td>Not blinded: 7 studies</td>
<td>2,231 / 1,603</td>
<td>1.21 (1.00, 1.46)</td>
<td>52.1</td>
<td></td>
</tr>
<tr>
<td>Unknown: 10 studies</td>
<td>2,232 / 2,247</td>
<td>1.22 (0.96, 1.55)</td>
<td>59.0</td>
<td></td>
</tr>
<tr>
<td><strong>Language of publication</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>English-language: 34 studies</td>
<td>12,872 / 12,248</td>
<td>1.14 (1.04, 1.24)</td>
<td>69.3</td>
<td>4.52 on 1 df (0.03)</td>
</tr>
<tr>
<td>Non-English-language: 7 studies</td>
<td>1,004 / 794</td>
<td>1.52 (1.09, 2.11)</td>
<td>53.1</td>
<td></td>
</tr>
<tr>
<td><strong>Hardy-Weinberg equilibrium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conforming HWE: 38 studies</td>
<td>12,015 / 11,918</td>
<td>1.19 (1.08, 1.31)</td>
<td>70.0</td>
<td>1.27 on 1 df (0.26)</td>
</tr>
<tr>
<td>Violating HWE: 3 studies</td>
<td>1,504 / 362</td>
<td>1.00 (0.84, 1.18)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Overall odds ratio</strong></td>
<td></td>
<td>1.17 (1.07, 1.28)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 1. Studies of the Glu298Asp polymorphism and risk of coronary heart disease grouped by study characteristics. The sizes of the boxes relate to the inverse of the variance and thus to study size. CI, confidence interval; HWE, Hardy-Weinberg equilibrium.
associations gradually attenuated over time and became more stable, as data accrued. However, for the -786T>C-CHD association, some degree of instability over time was observed (Web appendix figures 8, 9, and 10). Although other possible sources of bias (e.g., survival, classification, and selection) and confounding (cardiovascular risk factors) are possible, they are unlikely to be present (130). However, to confirm or refute a role of the NOS3 gene in CHD, future large-scale studies using a map-based “candidate gene” approach by genotyping tSNPs are warranted.

**High blood pressure**

Two linkage studies, in Caucasians, analyzed the sharing of alleles at the highly polymorphic dinucleotide repeat element in intron 13 of the NOS3 gene among hypertensive sibling pairs. Both studies failed to detect an excess of allele sharing that would implicate this region regarding susceptibility to hypertension (131, 132). Findings from an additional association substudy (using SNPs in introns 18 and 23) were also negative (131). Larger genome-wide linkage studies examining Glu298Asp, -786T>C, or intron 4 polymorphisms have yielded conflicting conclusions. As of January 2004, 18 studies (15 articles) were detected (42, 119–132). For details of the studies included, refer to Web appendix table 6.

The Glu298Asp polymorphism was the variant evaluated most frequently, with 12 studies (10 articles; 3,950 cases and 5,538 controls) (42, 135–143). Overall, in three of 12 studies, positive associations were reported (Shoji et al. (142): OR = 1.8, 95 percent CI: 1.1, 3.0; Miyamoto et al. (Kyoto) (140): OR = 2.3, 95 percent CI: 1.4, 3.9; and Miyamoto et al. (Kumamoto) (140): OR = 2.4, 95 percent CI: 1.4, 4.0) under a dominant model of inheritance. However, conflicting results have been reported, even in a larger study whose subjects were of the same ethnic background (143). The second most described NOS3 variant was -786T>C (four studies; 2,183 cases and 3,671 controls) (42, 143–145). Only one study in Caucasians (144) has been associated with an increased risk of hypertension (adjusted OR = 2.16, 95 percent CI: 1.3, 3.7) for individuals homozygous for the -786C allele compared with TT-genotype subjects; however, in the much larger, community-based case-control study in Japanese individuals by Tsujita et al. (143), a null association was found. Likewise, for the intron 4 polymorphism, conflicting results have been reported in six individually underpowered studies (total of 960 cases and 1,301 controls) published until January 2004 (136, 140, 142, 146–148). Subsequently, a large, population-based study in Caucasians found that the Glu298Asp polymorphism was not associated with either prevalent hypertension or difference in systolic or diastolic blood pressure by genotype (149).

In addition to the well-recognized difficulties in reliably identifying the small expected genotypic effect in complex disorders, there are additional difficulties in assessing blood pressure by genotype: 2006;164:921–935

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**TABLE 2. Genotypic odds ratios for coronary heart disease for the endothelial nitric oxide synthase gene Glu298Asp,† intron 4, and -786T>C polymorphisms**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Glu298Asp</th>
<th>Intron 4</th>
<th>-786T&gt;C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio</td>
<td>95% CI§</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>Additive model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random</td>
<td>1.17</td>
<td>1.07, 1.28</td>
<td>1.12</td>
</tr>
<tr>
<td>$\hat{\phi}$ (p for heterogeneity)</td>
<td>67.9% (&lt;0.0001)</td>
<td>55.4% (&lt;0.0001)</td>
<td>62.7% (&lt;0.0001)</td>
</tr>
<tr>
<td>Homozygous for rare allele vs. homozygous for common allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random</td>
<td>1.36</td>
<td>1.12, 1.66</td>
<td>1.24</td>
</tr>
<tr>
<td>$\hat{\phi}$ (p for heterogeneity)</td>
<td>60.1% (&lt;0.0001)</td>
<td>21.5% (0.148)</td>
<td>52.6% (0.003)</td>
</tr>
<tr>
<td>Heterozygous vs. homozygous for common allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random</td>
<td>1.07</td>
<td>0.98, 1.17</td>
<td>1.10</td>
</tr>
<tr>
<td>$\hat{\phi}$ (p for heterogeneity)</td>
<td>46.4% (0.001)</td>
<td>53.3% (&lt;0.0001)</td>
<td>54.2% (0.002)</td>
</tr>
<tr>
<td>Recessive model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random</td>
<td>1.34</td>
<td>1.11, 1.62</td>
<td>1.20</td>
</tr>
<tr>
<td>$\hat{\phi}$ (p for heterogeneity)</td>
<td>58% (&lt;0.0001)</td>
<td>14.8% (0.238)</td>
<td>40.2% (0.03)</td>
</tr>
<tr>
<td>Dominant model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random</td>
<td>1.15</td>
<td>1.04, 1.27</td>
<td>1.12</td>
</tr>
<tr>
<td>$\hat{\phi}$ (p for heterogeneity)</td>
<td>59.5% (&lt;0.0001)</td>
<td>55.8% (&lt;0.0001)</td>
<td>60.2% (&lt;0.0001)</td>
</tr>
</tbody>
</table>

* The rare allele is Asp and the common is Glu.
† The rare allele is a and the common is b.
‡ The rare allele is C and the common is T.
§ CI, confidence interval.
pressure or hypertension as outcomes. Most of the previous studies used certain threshold blood pressures (e.g., ≥140 mmHg systolic or ≥90 mmHg diastolic) to define hypertension, assuming the presence of a clear cutoff point in the blood pressure-CHD association. Nevertheless, it has become clear that the risk associated with blood pressure is continuous and graded over the whole range of usually encountered blood pressure levels (150), even in those individuals considered normotensive. Use of blood pressure as a continuous outcome together with a gene-based approach using tSNPs may therefore be a more informative approach for assessing the effect of the \( \text{NOS3} \) variation on blood pressure.

**Preeclampsia**

It has been proposed that enhanced synthesis of nitric oxide is responsible in part for the adaptive change in maternal hemodynamics status observed in normal pregnancy (151). Hypertensive disorders of pregnancy are characterized by an inappropriately high vascular resistance that might arise from reduced nitric oxide bioavailability (152, 153). Preeclampsia has a familial component, and women with disorders associated with endothelial dysfunction (e.g., hypertension, diabetes) are at greater risk of developing preeclampsia (154). Several authors have examined the role of the \( \text{NOS3} \) gene in the pathogenesis of preeclampsia. In a recent meta-analysis of genetic association studies (12 studies; 1,334 cases and 2,894 controls) published up to November 2005, including a new case-control study (155), we did not detect a significant increase in the risk of preeclampsia associated with the \( \text{Glu298Asp} \) polymorphism under an additive model (OR = 1.03, 95 percent CI: 0.79, 1.34). Similar results were observed for a recessive model (OR = 1.28, 95 percent CI: 0.76, 2.16) and a dominant model (OR = 1.12, 95 percent CI: 0.84, 1.49) (155). Regarding other \( \text{NOS3} \) polymorphisms (intron 4 and -786T>C), only a few case-control studies in different ethnic groups have evaluated the role of these polymorphisms (19, 156–158), and no increase in preeclampsia risk was observed (19).

**Stroke**

In a comprehensive meta-analysis of studies of all candidate genes for ischemic stroke in Caucasians published to January 2003, individuals homozygous for the Asp298 allele (three studies; 1,086 cases and 1,089 controls), in

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**FIGURE 2.** Studies of the intron 4 a/b variant and risk of coronary heart disease grouped by study characteristics. The sizes of the boxes relate to the inverse of the variance and thus to study size. CI, confidence interval; HWE, Hardy-Weinberg equilibrium.
comparison with Glu298 carriers, did not have an increased risk of ischemic stroke (OR = 0.98, 95 percent CI: 0.76, 1.26) (159). Since that analysis, two additional studies among Caucasians have been published (160, 161); in both, no significant associations between the Glu298Asp genotypes and stroke were observed. In one of the studies (161), only those subjects with cerebral small-vessel disease were evaluated, and, despite null associations for the NOS3 Glu298Asp, -786T>C, and intron 4 polymorphisms overall, for a subgroup of patients with lacunar infarction, a protective effect for the a allele of intron 4 was reported. However, the possibility of a false-positive result because of multiple comparisons has to be considered. In the non-Caucasian population, the studies have mainly focused on the role of the intron 4 polymorphism and have yielded contradictory results. In the Chinese population, Hou et al. (162) (364 cases and 516 controls) observed an increased risk of ischemic stroke (OR = 2.13, 95 percent CI: 1.98, 4.80) for carriers of the a allele after adjusting for potential confounders. On the other hand, two other studies, one in Japanese and the other mainly in Caucasians (240 cases and 1,604 controls), found no increase in risk of stroke for carriers of the a allele (163, 164). In Afro-Americans, a recent, small case-control study (110 cases and 206 controls) of young women reported an increased risk of stroke for the -786T>C variant (OR = 2.9, 95 percent CI: 1.3, 6.4) (165).

Detailed analysis of studies reporting carotid stenosis as an outcome (87, 140, 166–172) can be found in the online Appendix. In addition, detailed analysis of studies reporting coronary spasm (40, 173–178), restenosis (179–182), diabetes mellitus (166, 183–185), renal disease (28, 186–192), and rheumatologic disorders (193–195) can also be found there.

**GENE-ENVIRONMENT INTERACTIONS**

Several interactions between the NOS3 polymorphisms and environmental factors have been proposed. Smoking has been the main focus of attention, particularly in studies of the intron 4 and -786T>C polymorphisms in Asian populations. Wang et al. (15) reported that smoking was an effect modifier of the intron 4–coronary stenosis association. Likewise, Nasreen et al. (196) found that, among smokers, homozygosity for the -786C allele was associated with a lower cerebral blood flow as well as higher cerebral vascular resistance in comparison with -786T allele carriers. Flow-mediated dilation was compared between healthy individuals in relation to the NOS3 Glu298Asp polymorphism by Leeson et al. (30), and interactions with a proatherogenic...
risk factor (smoking) and an antiatherogenic factor (n-3 fatty acids) were investigated. Flow-mediated dilation was not related to genotype in the group as a whole or within sexes. However, among men, smoking was associated with lower flow-mediated dilation in Asp298 carriers but not in Glu298 homozygotes. In the whole group, n-3 fatty acid levels were positively related to flow-mediated dilation in Asp298 carriers but not in Glu298 homozygotes. Thus, the Glu298Asp polymorphism may be associated with differences in the response of the endothelium to both smoking and n-3 fatty acid status. These early findings suggestive of gene-environmental interactions with the different NOS3 polymorphisms may be associated with differences in the re-

erase (197, 198).

LABORATORY TESTS

All genetic association studies described in this review used genomic DNA extracted from blood. All involve amplification of genomic sequences containing the polymorphic sites. For determining the NOS3 Glu298Asp and -786T>C polymorphisms, the most frequently used method has been polymerase chain reaction and restriction fragment length polymorphism analysis (3). Other techniques have been a fluorescence or colorimetry-based allele-specific DNA-probe assay system (TaqMan; Applied Biosystems, Foster City, California) (12, 107). For the intron 4 polymorphism, the procedure used has been polymerase chain reaction. In most of the studies, quality control by DNA sequencing has been carried out in a small, random sample of the subjects genotyped in each study. Alternative methods for genotyping using melting curve analysis, in which the distinction of the different genotypes (wild type, mutant, and heterozygous) can be ascertained graphically by differences in their respective melting temperatures, have also been used (42).

POPULATION TESTING

We could not identify any published data on population testing of the NOS3 polymorphisms described in this review in relation to any of the associations described so far. The odds ratios for the Asp298 and 786C alleles are consistent with the genetic contribution to CHD being 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 persons (17). However, whether combined genotype analysis integrated with orthodox assessment of cardiovascular risk will enhance the prediction of CHD warrants further exploration (197, 198).

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

A considerable amount of evidence has accumulated evaluating the role of the NOS3 gene not only in cardiovascular disease but also in other complex disorders. An obstacle to evaluating, at the human level, the effects of the different NOS3 polymorphisms has been the lack of a reliable marker of the NOS3 gene function, partly due to the technical complexity in measuring nitric oxide production in humans. However, other than for CHD, no disease associations have been extensively described so far. Even for the positive associations with CHD, the likelihood of publication bias is substantial and cannot be excluded as a possible explanation. The complexity of the results observed for the association of each polymorphism with CHD, together with the absence of a definitive functional gene variant, strongly suggests that future studies evaluating the effect of the NOS3 gene on cardiovascular disease should use a “gene-based” approach. It seems unlikely that studies evaluating isolated NOS3 gene variants will produce a real advance in the understanding of the NOS3 gene and the role of nitric oxide. In addition, further and larger genetic association studies are needed in ethnic minorities with different allele/genotype frequencies of the NOS3 variants and different patterns of cardiovascular disease.

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A. D. H. is a co-inventor on a patent held by UCL for the use of ADMA (an inhibitor of nitric oxide synthase) as a diagnostic test for preeclampsia.

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