Genetic and Environmental Determinants of Plasma Nitrogen Oxides and Risk of Ischemic Heart Disease

Noor Jeerooburkhan, Lisa C. Jones, Sarah Bujac, Jacqueleine A. Cooper, George J. Miller, Patrick Vallance, Steve E. Humphries, Aroon D. Hingorani

Abstract—Endothelial dysfunction, caused in part by reduced NO bioavailability, is a feature of hypercholesterolemia, hypertension, smoking, and atherosclerosis. We examined whether cholesterol, blood pressure, smoking status, and polymorphisms in the endothelial NO synthase gene (NOS 3) influence NO production (as assessed by the plasma levels of nitrogen oxides, NOx) in middle-aged men. We also determined whether plasma NOx or NOS 3 genotype predicted the risk of ischemic heart disease (IHD). We studied 3052 men who were initially free of IHD and recruited from 9 UK primary care practices. Blood pressure, age, body mass index, serum cholesterol, and smoking status were assessed at baseline and annually over 8.1 years of follow-up, and all IHD events were recorded. DNA samples were screened for 4 NOS 3 gene polymorphisms: −786 T/C, −922 A/G, 894 G/T (which predicts a Glu298→Asp amino acid substitution in the mature protein), and a 27-bp tandem repeat in intron 4 (eNOS4a/4b). NOx was measured in plasma samples obtained on entry in 1121 participants from North Mymms and Chesterfield general practices, together with an additional 571 recruits selected at random. Genotype frequencies were in Hardy-Weinberg equilibrium, and linkage disequilibrium was detected between all the NOS 3 polymorphisms studied, with the strongest allelic association being detected between −922 A/G and −786 T/C polymorphisms in the gene promoter (Δ=0.90, P<0.001). Plasma NOx was lower in smokers than in nonsmokers in the North Mymms (10.8±4.5 versus 11.8±4.6 μmol/L, P=0.13), Chesterfield (8.4±3.6 versus 9.9±4.0 μmol/L, P=0.01), and random samples (10.7±5.1 versus 11.7±4.7 μmol/L, P=0.03). A weak but significant inverse relationship was detected between plasma NOx and serum cholesterol only in the North Mymms data set (r=−0.14, P=0.02). No relationship was detected between plasma NOx and any of the NOS 3 polymorphisms, nor was there any association between any NOS 3 polymorphism and risk of an IHD event in either smokers or nonsmokers. These data support the hypothesis that the endothelial dysfunction observed in the blood vessels of smokers is related to reduced NO bioactivity but indicate that NOS 3 genotype does not influence significantly the level of plasma NOx or the risk of IHD in this population sample of middle-aged British men. (Hypertension. 2001;38:1054-1061.)

Key Words: endothelium ■ nitric oxide ■ polymorphism ■ endothelial dysfunction ■ smoking

Nitric oxide is synthesized from l-arginine through the catalytic activity of the NO synthases (NOS).1 In the absence of inflammation, the constitutively expressed NOS isoforms, endothelial (eNOS) and neuronal, are likely to be the major contributors to whole-body NO production. In the vasculature, NO exerts a vasodilator influence,2 regulates regional blood flow and systemic blood pressure (BP),3 and confers thromboreisistant and atheroprotective properties to the endothelium.4 A reduction in its synthesis or availability might underlie impaired endothelium-dependent vasodilation observed in blood vessels of individuals with cardiovascular risk factors, including active and passive smokers5,6 and patients with hypertension7 or hypercholesterolemia.8 Furthermore, loss of NO-mediated effects may predispose to the development of atherosclerosis.9 Because of the short half-life of free NO,10 the result of its rapid oxidative breakdown to nitrite (NO2−) and nitrate (NO3−),11 it has not been possible to measure directly NO generation in disease states. However, the plasma level of NO2−+NO3− (NOx) has been used to provide an in vivo index of NO synthesis,11 and small studies have suggested changes with cardiovascular risk factors.

Although many environmental factors and disease states may alter NOS activity, genetic factors may also play a part. Polymorphisms have been identified in the gene (NOS 3) that encodes eNOS.12,13 Some have been associated with an increased risk of cardiovascular disorders,12,14–17 but the data are not conclusive.13,18,19 Furthermore, data are lacking or controversial as to whether individual NOS 3 polymorphisms influence NO generation in vivo. To address these issues and to explore both environmental and genetic influences on...
plasma NO\textsubscript{x} levels, we used a large-scale, population-based, prospective cohort study (with a nested case-control analysis) to test the hypothesis that \textit{NOS} 3 polymorphisms and ortho- and cardiovascular risk factors reduce the levels of plasma NO\textsubscript{x} in vivo and, in so doing, determine the risk of a future ischemic heart disease (IHD) event.

\textbf{Methods}

We recruited 3052 men who were 50 to 61 years of age and free of IHD to the second Northwick Park Heart Study (NPHS II) from 9 primary care practices in the United Kingdom.\textsuperscript{20} Subjects were asked not to smoke from the midnight before entry and to avoid heavy meals until the entry examination. BP and a variety of lipid and other measures were recorded at baseline and on annual visits over 9 years of follow-up. All IHD events, defined by World Health Organization criteria\textsuperscript{21} as fatal, nonfatal, and silent myocardial infarction (MI) and coronary revascularization, were ascertained from practice records, hospital attendances, and, for fatal events, coroners’ offices. To date, 159 men have developed events over a median follow-up of 8.1 years.

Plasma NO\textsubscript{x} was measured by a modified Griess reaction wherein plasma NO\textsubscript{x} is first converted to NO\textsubscript{3} by nitrate reductase and the NO\textsubscript{3} generated is quantified in terms of the diazonium product generated is quantified in terms of the diazonium product -(1-naphthalenyl)imidazo[4,5-b]pyridine.\textsuperscript{22} The assay was linear in the physiological range and precise, with an intra-assay coefficient of variation of 3.4% and interassay coefficient of variation of 4.0%. NO\textsubscript{x} measurements were made in 1121 men: 290 and 260 from the North Mymms and Chesterfield practices, respectively, and an additional 571 measures were made in 1121 men: 290 and 260 from the North Mymms and Chesterfield practices, respectively, and an additional 571 individuals selected by random sampling from the whole data set.

Genomic DNA was extracted from 2743 samples by the salting-out method. Polymerase chain reaction–restriction fragment length polymorphism was used for genotyping \textit{3 NOS} 3 polymorphisms, \(-922 A/G, -786 T/C\) and another sample of 532 individuals obtained at random from the North Mymms and Chesterfield practices, respectively, and an additional 571 individuals selected by random sampling from the whole data set.

Genomic DNA was extracted from 2743 samples by the salting-out method. Polymerase chain reaction–restriction fragment length polymorphism was used for genotyping \textit{3 NOS} 3 polymorphisms, \(-922 A/G, -786 T/C\), and the 894 G/T variant in exon 7, the only coding region polymorphism that predicts a Glu298Asp amino acid substitution in the mature protein.\textsuperscript{23} Primer pairs and allele-specific restriction endonucleases are shown in Table 1. The intron 4 VNTR (variable number tandem repeat) was genotyped with the method of Wang et al.\textsuperscript{22} An independent observer confirmed all genotypes, with discrepancies resolved by repeated polymerase chain reaction and enzyme digestion.

Statistical analyses were conducted with Intercooled Stata 5.0 software. Tests for Hardy-Weinberg equilibrium were performed by \(\chi^2\) analysis. Linkage disequilibrium coefficients between polymorphisms were estimated by log-linear analysis.\textsuperscript{24} Non–normally distributed variables were logarithmically transformed before analysis, with geometric means and approximate SDs reported. ANOVA was used to analyze continuous variables by genotype. Because of the heterogeneity of NO\textsubscript{x} values between practices, adjustment was made when appropriate. To assess the effect of genotype on IHD risk, survival analysis was performed with Cox’s proportional-hazards method. Statistical significance was taken to be \(P<0.05\).

We estimated that 50 homozygotes per group would be required to detect a 10% difference in group means for log NO\textsubscript{x} and 35 to 40 individuals to detect a similar difference in diastolic and systolic BPs with 90% power at \(P=0.05\). We also estimated that with 200 IHD events and at least a similar number of controls, we would have 90% power to detect a 2-fold excess risk of IHD among rare allele homozygotes assuming a recessive model and 90% power to detect a 1.5-fold excess risk among rare allele carriers assuming a dominant model at \(P=0.05\).

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

\textbf{Results}

\textbf{Demographic Characteristics of Study Participants}

Of the 4009 men invited for screening, 3052 were recruited; the remainder were excluded for the following reasons: refusal of venepuncture; evidence of previous MI on baseline ECG; missing results because of loss of blood samples during storage, transport, or laboratory processing; or unclear labeling of samples. A total of 159 IHD events were recorded over 8.1 years of follow-up among 2965 men for whom full follow-up data were available. The baseline characteristics of subjects who suffered an IHD event and those who did not are shown in Table 2.

\textbf{Associations Between Cardiovascular Risk Factors and Plasma NO\textsubscript{x}}

\textit{Smoking Status and Plasma NO\textsubscript{x}}

NO\textsubscript{x} levels were 8.5% lower in current smokers compared with nonsmokers in the North Mymms recruits (\(P=0.13\) and 17.5% lower in the Chesterfield subset (\(P=0.01\); Table 3). In another sample of 532 individuals obtained at random from the NPHS II cohort, plasma NO\textsubscript{x} was 8.5% lower among current smokers (\(P=0.03\)). Overall, plasma NO\textsubscript{x} was 10.6±4.8 \textmu mol/L in smokers and 11.7±4.7 \textmu mol/L in nonsmokers (\(P=0.0005\)).

\textit{Cholesterol and Plasma NO\textsubscript{x}}

There was a weak but significant inverse relationship between serum total cholesterol and plasma NO\textsubscript{x} in the North Mymms data set (\(r=-0.14, P=0.02\)) but not in the Chesterfield recruits (\(r=-0.08, P=0.21\)) or random sample (\(r=0.02, P=0.64\)). These correlations were unaffected by adjustment for serum creatinine.

\textit{BP and Plasma NO\textsubscript{x}}

No significant correlations were detected between systolic or diastolic BP and plasma NO\textsubscript{x} in any of the groups studied. Correlation coefficients (\(r\) for systolic BP were −0.03, 0.12, and −0.02 (\(P=NS\)) for the North Mymms, Chesterfield, and

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Variant} & \textbf{Primer Pairs Sense/Antisense} & \textbf{Mg\textsuperscript{2+}, mmol/L} & \textbf{\(T_m\), °C} & \textbf{Restriction Enzyme} & \textbf{Gel, %} & \textbf{Products, bp} \\
\hline
\(-922 A/G\) & 5\textsuperscript{'}-CAGCAGTGGGCTTTCCTC-3\textsuperscript{'} & 2.0 & 60 & \textit{Bst} & 7.5 & 28 110 \\
\(-922 A/G\) & 5\textsuperscript{'}-AGACTGATGGCCCTGGT-3\textsuperscript{'} & 1.5 & 61 & \textit{NgoMl} & 10 & 51 143 \\
\(-786 T/C\) & 5\textsuperscript{'}-AGTTCCTGATTGGCACCAGT-3\textsuperscript{'} & 1.2 & 56 & \ldots & 10 & 393 420 \\
\textit{Intron 4} & 5\textsuperscript{'}-CCACACCCTGACTGACTG-3\textsuperscript{'} & 2.0 & 63 & \textit{Dpn II} & 10 & 51 100 \\
\textit{VNTR} & 5\textsuperscript{'}-TCTTTCGTGGCCTGGTCAC-3\textsuperscript{'} & & & & & \\
\textit{Glu298Asp} & 5\textsuperscript{'}-CCCTCCATCCACCCAGATCAAC-3\textsuperscript{'} & & & & & \\
\textit{VNTR} & 5\textsuperscript{'}-AGGGAAGGTCTCCTGCACATGTCG-3\textsuperscript{'} & & & & & \\
\hline
\end{tabular}
\caption{Primer Pairs and PCR/MADGE Conditions for \textit{NOS} 3 Polymorphisms}
\end{table}
random data sets, respectively. Corresponding values for diastolic BP were −0.07, 0.01, and −0.04 (P=NS).

Allele Frequencies of NOS 3 Polymorphisms and Linkage Disequilibrium
A total of 2792 participants were genotyped for the −922 A/G polymorphism, 2720 for the −786 T/C polymorphism, 2710 for the intron 4 VNTR, and 2584 for the Glu298Asp variant (Table 4). The genotype frequencies were as predicted by Hardy-Weinberg equilibrium (P>0.05 for all analyses).

Pairwise linkage disequilibrium coefficients (Δ) were calculated for the 4 polymorphisms studied, all of which are located at the 5′ end of the NOS 3 gene (Figure 1). All comparisons achieved statistical significance (P<0.001). Allelic association was greatest between the 2 polymorphisms located in the promoter (Δ=0.90), and associations between polymorphisms in the gene promoter and the exon 7 polymorphism were present but weaker (Δ=0.4). The value for the exon 7–intron 4 association was only Δ =−0.27 despite the physical proximity of these 2 loci. Of interest, however, 34 of 37 individuals (92%) homozygous for the rare intron 4 variant that has previously been associated with IHD and MI12,25 were also homozygous for the polymorphisms at positions −786 and −922 in the gene promoter, raising the possibility that the intron 4 variant might act as a marker in linkage disequilibrium with potential functional variants in the regulatory region of the gene.26

Associations Between Genotype and Phenotype
Baseline biochemical and other demographic variables did not differ according to NOS 3 genotype (data not shown).

BP and NOS 3 Genotype
Systolic and diastolic BPs (Table 5) were higher in heterozygotes for the Glu298Asp polymorphism (P<0.002 by ANOVA). Such a pattern is difficult to explain by conventional models of inheritance and raises the possibility of a type I statistical error. However, because eNOS is a dimer,27 the possibility that Glu/Asp heterodimers have reduced activity compared with Glu/Glu and Asp/Asp homodimers cannot be excluded. No other NOS 3 polymorphism exerted a statistically significant influence on BP.

Plasma NOx and NOS 3 Genotype
The hypothesis that NOS 3 polymorphisms influence endothelial NO production was tested by measuring plasma NOx in a subset of 1121 subjects, 290 from the North Mymms practice, 260 from the Chesterfield practice, and 571 selected at random from the whole cohort (Table 6). None of the polymorphisms studied influenced plasma NOx even when corrected for serum creatinine.

NOS 3 Genotype, Plasma NOx, and IHD Risk
The risk of IHD over the duration of the study was not influenced by the level of plasma NOx recorded at entry (data not shown). However, these data were based on only 15 IHD events among the 955 subjects with NOx measurements and adequate follow-up data. Cox regression was used to assess the effect of genotype on risk of an IHD event, defined as

### Table 4. Genotype and Allele Frequencies of NOS 3 Polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype Frequency, n (%)</th>
<th>Allele Frequency, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−922 A/G</td>
<td>AA, 1089 (39.0)</td>
<td>A, 3473 (62.2)</td>
</tr>
<tr>
<td></td>
<td>AG, 1295 (46.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG, 408 (14.6)</td>
<td></td>
</tr>
<tr>
<td>−786 T/C</td>
<td>TT, 1026 (37.7)</td>
<td>T, 3352 (61.6)</td>
</tr>
<tr>
<td></td>
<td>CC, 394 (14.5)</td>
<td></td>
</tr>
<tr>
<td>Intron 4 VNTR</td>
<td>a, 2023 (74.6)</td>
<td>a, 4693 (86.6)</td>
</tr>
<tr>
<td></td>
<td>ab, 647 (23.9)</td>
<td></td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>Glu/Glu 114 (44.3)</td>
<td>Glu, 3438 (66.5)</td>
</tr>
<tr>
<td></td>
<td>Glu/Asp, 114 (44.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asp/Asp, 292 (11.3)</td>
<td>Asp, 1730 (33.5)</td>
</tr>
</tbody>
</table>

*Genotype frequencies did not differ significantly from those predicted under conditions of Hardy-Weinberg equilibrium (P>0.1 for all comparisons).
fatal, nonfatal, or silent MI and coronary surgery. There was no evidence of a relationship between any polymorphism and the risk of IHD when analyzed according to codominant or recessive models (Table 7). Kaplan-Meier survival plots of the effects of the Glu298Asp, −786 T/C, and intron 4 genotypes on IHD event-free survival are illustrated in Figure 2. There was also no evidence that smoking status modified the effect of genotype on IHD risk (data not shown).

**Discussion**

In this large, prospective, population-based study of middle-aged men, we have shown that plasma NO is lower in smokers than nonsmokers but is not significantly influenced by polymorphisms in the NOS 3 gene nor by prevailing levels of BP or cholesterol. Moreover, neither plasma NO nor NOS 3 genotype was predictive of future IHD events in this cohort.

Cardiovascular Risk Factors, NO Pathway, and In Vivo Measures of NO Synthesis

A defect in endothelium-dependent vasodilation has been demonstrated in arteries from individuals with essential hypertension and hypercholesterolemia and from those exposed to cigarette smoke, as well as in individuals with established atherosclerosis. This abnormality is considered a marker for generalized impairment of the vasculoprotective actions of the endothelium and may be the result of a reduction in NO synthesis or availability.

Plasma NO reflects endogenous NO synthesis, although nitrate from the diet also makes a significant contribution to the plasma pool. Nitrate and nitrite are removed via the urine and feces; thus, the plasma level of NO reflects the balance between dietary intake and endogenous synthesis on one hand and urinary excretion on the other. When dietary intake is controlled and with the assumption of constancy of renal

**Table 5. BP and NOS 3 Genotype in Men From the NPHS II Study**

<table>
<thead>
<tr>
<th>NPHS II</th>
<th>−922 (A/G)</th>
<th>−786 (T/C)</th>
<th>Intron 4 VNTR</th>
<th>Glu298Asp</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>133.2</td>
<td>133.7</td>
<td>133.5</td>
<td>132.2</td>
</tr>
<tr>
<td>AG</td>
<td>133.3</td>
<td>133.4</td>
<td>133.9</td>
<td>135.4</td>
</tr>
<tr>
<td>GG</td>
<td>135.5</td>
<td>133.2</td>
<td>131.5</td>
<td>131.8</td>
</tr>
<tr>
<td>P</td>
<td>0.95</td>
<td>0.85</td>
<td>0.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TT</td>
<td>133.7</td>
<td>133.2</td>
<td>131.5</td>
<td>135.4</td>
</tr>
<tr>
<td>TC</td>
<td>133.4</td>
<td>133.9</td>
<td>131.5</td>
<td>131.8</td>
</tr>
<tr>
<td>CC</td>
<td>133.9</td>
<td>131.5</td>
<td>0.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>P</td>
<td>0.95</td>
<td>0.85</td>
<td>0.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>aa</td>
<td>133.5</td>
<td>133.5</td>
<td>132.2</td>
<td>135.4</td>
</tr>
<tr>
<td>ab</td>
<td>133.2</td>
<td>131.5</td>
<td>131.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>bb</td>
<td>131.5</td>
<td>131.8</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>P</td>
<td>0.95</td>
<td>0.85</td>
<td>0.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>(18.4)</td>
<td>(18.6)</td>
<td>(18.6)</td>
<td>(18.7)</td>
</tr>
<tr>
<td></td>
<td>(18.6)</td>
<td>(18.2)</td>
<td>(18.0)</td>
<td>(18.1)</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>(10.8)</td>
<td>(10.8)</td>
<td>(10.6)</td>
<td>(11.1)</td>
</tr>
</tbody>
</table>

Values in parentheses are SD. Values are adjusted for age, BMI, and alcohol intake.
excretory capacity, the level of plasma nitrogen oxides, measured with the Griess reaction, has been taken as an index of enzymatic NO synthesis. Plasma NOx is markedly elevated in sepsis and inflammation, 28 but large-scale studies of this parameter in disorders in which the NO pathway appears downregulated are more limited. Some studies indicate a reduction in circulating or urinary nitrogen oxides in essential hypertension29 or the hypertensive disorders of pregnancy, 30 but studies have been relatively small and the findings inconsistent,31 possibly because of the difficulty in applying the Griess assay for NOx to large numbers of subjects. However, the technique developed in the present study allowed simultaneous analysis of large numbers of plasma samples in 96-well microtiter plates. The assay was reliable and reproducible with interassay and intra-assay coefficients of variation of 3.4% and 4.0%, respectively. The distribution of plasma NOx levels determined in this cohort closely matched those recorded in other studies. 29

Influence of Cardiovascular Risk Factors on Plasma NOx

A major finding of the present study is that in 3 subsets of the NPHS II cohort, recruits form the Chesterfield and North Mymms general practices and in a third data set of randomly ascertained individuals, plasma NOx levels were 10% to 20% lower in current smokers than nonsmokers. This result is particularly striking because cigarette smoke itself contains NO,32 and, over the short term, cigarette smoking might be expected to result in an elevation rather than a reduction in the plasma level of NOx.33 However, findings from the present study suggest strongly that long-term cigarette consumption is associated with depressed plasma levels of NOx in vivo, a finding supported by observations that salivary nitrate is also reduced in smokers compared with nonsmokers.34 The reduced level of plasma NOx in smokers may explain the well-documented association between both active and passive cigarette smoking and impaired endothelium-dependent vasodilation in vivo.5,6 A variety of studies lend insight into the mechanism by which smoking might decrease endothelial NO generation and therefore decrease the plasma levels of NOx. These mechanisms include (1)

### Table 6. Plasma NOx According to NOS 3 Genotype in North Mymms, Chesterfield, and Random Data Sets

<table>
<thead>
<tr>
<th>Genotype</th>
<th>North Mymms</th>
<th>Chesterfield</th>
<th>Random</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AG</td>
<td>GG</td>
</tr>
<tr>
<td>NOx, μmol/L</td>
<td>11.3</td>
<td>9.8</td>
<td>12.2</td>
</tr>
<tr>
<td>NOx/creatinine</td>
<td>0.115</td>
<td>0.106</td>
<td>0.128</td>
</tr>
</tbody>
</table>

Values are adjusted for age, smoking, cholesterol, BP, and practice. Values in parentheses are SD.

### Table 7. Influence of NOS 3 Genotype on IHD Risk

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Hazard Ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-dominant model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−922 (A/G)</td>
<td>AA</td>
<td>1</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>0.80 (0.56–1.13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>0.69 (0.39–1.22)</td>
<td></td>
</tr>
<tr>
<td>−786 (T/C)</td>
<td>TT</td>
<td>1</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>0.78 (0.54–1.12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>0.71 (0.41–1.23)</td>
<td></td>
</tr>
<tr>
<td>Intron 4 VNTR</td>
<td>aa</td>
<td>1</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>ab</td>
<td>1.15 (0.68–1.51)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bb</td>
<td>1.04 (0.26–4.21)</td>
<td></td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>Glu/Glu</td>
<td>1</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Glu/Asp</td>
<td>1.0 (0.68–1.51)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asp/Asp</td>
<td>0.60 (0.30–1.22)</td>
<td></td>
</tr>
</tbody>
</table>

Recessive model

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Hazard Ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>−922 (A/G)</td>
<td>AA + AG</td>
<td>1</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>0.71 (0.42–1.22)</td>
<td></td>
</tr>
<tr>
<td>−786 (T/C)</td>
<td>TT + TC</td>
<td>1</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>0.81 (0.48–1.36)</td>
<td></td>
</tr>
<tr>
<td>Intron 4 VNTR</td>
<td>aa + ab</td>
<td>1</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>bb</td>
<td>1.03 (0.26–4.18)</td>
<td></td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>Glu/Glu + Glu/Asp</td>
<td>1</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Asp/Asp</td>
<td>0.60 (0.31–1.19)</td>
<td></td>
</tr>
</tbody>
</table>
increased formation of oxygen-derived free radicals and oxidized LDL cholesterol, which attenuates eNOS activity; (2) a reduction in eNOS protein expression and activity; and (3) a smoking-induced reduction in the availability of tetrahydrobiopterin (BH4), a cofactor for NOS.

Elevations in serum cholesterol are also associated with impaired endothelium-dependent vasodilation, but in this study, apart from a weak inverse correlation between cholesterol and NOx in the North Mymms data set, there was no consistent correlation between serum total cholesterol and plasma NOx even after correction for serum creatinine. The failure to detect a consistent relationship suggests either that the effect of cholesterol on endothelial function is mediated independently of NO synthesis (possibly via other endothelial dilator systems or by increased generation of oxidative free radicals) or that a small effect of cholesterol on NO synthesis was masked by the influence of dietary nitrate. Studies indicate that dietary nitrate is cleared from the plasma pool within 12 hours of a meal. Thus, in this study in which subjects did not fast but had been asked to consume no more than a light meal on the morning of recruitment, it is possible that a small effect of cholesterol on endothelial NO synthesis was masked by the confounding effect of dietary nitrate intake on the morning of the study. Likewise, no clear correlation was observed between BP and plasma NOx in this study, a result that is at odds with a previous study of Japanese subjects. However, because hypertension has been shown to depress endothelium-dependent vasodilation in some but not all studies, this suggests that the effect of BP on endothelial function is variable and that when seen may result from processes that are either dependent or independent of endothelial NO synthesis.

There was no discernible influence of NOx level at study entry on future risk of an IHD event. Although this finding must be interpreted with caution because it rests on only a small number of events, a possible explanation for this observation is that plasma NOx levels might vary with time as result of the changes in the dietary intake of nitrate or in renal excretory capacity. If this were the case, relationships between plasma NOx and, for example, smoking might be seen when both are measured at the same time point, but an NOx measurement at a single time point would have little long-term predictive value. Indeed, this finding urges caution in the interpretation of results of previous smaller studies in which plasma NOx levels have apparently been related to disease or risk factor status. When NOx levels were measured in 86 individuals under the same circumstances on 2 occasions a year apart, there was little correlation between the 2 values within the same individual (r=0.23, P=0.17). This poor correlation was not due to poor reproducibility of the assay itself, which, in our laboratory, exhibited an intra-assay coefficient of variation of 3.4% and an interassay coefficient of variation of 4.0%.

**NOS 3 Polymorphisms, Plasma NOx, and IHD Risk**

A number of case-control studies have demonstrated an association between polymorphisms in the NOS 3 gene and a variety of cardiovascular end points, including hypertension, MI, coronary artery disease, and coronary artery spasm. These positive findings, however, are not universal. The limitations of case-control designs and of genetic studies involving relatively small populations have been discussed previously. In the present study, we aimed to overcome some of the limitations of previous studies by (1) performing the largest population-based study of NOS 3 polymorphisms conducted to date, (2) using a group of men initially free from IHD to investigate the genetic influences on IHD events in a prospective manner, and (3) examining all of the polymorphisms at the NOS 3 locus that have been associated previously with cardiovascular disease. The study also allowed us to reassess the relationship between NOS 3 polymorphisms and BP.

In our study, we did not find any influence of NOS 3 gene polymorphisms on risk of IHD over the 8 years of follow-up in a group of 2965 men ascertained from 9 different regions of the United Kingdom (Figure 2). There was also no interaction between NOS 3 genotype and any of the cardiac risk factors studied. In particular, there was no interaction between smoking status and NOS 3 genotype in the determi-
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of IHD risk, as has been reported by a previous study. We also did not see any relationship between any of the polymorphisms studied and plasma NO\textsubscript{x}, in contrast to 2 previous reports. The lack of a relationship between plasma NO\textsubscript{x} and the −786 T/C polymorphism runs contrary to the observation that the NOS 3 gene promoter containing the C allele exhibits reduced transcriptional activity compared with the promoter containing the T allele. It also runs contrary to the observation that eNOS Asp298 has an enhanced susceptibility to intracellular proteolytic cleavage compared with eNOS Glu298. However, because plasma NO\textsubscript{x} is likely to reflect NO synthesis via both eNOS and the neuronal NOS isoform, it is possible that small differences in NO generation resulting from genetic differences in the NOS 3 gene cannot be detected sensitively by measurement of steady-state plasma NO\textsubscript{x}. Dietary contributions to the plasma NO\textsubscript{x} pool may also have limited even further the ability to detect such a relationship. Additional studies are currently underway in individuals with defined NOS 3 variants in whom enzymatic generation of NO will be measured with an L-arginine tracer technique to circumvent the problem of dietary contribution to the plasma NO\textsubscript{x} pool. Basal and stimulated endothelial NO generation is also being assessed in the same individuals to define whether there is a deficiency of local NO generation in individuals with NOS 3 variants.

In a prior study conducted in the East Anglian region of the United Kingdom, we identified a positive association between the Asp298 variant and angiographic coronary artery disease and MI. The failure to detect an influence of this variant on the risk of IHD in the present study might have arisen for a number of reasons. The first trial was a conventional case-control study conducted among a cross section of disease sufferers and control subjects, whereas the present study represented a prospective observational study with a nested case-control design. Thus, the number of cases accrued at the end of 8 years of follow-up could only be estimated. The initial power calculations were based on a projection of 200 IHD cases rather than the 159 that were observed; thus, the study had slightly less power than envisaged originally. Nevertheless, we still had 90% power to detect a 2.2-fold excess risk of IHD among rare allele homozygotes assuming a recessive model and 90% power to detect a 1.6-fold excess risk among rare allele carriers assuming a dominant model at $P = 0.05$. In previous studies, excess risks of cardiovascular end points have been confined to homozygotes for NOS 3 variants, and the odds ratios for IHD or other end points in such individuals have ranged from 2 to 4, suggesting that our study had adequate power to detect this level of risk. Our negative findings might therefore indicate either that some previous smaller-scale studies might have generated falsely positive results or that the true level of risk is somewhat more modest than originally suspected. An alternative explanation for the discrepant results of the 2 studies is that the effect of NOS 3 polymorphisms might become apparent only in individuals of particular ethnic or genetic backgrounds in whom the facilitatory effect of additional genetic loci is present. Although in both studies the individuals were overtly white, subtle levels of ethnic admixture cannot be excluded even within such apparently homo-
genous groups. The Cambridge Heart Antioxidant Study (CHAOS) trial was conducted in the relatively stable and therefore more ethnically homogenous population of the East Anglia, which might explain why a positive association between the Glu298Asp polymorphism and IHD was observed in that study.

Systolic and diastolic BPs were higher in heterozygotes for the Glu298Asp polymorphism ($P < 0.0001$ and $P = 0.002$, respectively, by ANOVA), but such a pattern is difficult to explain by conventional models of inheritance and raises the possibility of a type I statistical error. Another potential explanation for such findings is that Glu/Asp heterodimers have reduced enzymatic activity compared with Glu/Glu and Asp/Asp homodimers. This has not been tested formally and warrants further investigation. No other NOS 3 polymorphism exerted a statistically significant influence on BP.

In summary, we have found evidence in a large cohort of male subjects that cigarette smoking but not the prevailing level of BP or serum cholesterol is associated with a reduction in NO generation in vivo. Such a finding endorses the view that the endothelial dysfunction seen after exposure to smoking is due to a reduction in endothelial NO generation. However, we failed to detect an influence of polymorphisms located in the promoter or coding region of the NOS 3 gene in determining plasma levels of NO\textsubscript{x} or the future risk of an IHD event. This finding in a large study of UK men suggests that NOS 3 gene polymorphisms are unlikely to account for major differences in susceptibility to IHD in the general UK population, although an important effect in individuals from particular regions or ethnic backgrounds cannot be excluded.

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References

Genes, Environment, Nitric Oxide, and IHD


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