Common Genetic Variation at the Endothelial Nitric Oxide Synthase Locus and Relations to Brachial Artery Vasodilator Function in the Community

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Background—Sequence variants at the endothelial nitric oxide synthase (NOS3) locus have been associated with endothelial function measures, but replication has been limited.

Methods and Results—In reference pedigrees, we characterized linkage disequilibrium structure at the NOS3 locus using 33 common single nucleotide polymorphisms (SNPs). Eighteen SNPs that capture underlying common variation were genotyped in unrelated Framingham Heart Study participants (49.5% women; mean age, 62 years) with measured brachial artery flow-mediated dilation (n=11005) or hyperemic flow velocity (n=1043). Within 3 defined blocks of strong linkage disequilibrium that spanned NOS3, 11 SNPs captured >80% of common haplotypic variation. Among men, there were nominally significant associations between 8 NOS3 SNPs (minimum \( P = 0.002 \)) and between haplotypes (minimum \( P = 0.002 \)) and either flow-mediated dilation or hyperemic flow velocity. In women, we did not observe significant associations between NOS3 SNPs or haplotypes and endothelial function measures. To correct for multiple testing, we constructed 1000 bootstrapped null data sets and found that empirical probability values exceeded 0.05 for both phenotypes.

Conclusions—A parsimonious set of SNPs captures common genetic variation at the NOS3 locus. A conservative interpretation of our results is that, accounting for multiple testing, we did not observe statistically significant relations between NOS3 sequence variants and endothelial function measures in either sex. The nominal associations of select NOS3 variants with endothelial function in men (unadjusted for multiple testing) should be viewed as hypothesis-generating observations and may merit testing in other cohorts and experimental designs. (Circulation. 2005;112:1419-1427.)

Key Words endothelium ● epidemiology ● genetics ● nitric oxide synthase

The endothelial isoform of nitric oxide synthase (NOS) (encoded by the NOS3 gene) converts l-arginine to nitric oxide (NO), a potent vasodilator and inhibitor of inflammation and platelet activity.1 In humans, flow-mediated dilation (FMD) and reactive hyperemia have been shown to depend on the bioavailability of endothelium-derived NO.2,3 Impaired endothelium-dependent vasodilation predicts future cardiovascular events (reviewed in detail elsewhere),3 suggesting that loss of endothelium-derived NO contributes to the pathogenesis and clinical expression of atherosclerosis.6 For these reasons, there is intense interest in defining the mechanisms of endothelial dysfunction, including the possible contributory role of altered expression and/or activity of endothelial NOS.

Experimental and clinical studies suggest that genetic variation in NOS may influence the bioavailability of NO. In mice, targeted deletion of NOS3 leads to endothelial dysfunction and hypertension.7 Young individuals with a parental
history of premature cardiovascular disease have impaired brachial artery FMD,8,9 and we recently reported that brachial FMD is modestly heritable in the community.10 Other investigators have evaluated the relations of specific NOS3 genetic variants to endothelial function in small- to modest-sized referral samples, but these studies have yielded inconsistent results.11–16

It is now possible to comprehensively study single nucleotide polymorphisms (SNPs) and haplotypes (the combination of SNP alleles observed on a chromosome) at a genetic locus.17 SNPs at a locus may be correlated (termed linkage disequilibrium [LD]).18 and within regions of high LD, there are a limited number of common haplotype patterns.19 A parsimonious subset of SNPs (termed tag SNPs) can be chosen that reliably predict these common haplotypes and therefore capture the underlying common genetic variation within regions of strong LD.20,21 By testing an efficient set of tag SNPs and (within regions of strong LD) their common haplotypes, potential causal variants may be evaluated for an association with phenotype, either directly, or indirectly via LD.22

Thus, we sought to (1) define the LD pattern for common SNP variants at the NOS3 locus in reference pedigrees and identify a set of SNPs that capture the underlying common variation and (2) genotype these SNPs in a large community-based sample and test the hypothesis that common variation in the NOS3 gene is associated with brachial artery FMD and reactive hyperemia.

Methods

Study Participants

The design of the Framingham Heart Study (FHS) Offspring cohort has been described.23 Among the 3539 participants who attended the seventh examination of the Framingham Offspring Study, 2883 participants had adequate FMD measurements10 and 2031 had adequate hyperemic flow velocity measurements24 as described previously. Separately, during the sixth examination (1995–1998), 1809 unrelated individuals provided blood samples for DNA extraction.25 FHS randomly selected these unrelated individuals for DNA collection without regard to any phenotype feature. In the present study, everyone who had FMD or hyperemic flow velocity measured was eligible provided they had DNA available. This resulted in 1446 eligible for genotype–FMD and 1043 for genotype–reactive hyperemia association analyses. The Boston Medical Center institutional review board approved the study; participants gave written informed consent.

Noninvasive Measures of Endothelial Function

We a priori selected FMD and hyperemic flow velocity as phenotypes to be consistent with prior publications.10,26 We selected FMD to assess conduit artery endothelial function. Because hyperemic flow is the principal stimulus for FMD and is an indicator of microvascular function, we investigated hyperemic flow velocity. The methodology and reproducibility for measuring brachial artery FMD (expressed as percent increase in diameter) and mean hyperemic flow velocity (cm/s) have been described.10,24 Briefly, using a Toshiba SSH-140A ultrasound system and commercially available software (Brachial Analyzer version 3.2.3, Medical Imaging Applications), investigators blinded to participant clinical and genetic status determined brachial artery diameter at baseline and 1 minute after reactive hyperemia induced by 5-minute forearm cuff occlusion. Mean hyperemic flow velocity was analyzed with the use of semiautomated signal averaging (Cardiovascular Engineering).

Genotyping Methods

Genotyping was performed at the Broad Institute of the Massachusetts Institute of Technology/Harvard University by using matrix-assisted laser desorption ionization–time of flight mass spectrometry (Sequenom) to resolve allele-specific single-base extension products. The genotyping protocol has been described.27

SNP Selection and Genotyping in Reference Pedigrees

We surveyed common genetic variation in the region spanning NOS3 (chromosome 7; accession number NM_000603). We successfully genotyped common SNPs (minor allele frequency ≥5%) with an average density of 1 SNP every 1.2 kilobases (kb), such that there were at minimum of 6 common SNPs per block of strong LD and gaps between blocks were <2 kb. We selected 139 markers from the public National Center for Biotechnology Information SNP map (http://www.ncbi.nlm.nih.gov/SNP) and the Celera variation database (http://www.celera.com). We genotyped these SNPs in a panel of 12 multigenerational pedigrees containing 93 individuals of western and northern European ancestry from Utah–Centre d’Etude du Polymorphisme Humain (CEPH) panel (Coriell Institute for Medical Research, Camden, NJ).27

We developed working assays for 33 polymorphic common SNPs (Table I in the online-only Data Supplement for CEPH minor allele frequencies) as defined by the following criteria: (1) at least 75% genotyping call success; (2) Hardy-Weinberg equilibrium P>0.01; and (3) mendelian transmission errors in CEPH pedigrees ≤1.

LD Structure in Reference Pedigrees and Identification of Haplotype Blocks

For each pair of markers, D’ (LD strength estimate)28 and logarithm of the odds score (LD significance estimate)29 were calculated. With the use of the “spine of LD” setting in Haplovew software, haplotype blocks were defined on the basis of each end marker of a block having a D’ >0.8, with all intervening pairwise marker comparisons, allowing for 1 comparison exception (version 2.03; http://www.broad.mit.edu/personal/jcbarret/haplovew/).30

Tag SNP Selection

In the reference population, we used tag SNPs selection software (http://www-rcf.usc.edu/~stram), which calculates the squared correlation, R2, between haplotypes within LD blocks and haplotypes predicted with the use of only a subset of SNPs.31 We selected 11 of the 33 SNPs (3, 6, and 2 SNPs for blocks 1, 2, and 3, respectively) as tag SNPs; they represented the minimum subset of SNPs that was required to predict all common haplotypes (≥5% frequency) within each block, with R2 ≥0.90. Using customized software (http://www.broad.mit.edu/mpg/tagger/) that assesses the ability of tag SNPs or their multimarker combinations to capture other SNPs, we separately evaluated the ability of tag SNPs to capture common variation at the locus, independent of block designation.

Genotyping in the FHS

In the Framingham sample we genotyped the 11 tag SNPs, the previously reported common missense variant, Glu298Asp (rs1799983),11,13–16 the previously studied T→C promoter variant (rs2070744), and 5 SNPs that were redundant in CEPH pedigrees (to help assess LD block structure similarity between CEPH and FHS samples; data not shown). Thus, 18 NOS3 SNPs were genotyped in FHS (Table I in the online-only Data Supplement). All SNPs were in Hardy-Weinberg equilibrium in the FHS sample (χ2 test P>0.05).

Statistical Analysis

Using a general model of inheritance, we conducted multivariable linear regression analyses (SAS Proc GLM11) to test the null hypothesis that FMD and hyperemic flow velocity means did not differ by SNP genotype. In exploratory analyses, because of prior reports,11,12 we evaluated specific interaction terms in the multivari-
able-adjusted model, including sex for all 18 SNPs and smoking for the Glu298Asp variant. Because of previously reported sex differences and statistically significant sex-phenotype-genotype interactions observed in our data, we report sex-specific analyses.

Haplotype-based association analyses were conducted with the use of weighted-regression as implemented in the haplo.stats program. Haplotype frequencies in Framingham participants were estimated with the use of the expectation-maximization algorithm. A global score statistic tested whether trait differences existed among all haplotypes simultaneously; a haplotype-specific score statistic tested whether trait differences existed between a single haplotype versus all other haplotypes combined.

In both individual SNP and haplotype analyses, we adjusted for covariates previously associated with FMD or hyperemic flow response in our cohort, including mean arterial pressure, brachial artery pulse pressure, heart rate, body mass index, total/HDL cholesterol ratio, fasting glucose, diabetes, smoking within past 6 hours, prevalent cardiovascular disease, hormone replacement therapy, hypertension (systolic blood or diastolic pressure ≥140/90 mm Hg or antihypertensive medication use), lipid-lowering medication, and walk test (before or after FMD determination).

To account for multiple statistical testing, we constructed null data sets through bootstrap resampling; genotypes and phenotypes were sampled randomly with replacement. For each of the 18 SNPs, we ran 6 regression models (male, female, and sex-pooled, age-adjusted, and multivariable-adjusted models) 1000 times and evaluated the distribution of minimum probability values. We obtained an empirical probability value for each phenotype by comparing the minimum nominal probability value with the distribution of probability values from the null data sets.

**Results**

**LD Pattern at the NOS3 Locus in Reference Pedigrees**

The LD structure at the NOS3 locus was defined by genotyping 33 common SNPs (minor allele frequency ≥5%) in CEPH pedigrees (Table I in the online-only Data Supple-
ment). The mean interval between SNPs was 1.2 kb, and the SNP map spanned 39 kb in genomic distance (7 kb upstream, 23 kb NOS3 coding region, and 9 kb downstream). Three blocks of strong LD were evident: The blocks encompassed 14.2, 10.1, and 3.2 kb for blocks 1, 2, and 3, respectively (Figure). Both interblock distances were 0.5 kb. Within each block, there was limited haplotypic diversity. In blocks 1, 2, and 3, there were 4, 6, and 3 common haplotypes (frequency ≥5%) capturing 97%, 83%, and 98% of chromosomes, respectively (Figure).

### Adequacy of SNP Selection
To examine the extent to which SNPs genotyped in our association study predicted unmeasured SNPs without regard to block structure, we calculated pairwise correlation ($r^2$). In pairwise correlation between the 18 NOS3 SNPs genotyped in FHS and the remaining 15 unmeasured SNPs, we observed that 30 of the 33 common SNPs (91%) were predicted with an $r^2 \geq 0.70$ (mean $r^2 = 0.90$).

We compared the correlation between SNPs genotyped in FHS and NOS3 SNPs from the SeattleSNPs Program for Genomics Applications resequencing effort. SeattleSNPs identified 37 common NOS3 SNPs over a 25-kb genomic span in 23 individuals of European ancestry. Thirteen SNPs overlapped between the 37 identified by SeattleSNPs resequencing and the 18 SNPs genotyped in FHS. These 13 SNPs genotyped had strong correlation ($r^2 > 0.70$) with the majority (76% [28 of 37 SNPs]) of SeattleSNPs variants (mean $r^2 = 0.72$). Hence, we infer that the 18 SNPs genotyped in our association study captured most of the common genetic variation at the NOS3 gene.

### FHS Participants
We examined 1446 (49.5% women) middle-aged to elderly FHS participants (mean age, 62 ± 9 years; Table 1) with NOS3 genotype and FMD (1043 had hyperemic flow velocity measurements). The mean FMD and hyperemic flow velocity were 3.36 ± 3.08% and 53.8 ± 21.9 cm/s in women and 2.32 ± 2.38% and 47.1 ± 20.4 cm/s in men, respectively. The age- and sex-adjusted correlation between FMD and hyperemic flow velocity was 0.41 ($P = 0.0001$).

### Associations of Single SNPs With FMD or Hyperemic Flow Velocity
In men, 4 sequence variants showed modest nominally significant (ie, $P < 0.05$, not corrected for multiple testing) associations with FMD (Table 2). For example, individuals homozygous or heterozygous for the minor allele of SNP 10 (rs1800781) had higher FMD versus major allele homozygotes (3.37 ± 0.72%, 2.66 ± 0.18%, 2.21 ± 0.10% for AA, AG, and GG genotypes, respectively; $P = 0.027$).

In men, 5 sequence variants showed modest nominally significant associations with hyperemic flow velocity (Table 3). For example, individuals heterozygous for the minor allele of SNP 16 (rs3918174) had higher hyperemic flow velocity compared with major allele homozygotes (51.9 ± 1.54 cm/s versus 45.9 ± 0.88 cm/s for AG versus AA; general model $P = 0.002$). SNP 16 explains ≈2% of the variability in hyperemic flow velocity in men.

### Table 1. FHS Sample Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Men* (n=730)</th>
<th>Women* (n=716)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>62 ± 9</td>
<td>61 ± 9</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>95 ± 11</td>
<td>88 ± 12</td>
</tr>
<tr>
<td>Pulse pressure, mm Hg</td>
<td>53 ± 15</td>
<td>56 ± 15</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>62 ± 11</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>28.9 ± 4.7</td>
<td>27.3 ± 5.5</td>
</tr>
<tr>
<td>Total/HDL cholesterol ratio</td>
<td>4.5 ± 1.4</td>
<td>3.6 ± 1.2</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>111 ± 33</td>
<td>102 ± 26</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>Smoking within past 6 h, %</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Prevalent cardiovascular disease, %</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>Hormone replacement therapy, %</td>
<td>. . .</td>
<td>38</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>51</td>
<td>43</td>
</tr>
<tr>
<td>Lipid-lowering medication, %</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>Walk test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before brachial testing, %</td>
<td>36</td>
<td>38</td>
</tr>
<tr>
<td>After brachial testing, %</td>
<td>33</td>
<td>41</td>
</tr>
<tr>
<td>FMD, %</td>
<td>2.32 ± 2.38</td>
<td>3.36 ± 3.08</td>
</tr>
<tr>
<td>Hyperemic flow velocity, cm/s</td>
<td>47.1 ± 20.4</td>
<td>53.8 ± 21.9</td>
</tr>
</tbody>
</table>

Values for continuous measures, mean ± SD.

* $n = 1043$ for hyperemic flow velocity phenotype.

Notably, we did not observe any significant association between the previously reported T → C promoter variant (SNP 9: rs2070744) or Glu298Asp (SNP 15: rs1799983) coding variant and either FMD or hyperemic flow velocity. In women, we did not observe significant association between any of the 18 NOS3 SNPs and either phenotype.

### Effect Modification by Gender or Smoking
The difference in association between hyperemic flow velocity and genotype in the 2 sexes was nominally significant in an interaction model (interaction term $P < 0.05$ for 4 SNPs: SNPs 10, 11, 16, 29). There was not a significant sex × SNP interaction for FMD for the 18 SNPs tested. Given a previous report, tested but did not observe any significant interaction between Glu298Asp (SNP 15: rs1799983) genotype and smoking for either phenotype ($P > 0.05$).

### Accounting for Multiple Testing
For association analyses involving FMD, 18 SNPs, and 6 models for each SNP, our minimum nominal $P = 0.008$ (in men, age-adjusted model for SNP 10). With 1000 resampling procedure runs, this corresponds to an empirical $P = 0.34$ for FMD. For hyperemic flow velocity, our minimum nominal $P = 0.0021$ (in men, multivariable-adjusted model for SNP 16), corresponding to an empirical $P = 0.14$.

### Associations of Haplotypes With FMD or Hyperemic Flow Velocity
In women, we did not observe any significant association between NOS3 haplotypes and FMD or hyperemic flow velocity. Among men, global haplotype association tests were not significant in either block 1 or block 2 (Tables 4 and 5).
TABLE 2. Association of Selected NOS3 SNPs and FMD in Men and Women*

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Least-Squares Mean (SE)</td>
<td>Nominal P</td>
</tr>
<tr>
<td>SNP 6: hCV3219467</td>
<td>CC</td>
<td>2.45 (0.12)</td>
<td>0.040</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>2.33 (0.14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>1.48 (0.36)</td>
<td></td>
</tr>
<tr>
<td>SNP 7: rs1800783</td>
<td>TT</td>
<td>2.15 (0.14)</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>2.32 (0.12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>2.81 (0.23)</td>
<td></td>
</tr>
<tr>
<td>SNP 9: rs2070744 (T&lt;sup&gt;G&lt;/sup&gt;→C)</td>
<td>TT</td>
<td>2.10 (0.15)</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>2.34 (0.13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>2.74 (0.22)</td>
<td></td>
</tr>
<tr>
<td>SNP 10: rs1800781</td>
<td>GG</td>
<td>2.21 (0.10)</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>2.66 (0.18)</td>
<td></td>
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<tr>
<td></td>
<td>AA</td>
<td>3.37 (0.72)</td>
<td></td>
</tr>
<tr>
<td>SNP 14: rs1007311</td>
<td>AA</td>
<td>2.62 (0.15)</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>2.14 (0.12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>2.44 (0.20)</td>
<td></td>
</tr>
<tr>
<td>SNP 15: rs1799983 (Glu298Asp)</td>
<td>GG</td>
<td>2.48 (0.13)</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>2.14 (0.12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>2.52 (0.24)</td>
<td></td>
</tr>
</tbody>
</table>

Values are multivariable-adjusted least-squares means for FMD. See Table II in online-only Data Supplement for a display of association results for all 18 SNPs tested.

*Displayed are SNPs associated with FMD phenotype (nominal P<0.05) and previously studied Glu298Asp and T<sup>G</sup>→C variants. 
†Phenotypes adjusted for age, mean arterial pressure, brachial artery pulse pressure, heart rate, body mass index, total/HDL cholesterol ratio, fasting glucose, diabetes, smoking within past 6 h, prevalent cardiovascular disease, hormone replacement therapy, hypertension medication, lipid-lowering medication, and walk test.
‡P for genotype-phenotype association using general model of inheritance.

However, given that global haplotype tests may miss more subtle associations, we compared individual haplotypes versus all other haplotypes combined. Among men, haplotype 1D in block 1 (12% frequency) showed a nominally significant association with higher FMD and hyperemic flow velocity (minimum haplotype P = 0.008; Table 4). The minor A allele of SNP 10 (rs1800781), present exclusively on haplotype 1D (Figure), was nominally significantly associated with both FMD and hyperemic flow velocity in men (Tables 2 and 3); thus, the single SNP and haplotype findings represent the same result.

Among men, haplotype 2C in block 2 (8% frequency) showed nominal significance for association with both phenotypes (haplotype P = 0.04 and 0.03, respectively; Table 5). Haplotype 2F (5% frequency) showed a nominally significant association with higher FMD (haplotype P = 0.006). The minor G allele of SNP 16 (rs3918174) was present on haplotypes 2C and 2F.

Among men, the global haplotype association test was nominally significant in block 3 (global P = 0.006 and 0.02 for FMD and hyperemic flow velocity, respectively; Table 6). Men with haplotype 3B (33% frequency) had higher FMD and greater hyperemic flow velocity compared with all other haplotypes combined (haplotype P = 0.0007 and 0.01, respectively). The minor T allele of SNP 29 (rs1065299) was present on haplotype 3B; hence, SNP 29 represents the same association as haplotype 3B.

Discussion

Principal Findings

In reference pedigrees, we characterized LD structure across a 39-kb genomic segment spanning the NOS3 gene using a dense SNP map and found 3 blocks of SNPs in strong LD. A subset of 11 tag SNPs captured a majority of common variation at the locus. With 33 common SNPs in reference pedigrees and the genotyping of 18 NOS3 SNPs in our association study, we have extended prior work by comprehensively studying the sex-specific association of common NOS3 genetic variation with endothelial function measures in a large community-based sample.

In women, we found no evidence of an association between endothelial function and genetic variation at the NOS3 locus. In men, however, we identified modest associations with both specific haplotypes and SNPs residing on these haplotypes. The first involves variants in the promoter region (SNPs 6 and 7). The second involves 1 of 3 intronic SNPs that are near-perfect proxies for each other: SNPs 10, 11, 16. The third possible site is SNP 29, which is located in intron 24. Although these previously unreported associations were non-
inally significant, the empirical probability value exceeded 0.05 when the multiple statistical tests performed for this locus were taken into account. Therefore, we view our findings as hypothesis generating, underscoring the need for additional studies to test our observations.

Comparison With Prior Studies

Prior association studies examining the relation between endothelial function and NOS3 variants have focused on Glu298Asp, T786>C (SNP 9: rs2070744 in our study; Table I in the online-only Data Supplement), and a variable number tandem repeat (VNTR) in intron 4.11–16 In general, those studies were modest in size (n<250) and yielded mixed results.11–16 In particular, the Glu298Asp variant has been a focus because of initial suggestions that the mutation led to differential susceptibility to intracellular cleavage37; however, a more recent in vitro study has challenged this assertion.38 Leeson et al11 evaluated 248 young participants and reported that the Glu298Asp genotype did not relate to FMD in the overall sample, a finding that is consistent with our results.

| TABLE 3. Association of Selected NOS3 SNPs and Hyperemic Flow Velocity in Men and Women* |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Men             | Women           |                  |                  |                  |
|                  | Genotype n Least-Squares Mean (SE) Nominal P | Genotype n Least-Squares Mean (SE) Nominal P |
| SNP 9: rs2070744 | TT 161 46.4 (1.36) 0.78 | TT 180 52.4 (1.32) 0.18 |
| (T786>C)         | TC 222 47.5 (1.15) 0.010 | TC 253 54.0 (1.12) 0.004 |
|                  | CC 69 47.7 (2.07) 0.004 | CC 54 57.5 (2.44) 0.004 |
| SNP 10: rs1800781| GG 346 45.9 (0.90) 0.004 | GG 376 54.4 (0.92) 0.004 |
|                  | AG 119 51.4 (1.55) 0.004 | AG 132 52.5 (1.58) 0.004 |
|                  | AA 6 46.2 (6.90) 0.004 | AA 8 48.1 (6.43) 0.004 |
| SNP 11: rs3918169| AA 353 45.9 (0.89) 0.004 | AA 368 54.2 (0.93) 0.004 |
|                  | AG 122 51.7 (1.52) 0.004 | AG 142 53.4 (1.50) 0.004 |
|                  | GG 6 41.1 (6.92) 0.004 | GG 8 48.5 (6.31) 0.004 |
| SNP 15: rs1799883| GG 196 48.1 (1.22) 0.004 | GG 226 53.6 (1.18) 0.004 |
| (Glu298Asp)      | GT 224 46.9 (1.14) 0.004 | GT 232 53.7 (1.16) 0.004 |
|                  | TT 63 45.4 (2.16) 0.004 | TT 46 54.7 (2.65) 0.004 |
| SNP 16: rs3918174| AA 356 45.9 (0.88) 0.004 | AA 373 54.2 (0.92) 0.004 |
|                  | AG 119 51.9 (1.54) 0.004 | AG 132 53.0 (1.56) 0.004 |
|                  | GG 6 39.2 (6.89) 0.004 | GG 6 47.6 (7.31) 0.004 |
| SNP 22: rs891511 | GG 179 45.2 (1.27) 0.004 | GG 176 54.1 (1.35) 0.004 |
|                  | AG 190 50.1 (1.23) 0.004 | AG 217 53.7 (1.22) 0.004 |
|                  | AA 37 49.9 (2.82) 0.004 | AA 48 53.1 (2.60) 0.004 |
| SNP 29: rs1065299| GG 213 45.2 (1.14) 0.004 | GG 209 53.9 (1.23) 0.004 |
|                  | GT 223 48.2 (1.12) 0.004 | GT 240 54.6 (1.15) 0.004 |
|                  | TT 46 52.3 (2.48) 0.004 | TT 61 51.0 (2.30) 0.004 |

See Table 2 for genetic model. All phenotypes have been adjusted for covariates in Table 2 legend. See Table III in online-only Data Supplement for display of association results for all 18 SNPs.

*Displayed are SNPs associated with FMD phenotype (nominal P<0.05) and previously studied Glu298Asp and T786>C variants.

| TABLE 4. Relations of NOS3 Block 1 Haplotypes to FMD and Hyperemic Flow Velocity in FHS Men* |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Haplotype Frequency | Haplotype Homework | Global P | Haplotype Frequency | Haplotype Homework | Global P |
| SNP 9: rs2070744| TT 0.33 0.75 0.08 | 0.33 0.48 0.14 |
| SNP 10: rs1800781| TT 0.26 0.13 0.08 | 0.26 0.79 0.08 |
| SNP 11: rs3918169| TT 0.25 0.64 0.08 | 0.25 0.30 0.08 |
| SNP 15: rs1799883| TT 0.12 0.008 0.08 | 0.12 0.01 0.08 |

All phenotypes have been adjusted for covariates in Table 2 legend.

*n=2892 and n=2086 chromosomes for FMD and hyperemic flow velocity, respectively.

†Haplotype P compares the specific haplotype vs all others combined.
One study observed a relation between the T→C promoter variant and forearm blood flow responses during acetylcholine infusion in hypertensive patients, but other studies found no relation between this polymorphism and FMD. Cattaruzza et al evaluated the response of endothelial cells (isolated from umbilical cord vein samples) to 24 to 36 hours of laminar shear stress and reported that in samples with the CC genotype, NOS3 mRNA and protein levels did not increase in response to shear stress. We observed that the T→C variant (rs20720744, SNP 9) was not associated with FMD or hyperemic flow velocity, although the nominal P was of borderline significance (P=0.06). The intron 4 VNTR polymorphism has been related to lower FMD in diabetic patients but not in a cohort of black subjects. We did not directly test the intron 4 VNTR; however, this polymorphism has been previously shown to be in LD with the T→C variant.

In summary, differences in phenotype and variants studied limit direct comparison of our findings to prior work. However, the cumulative evidence argues against an effect of Glu298Asp coding variant on FMD or hyperemic flow. Similar to prior work, our study leaves open the possibility that endothelial function might relate to T→C, intron 4 VNTR, or a SNP in LD.

SNPs and Haplotypes

In addition to the much larger sample size, the present study differed from prior work by including haplotype analyses. Testing haplotypes in association studies may offer several advantages. For example, specific multimarker combinations may be better predictors of unmeasured variants than an individual SNP. In addition, SNP alleles interacting in cis may produce a greater effect than any individual variant. The associations of 5-lipoxygenase activating protein haplotypes and myocardial infarction or β2-adrenergic receptor haplotypes and bronchodilator responsiveness provide examples of haplotypes apparently providing information beyond testing specific SNPs. However, in the present study the haplotypes and SNP analyses provided similar insights.

Role of Multiple Testing

To facilitate discovery, it has been suggested that candidate gene association studies transition from testing single variants to gene-based approaches in which all common variation within a gene is considered jointly. We undertook such an approach and identified several modest nominal associations. However, considering multiple variants at a locus may increase type I error. Methods to determine whether putative associations identified are bona fide or statistical fluctuations include stringent statistical criteria to correct for multiple testing and replication.

We constructed bootstrapped null-distribution data sets to guide the interpretation of our results. After consideration of the number of SNPs and analytical models studied, our strongest result was observed by chance >5% of the time for both phenotypes. Thus, despite observing a minimum nominal P=0.002, declaration of a bona fide association would be premature. Instead, we regard our putative associations as intriguing hypotheses (specifically SNPs 6, 7, 10, 11, 16, and 29) for independent replication studies. Replication would require determination of vascular function and NOS3 genotype determination, a similar-sized or larger sample, and

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**TABLE 5. Relations of NOS3 Block 2 Haplotypes to FMD and Hyperemic Flow Velocity in FHS Men**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>rs1007311</th>
<th>rs3918174</th>
<th>rs1800780</th>
<th>rs3918188</th>
<th>rs891511</th>
<th>rs3918196</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplotype Frequency</td>
<td>Haplotype Frequency</td>
<td>Haplotype Frequency</td>
<td>Haplotype Frequency</td>
<td>Haplotype Frequency</td>
<td>Haplotype Frequency</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>FMD</td>
<td>0.28</td>
<td>0.84</td>
<td>0.09</td>
<td>0.29</td>
<td>0.39</td>
<td>0.07</td>
</tr>
<tr>
<td>Hyperemic Flow Velocity</td>
<td>0.26</td>
<td>0.18</td>
<td>0.26</td>
<td>0.08</td>
<td>0.03</td>
<td>0.82</td>
</tr>
</tbody>
</table>

For sample sizes, see Table 4. All phenotypes have been adjusted for covariates in Table 2 legend.

*Haplotype P compares single haplotype vs all others combined.

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**TABLE 6. Relations of NOS3 Block 3 Haplotypes to FMD and Hyperemic Flow Velocity in FHS Men**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>rs753482</th>
<th>rs1065299</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplotype Frequency</td>
<td>Haplotype Frequency</td>
<td>Haplotype Frequency</td>
</tr>
<tr>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>FMD</td>
<td>0.45</td>
<td>0.002</td>
</tr>
<tr>
<td>Hyperemic Flow Velocity</td>
<td>0.45</td>
<td>0.02</td>
</tr>
</tbody>
</table>

For sample sizes, see Table 4. All phenotypes have been adjusted for the covariates in Table 2 legend.

*Haplotype P compares single haplotype vs all others combined.**
significant association findings with the same alleles with the same direction of effect.

Study Limitations and Strengths

Our study has several limitations. There remains the possibility that multiple rare NOS3 mutations influence endothelial function measures. The allelic spectrum for complex traits such as FMD and hyperemic flow velocity is unknown. Because most human heterozygosity is accounted for by common variants and susceptibility alleles for common disease (and related subclinical disease measures) may not be under strong negative selection, common variants may contribute to common disease traits. Approach is most suitable for the discovery of such variants.

Second, the optimal methodology to select a nonredundant tag SNP set at a locus is still evolving. We tested the performance of our set using several approaches; the tag SNPs we selected captured the majority of common variation at the NOS3 locus. Third, our cohort was middle-aged to elderly and white, potentially limiting generalizability to individuals who are younger or of other ethnicities/races.

Fourth, insufficient sample size or random measurement error may have limited our power to detect phenotype-genotype association. Although accepted as an in vivo measure of endothelial function, measurement of brachial artery FMD is subject to a number of well-recognized pitfalls and limitations. In particular, using below-elbow blood pressure cuff position may have led to low FMD values and less biological variability. To address this issue, we performed the following power calculations on the basis of our data. We set type I error at α=0.0028 (ie, α=0.05/18 due to 18 SNPs), fixed sex-specific sample sizes at 700 for FMD (600 for hyperemic flow velocity), and assumed that covariates explained 15% of interindividual variation in FMD (35% for hyperemic flow velocity). Given these conditions, we had 80% power to detect any SNP that is expected to account for 2.1% of interindividual variability in FMD (for hyperemic flow velocity, 1.9%). To place our power to detect phenotype-genotype associations in context, systolic blood pressure and age explained 3.2% to 12.4% of variance in FMD and 6.0% to 26.3% for hyperemic flow velocity, whereas cardiovascular disease explained 1.8% of variance in hyperemic flow velocity among men. If the true effects of SNPs on phenotypes were smaller, we would have had lower power and we may have failed to detect true associations.

Finally, by controlling the false-positive rate through empirical methods, we may have increased our false-negative rate. Rigid adherence to an empirical P<0.05 significance threshold across a study could be overly conservative and obscure some true positive associations.

Study strengths distinguishing the present investigation include the characterization of NOS3 genetic variation in reference pedigrees, genotyping of a comprehensive set of common variants in a large community-based cohort with routinely ascertained phenotypes and covariates, sex-specific analyses, the inclusion of both single variant and haplotype association, and multiple statistical testing assessments.

Conclusion

We created a dense SNP map at the NOS3 locus and defined a parsimonious set of SNPs that captured common genetic variation. Overall, we did not observe statistically significant relations of NOS3 sequence variants with endothelial function measures in men or women in our sample after accounting for multiple testing. The nominal associations of select NOS3 variants with endothelial function in men may provide hypotheses for further testing in other samples or in different experimental designs. In addition, our results provide the tools necessary to comprehensively test common genetic variation at NOS3 for association with other phenotypes potentially influenced by NO such as hypertension and incident coronary heart disease.

Acknowledgments

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Common Genetic Variation at the Endothelial Nitric Oxide Synthase Locus and Relations to Brachial Artery Vasodilator Function in the Community

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