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NOS3 polymorphisms, cigarette smoking, and cardiovascular disease risk:

The Atherosclerosis Risk in Communities study

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Abstract

Objective—Endothelial nitric oxide synthase (*NOS3*) activity and cigarette smoking significantly influence endothelial function. We sought to determine whether cigarette smoking modified the association between *NOS3* polymorphisms and risk of coronary heart disease or stroke.

Methods—All 1085 incident coronary heart disease cases, all 300 incident ischemic stroke cases, and 1065 reference individuals from the Atherosclerosis Risk in Communities study were genotyped for the *T-786C* and *E298D* polymorphisms in *NOS3*. Using a case–cohort design, associations between genotype/haplotype and disease risk were evaluated by multivariable proportional hazards regression. Multiplicative scale interaction testing evaluated the influence of cigarette smoking history at baseline on these associations.

Results—In Caucasians, association between *E298D* genotype and risk of coronary heart disease was significantly modified by current smoking status (interaction $P = 0.013$), with the highest risk observed in smokers carrying the variant *D298* allele relative to nonsmokers carrying two *E298* alleles (adjusted hazard rate ratio 2.07, 95% confidence interval 1.39−3.07). In African-Americans, association between *T-786C* genotype and risk of ischemic stroke was significantly modified by pack-year smoking history (interaction $P = 0.037$), with the highest risk observed in ≥ 20 pack-year smokers carrying the variant *C-786* allele relative to < 20 pack-year smokers carrying two *T-786* alleles (adjusted hazard rate ratio 4.03, 95% confidence interval 1.54−10.6).

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Conclusions—An interaction between the *E298D* and *T-786C* polymorphisms in *NOS3*, cigarette smoking, and risk of incident coronary heart disease and ischemic stroke events appears to exist, suggesting a potential complex interplay between genetic and environmental factors and cardiovascular disease risk.

Keywords

coronary artery disease; endothelial nitric oxide synthase; polymorphism; smoking; stroke

Introduction

Ischemic cardiovascular disease is a major public health problem. It is estimated that 1.2 million Americans will experience an acute coronary heart disease (CHD) event and 0.7 million will experience an acute stroke event this year [1]. Endothelial dysfunction is integrally involved in the pathogenesis of ischemic cardiovascular disease, and has been associated with an increased risk of myocardial infarction and stroke [2,3].

Nitric oxide is synthesized by endothelial nitric oxide synthase (eNOS, *NOS3*) in the vasculature, where it possesses potent vasodilatory [4] and anti-inflammatory [5] effects. Impairment in eNOS-mediated nitric oxide biosynthesis contributes to endothelial dysfunction and promotes the development of atherosclerosis [6,7]. Cigarette smoking also contributes to endothelial dysfunction and atherosclerosis [8], eliciting many of its deleterious endothelial effects via attenuation of nitric oxide biosynthesis and activity [9,10].

Multiple genetic polymorphisms in *NOS3* have been discovered, including the *T-786C* and *E298D* variants that have been associated with reduced nitric oxide biosynthesis [11,12]. Numerous clinical studies evaluating potential associations between *NOS3* polymorphisms and cardiovascular disease risk have reported inconsistent results [13]; however, few have specifically evaluated the contribution of cigarette smoking, even though smoking may modify the influence of these polymorphisms on endothelial function [14−17]. The primary aim of our investigation was to determine whether exposure to cigarette smoke significantly modified the association between presence of the *T-786C* or *E298D* polymorphisms in *NOS3* and risk of incident CHD or ischemic stroke events in individuals enrolled in the biethnic Atherosclerosis Risk in Communities (ARIC) study.

Methods

Study population

Participants were selected from the ARIC study, a longitudinal, population-based cohort study of 15 792 men and women aged 45−64 years from four US communities (Forsyth County, North Carolina; Jackson, Mississippi; Minneapolis, Minnesota; and Washington County, Maryland) enrolled between 1987 and 1989 [18]. Since enrollment, participants have been followed prospectively via annual phone interviews, clinic examinations approximately every 3 years through 1998, and ongoing abstraction of hospital and death certificate records. The study protocol was approved by the Institutional Review Board of each center, and consent was obtained from each participant.

Ascertainment of incident coronary heart disease and stroke cases

All incident cases that occurred between baseline and December 31, 1998 were evaluated (median follow-up 9.1 years), excluding participants with a history of CHD or stroke at baseline. Incident CHD (n = 1085) was defined as (1) definite or probable myocardial infarction (n = 520), (2) electrocardiographic evidence of silent myocardial infarction (n = 112), (3)

definite CHD death (n = 110), or (4) coronary revascularization procedure (n = 343). Incident stroke was defined as a definite or probable ischemic stroke (n = 300).

The ascertainment of cases and criteria for classification have been previously described [19, 20]. All potential events were systematically reviewed and adjudicated by the ARIC Morbidity and Mortality Classification Committee [18−20]. Hospitalized myocardial infarction was classified as definite or probable on the basis of chest pain symptoms, cardiac enzyme levels, and electrocardiographic changes. Definite CHD death was classified on the basis of chest pain symptoms, underlying cause of death, hospitalization records, and medical history. Coronary revascularization procedures included coronary artery bypass grafting and percutaneous coronary intervention. Using the National Survey of Stroke criteria [21], a stroke event was classified as definite or probable if there was (1) rapid onset of neurological symptoms that lasted > 24 h or led to death within 24 h, (2) no evidence of pathology that could have mimicked stroke, and (3) one major (e.g. aphasia or hemiparesis) or two minor (e.g. diplopia or dysarthria) neurological deficits. Qualifying cases were further classified as ischemic (thrombotic brain infarction, cardioembolic stroke) or hemorrhagic (subarachnoid hemorrhage, intracerebral hemorrhage) on the basis of neuroimaging studies and autopsy, when available [20].

Baseline measurements

Detailed demographic, clinical, and biochemical data were obtained from each participant at baseline. Race was self-reported. Prevalent CHD and stroke were defined as history of a physician-diagnosed event. Hypertension was defined as systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or current antihypertensive medication use. Diabetes was defined as fasting blood glucose ≥ 7.0 mmol/l, nonfasting blood glucose ≥ 11.1 mmol/l, physician diagnosis, or pharmacologic treatment. Detailed information on cigarette smoking was obtained through an interview-administered questionnaire, and was defined by current smoking status (yes/no) and pack-year smoking history (≥ 20 or < 20 pack-years).

Cohort random sample

A random sample of all ARIC participants without the history of CHD or stroke at baseline was assembled to serve as the reference group for the case–cohort comparisons [n = 1065; 85 (57 Caucasians, 28 African-Americans) and 29 (12 Caucasians, 17 African-Americans) of which are also incident CHD and stroke cases, respectively]. Sampling of the cohort was stratified on age (≥ 55 or < 55 years), sex, and race (Caucasian or African-American). Sampling proportions varied across each stratum.

Genotyping

Genomic DNA from all incident CHD and ischemic stroke cases and the cohort random sample was genotyped for the T > C polymorphism at nucleotide position −786 in the proximal promoter (*T-786C*) and the G > T polymorphism at nucleotide position 5246 in exon 7 (*E298D*) of *NOS3*, using multiplex matrix-assisted laser desorption/ionization time-of-flight mass spectrometry methods, as described [22]. Briefly, targeted sequences were amplified via polymerase chain reaction using specific forward (*T-786C*: 5′-ACGTTGGATGACTGTAGTTTCCCTAGTCCC-3′; *E298D*: 5′-ACGTTGGATGACCTCAAGGACCAGCTCGG-3′) and reverse (*T-786C*: 5′-ACGTTGGATGAGGTCAGCAGAGAGACTAGG-3′; *E298D*: 5′-ACGTTGGATGAAACGGTCG CTTCGACGTGC-3′) primers for each polymorphism, followed by extension reactions utilizing specific oligonucleotide primers that annealed immediately upstream of each polymorphic site and extended the amplification product by a single base pair in the forward direction (*T-786C*: 5′-CATCAAGCTCTTCCCTGGC-3′; *E298D*: 5′-GCTGCAGGCCCCAGATGA-3′). The mass of the extension reaction products was determined and translated into genotype using the MassARRAY RT software (Sequenom

Inc., San Diego, California, USA). Blind replicates were included for quality control. Missing genotypes were present in < 5% of individuals.

Data analysis

Inverse sampling fractions from each stratum were used as weights in variance estimation of adjusted covariate means and proportions by linear and logistic regression, respectively. Cohort random sample allele frequencies were evaluated for deviation from Hardy–Weinberg equilibrium, and pairwise linkage disequilibrium statistics were calculated (Haploview 3.2; Broad Institute of Harvard and MIT Cambridge, Massachusetts, USA) [23]. Hazard rate ratios (HRRs) and 95% confidence intervals (CIs) for the development of incident CHD or ischemic stroke in relation to genotype were calculated by weighted proportional hazards regression, using Barlow's method to account for the stratified random sampling and case–cohort design [24]. Model 1 included age, sex, and study center as covariates. Model 2 also included significant clinical covariates, which were current smoking status, diabetes, hypertension, high-density lipoprotein cholesterol, total cholesterol, and body mass index for the CHD end point, and current smoking status, diabetes, and hypertension for the stroke end point. Assuming an autosomal dominant mode of inheritance, individuals with one or two variant alleles were combined for comparison with wild-type individuals. All analyses were completed separately in Caucasians and African-Americans. Assuming a case–control design and type I error $\alpha = 0.05$, we had approximately 99 and 90% power for the CHD end point and 90 and 80% power for the stroke end point to detect an odds ratio of 2.0 in Caucasians and African-Americans, respectively.

Haplotypes and their frequencies were estimated using the phase reconstruction method (PHASE 2.1), which assigned the most probable haplotype pair to each individual [25], and compared across case status by χ^2. Associations between haplotype (0 or 1) and risk of CHD or stroke were evaluated using Barlow's method [24], by simultaneously modeling each haplotype relative to the most common haplotype using the model 2 covariate adjustment strategy.

Gene–environment interaction testing was completed on a multiplicative scale between genotype and current smoking status (yes/no) or pack-year smoking history (≥ 20 or < 20 pack-years) at baseline using a Wald χ^2 test for significance of the estimated β-coefficient for the interaction term [26]. Haplotype–smoking interaction testing was also completed in Caucasians for the reconstructed haplotype containing both the variant *C-786* and *D298* alleles; however, this was not completed in African-Americans because of sample size limitations. As interaction hypothesis testing on a multiplicative scale is underpowered, the critical value for statistical significance was set to $\alpha = 0.15$, two-sided [27]. To minimize the impact of the multiple statistical tests conducted in our analysis, we estimated the false discovery rate (FDR) *q*-value for each interaction test separately for the CHD and stroke end points (QVALUE). The FDR *q*-value is defined as the proportion of statistical tests deemed significant that are actually false-positives [28]. Stratified weighted proportional hazards regression was also completed according to baseline current smoking status or pack-year smoking history using the model 2 covariate adjustment strategy (minus current smoking status) to further explore potential interactions.

Results

Study population

Significant baseline differences in various risk factors were observed between CHD and stroke cases and noncases included in the cohort random sample (Table 1). Incident CHD and stroke cases were significantly older and more likely to be men, smokers, diabetic, hypertensive and

have abnormal fasting lipid panels compared with noncases. Stroke cases were also more likely to be African-Americans. In the cohort random sample, 71.5% of current smokers at baseline also had a ≥ 20 pack-year history.

NOS3 genotype

In the cohort random sample, the *T-786C* (0.371 versus 0.151) and *E298D* (0.330 versus 0.112) minor allele frequencies were significantly different in Caucasians and African-Americans, respectively ($P < 0.001$). Minimal evidence of pairwise linkage disequilibrium was observed between these polymorphisms in both Caucasians ($D' = 0.47$, $r^2 = 0.19$) and African-Americans ($D' = 0.41$, $r^2 = 0.12$). The allelic distribution of each polymorphism was in Hardy–Weinberg equilibrium ($P > 0.05$).

E298D polymorphism

The variant *D298* allele was not significantly associated with CHD or ischemic stroke incidence in either Caucasians or African-Americans (Tables 2 and 3), nor were these relationships modified by sex. In Caucasians, the relationship between *E298D* genotype and CHD risk was, however, significantly modified by baseline current smoking status (interaction $P = 0.013$, $q = 0.078$) and tended to be modified by pack-year smoking history (interaction $P = 0.126$, $q = 0.247$), with the highest risk of CHD observed in smokers carrying the variant *D298* allele (Table 4). In the stratified analysis, current smokers with the variant *D298* allele were at significantly greater risk of CHD than smokers with two *E298* alleles (HRR 1.84, 95% CI 1.10 −3.09, $P = 0.021$); however, no association between the variant *D298* allele and CHD risk was observed in nonsmokers (HRR 0.81, 95% CI 0.59−1.10, $P = 0.168$). Current smoking status (interaction $P = 0.762$) and pack-year history (interaction $P = 0.641$) did not modify the relationship between *E298D* genotype and CHD risk in African-Americans (Table 4). Moreover, neither cigarette smoking exposure estimate modified the relationship between *E298D* genotype and risk of ischemic stroke.

T-786C polymorphism

In Caucasians, the variant *C-786* allele tended to be more common in CHD cases compared with noncases (62.7 versus 57.7%, $P = 0.079$) (Table 2), and was associated with a trend toward greater CHD risk relative to *T-786* homozygotes (HRR 1.24, 95% CI 0.96−1.62, $P = 0.106$) (Table 3); although, this did not attain statistical significance. Pack-year smoking history appeared to modify this association, such that in the stratified analysis ≥ 20 pack-year smokers carrying the variant *C-786* allele were at significantly greater risk of CHD relative to ≥ 20 pack-year smokers with two *T-786* alleles (HRR 1.52, 95% CI 1.01−2.27, $P = 0.042$). No association was observed between the variant *C-786* allele and CHD risk in < 20 pack-year smokers (HRR 1.06, 95% CI 0.75− 1.51, $P = 0.731$). In African-Americans, no association between the variant *C-786* allele and risk of CHD was observed ($P = 0.421$) (Table 3); however, this relationship tended to be modified by current smoking status (interaction $P = 0.142$, $q = 0.444$) (Table 5).

In African-Americans, the variant *C-786* allele tended to be more common in incident stroke cases compared with noncases (39.5 versus 30.3%, $P = 0.099$) (Table 2), and was associated with a trend toward greater risk of stroke relative to *T-786* homozygotes (HRR 1.63, 95% CI 0.93− 2.88, $P = 0.090$) (Table 3); although this did not attain statistical significance. This relationship was not modified by sex. Cigarette smoking exposure, however, significantly modified this association (interaction $P = 0.037$, $q = 0.148$), such that the greatest risk of ischemic stroke was observed in ≥ 20 pack-year smokers carrying the variant *C-786* allele (Table 5). In ≥ 20 pack-year smokers, stratified analysis demonstrated that the *C-786* allele was more common in stroke cases compared with noncases (41.9 versus 23.4%, $P = 0.083$), and was associated with significantly higher risk of stroke relative to ≥ 20 pack-year smokers with two *T-786* alleles (HRR 4.23, 95% CI 1.34−13.4, $P = 0.014$). No relationship between

the variant *C-786* allele and risk of stroke was observed in < 20 pack-year smokers (HRR 1.08, 95% CI 0.56−2.10, *P* = 0.813). A similar association was also observed in current smokers carrying the variant *C-786* allele (HRR 2.65, 95% CI 0.99−7.1, *P* = 0.052), but not nonsmokers (HRR 1.16, 95% CI 0.55−2.46, *P* = 0.694). In Caucasians, the variant *C-786* allele was not significantly associated with the risk of ischemic stroke (*P* = 0.665) (Table 3), nor was this relationship modified by current smoking status (interaction *P* = 0.892) or pack-year history (interaction *P* = 0.488) (Table 5).

NOS3 haplotypes

Four potential haplotypes were reconstructed, and in Caucasians only haplotype *CG* (uniquely tagged by the variant *C-786* allele) was significantly more frequent in CHD cases compared with noncases (16.1 versus 13.1%, *P* = 0.034) (Table 6) and was associated with a trend toward greater CHD risk relative to the most common haplotype *TG* (HRR 1.34, 95% CI 0.97−1.85, *P* = 0.072); although this did not attain statistical significance. No significant differences in haplotype copy frequency were observed across CHD case status in African-Americans, or across stroke case status in Caucasians and African-Americans (Table 6).

Haplotype *CT* (uniquely tagged by both the variant *C-786* and *D298* alleles) was not significantly associated with risk of CHD relative to haplotype *TG* (HRR 1.16, 95% CI 0.88 −1.53, *P* = 0.296). Although no statistically significant interaction between haplotype *CT* and either current smoking status (interaction *P* = 0.179) or pack-year history (interaction *P* = 0.206) was observed, smokers carrying at least one copy of haplotype *CT* appeared to demonstrate higher risk of CHD than nonsmokers without this haplotype (Table 7). In the stratified analysis, ≥ 20 pack-year smokers carrying haplotype *CT* were at significantly higher risk of developing CHD relative to ≥ 20 pack-year smokers with two haplotype *TG* copies (HRR 1.55, 95% CI 1.02−2.36, *P* = 0.041). No association between haplotype *CT* and CHD risk was, however, observed in < 20 pack-year smokers (HRR 0.91, 95% CI 0.62−1.33, *P* = 0.469). A similar but nonsignificant association was also observed in current smokers carrying haplotype *CT* (HRR 1.73, 95% CI 0.99−3.02, *P* = 0.054), but not nonsmokers (HRR 1.01, 95% CI 0.72−1.42, *P* = 0.937).

Haplotype *CT* was not significantly associated with risk of ischemic stroke relative to haplotype *TG* (HRR 1.03, 95% CI 0.67−1.58, *P* = 0.642). Moreover, no significant interaction between haplotype *CT* and either current cigarette smoking status (interaction *P* = 0.309) or pack-year history (interaction *P* = 0.610) was observed (Table 7). In the stratified analysis, statistically significant associations were not observed between haplotype *CT* and risk of ischemic stroke in either current smokers (HRR 2.09, 95% CI 0.76−5.77, *P* = 0.156) or nonsmokers (HRR 0.85, 95% CI 0.52−1.41, *P* = 0.536) relative to haplotype *TG*.

Discussion

Our analysis demonstrated no statistically significant associations between the variant *C-786* or *D298* alleles and risk of either incident CHD or ischemic stroke in Caucasians and African-Americans enrolled in the ARIC study; although a trend towards higher risk was observed with the variant *C-786* allele for CHD in Caucasians and stroke in African-Americans. Importantly, our analysis identified a potential interaction between genetic variation in *NOS3*, cigarette smoking history, and risk of incident CHD and ischemic stroke events in these individuals. Specifically, the variant *C-786* and *D298* alleles appeared to be associated with significantly higher risk of developing CHD or ischemic stroke in current cigarette smokers and/or those with a ≥ 20 pack-year history at baseline, whereas these variant alleles were not significantly associated with disease risk in the absence of the smoking exposure. This interaction was particularly evident with *E298D* genotype and CHD risk in Caucasians, and *T-786C* genotype and ischemic stroke risk in African-Americans.

The role of endothelial dysfunction in the pathogenesis of cardiovascular disease has become increasingly appreciated. Endothelial dysfunction is typically manifested by impairment in endothelial-dependent vasodilation, and is associated with increased risk of myocardial infarction and stroke [2,3]. The vasodilatory, anti-inflammatory, and anti-proliferative properties of nitric oxide are vital mediators of this process, such that impairment in nitric oxide biosynthesis and/or activity elicits a substantial worsening of endothelial function and promotes the development of atherosclerosis [4–7]. Cigarette smoking also contributes significantly to endothelial dysfunction and atherosclerosis [8], in part through its reduction of eNOS-mediated nitric oxide biosynthesis via enhanced oxidative stress and eNOS uncoupling [9,10].

Numerous investigations have evaluated associations between *NOS3* polymorphisms and cardiovascular disease. Most have focused on the *T-786C* and *E298D* polymorphisms owing to associations between the variant alleles and reduced nitric oxide biosynthesis [11,12]; however, results have been inconsistent [13]. Importantly, few investigations have evaluated incident clinical events. Moreover, few have considered the potential influence of cigarette smoking despite their potential synergistic contribution to worsening endothelial function. For instance, the variant *C-786* allele has been significantly associated with risk of coronary spasm [15] and decreased cerebral blood flow [16] in smokers, but not nonsmokers. The variant *D298* allele has also been associated with significantly lower endothelial-dependent vasodilation in smokers, with no significant effect in nonsmokers [17]. Interestingly, the *D298* allele did not influence endothelial-dependent vasodilation in healthy volunteers despite being associated with reduced systemic nitric oxide levels [29]. These data suggest that presence of established underlying endothelial dysfunction, as observed in cigarette smokers, may be necessary for these polymorphisms to significantly attenuate endothelial function and predispose patients to increased cardiovascular risk. Our findings demonstrating a potential interaction between the *T-786C* and *E298D* polymorphisms, cigarette smoking, and risk of CHD and stroke events are consistent with this hypothesis.

We observed that associations between the variant *D298* and *C-786* alleles and CHD incidence may be significantly modified by exposure to cigarette smoke, such that smokers carrying these variant alleles were at significantly higher risk of developing CHD compared with nonsmokers without these variants. Smokers carrying the reconstructed haplotype containing both variant alleles also appeared to be at greater risk of CHD. Moreover, pack-year history significantly modified the association between the *C-786* allele and ischemic stroke risk in African-Americans. Collectively, we believe that these data suggest potential existence of a complex interplay between *NOS3* polymorphisms, cigarette smoking exposure, endothelial function, and cardiovascular disease risk.

Although our study evaluated rigorously ascertained incident events, we are unable to elucidate mechanisms underlying the observed statistical interactions. We also cannot rule out that the *T-786C* and *E298D* polymorphisms are simply markers in linkage disequilibrium with the true causative loci. We confirmed that these polymorphisms are, however, not in significant linkage disequilibrium with each other [13]. A previous epidemiological study reported a significant interaction between the *NOS3* 27-base pair repeat polymorphism in intron 4, smoking history, and risk of angiographically documented CHD [30]. Although we did not evaluate this polymorphism in our population, previous studies have demonstrated that it is not in significant linkage disequilibrium with either the *E298D* or *T-786C* polymorphisms [13]. Our analysis also identified a potential haplotype–smoking interaction. As haplotypes are inferred, some uncertainty exists in the haplotype assigned to each individual. Only two polymorphisms were, however, included in haplotype reconstruction, posterior haplotype probabilities exceeded 0.83 in all individuals (approximately 75% had a probability of 1.0), and similar haplotype distributions were observed using the expectation-maximization algorithm [23]. Unfortunately, validation of reported smoking status via biomarkers of tobacco exposure, such

as urinary cotinine, was not completed in the ARIC study; however, misclassification of smoking status was expected to be minor and more likely to involve a smoker who failed to report cigarette consumption in small amounts. Moreover, previous ARIC analyses evaluating smoking and risk of cardiovascular disease outcomes have been consistent with other populations [31]. Thus, it is unlikely that misclassification bias, if present, would have a detectable impact on our estimates.

We acknowledge it is difficult to gauge the statistical significance of these findings considering the number of comparisons completed in our analysis, and recognize the undesirable consequences of reporting false-positive findings. Moreover, some of our reported HRRs were imprecise because of small sample sizes and limited power, particularly in our African-American and stroke subsets. Even though statistical significance was, however, not demonstrated with every comparison, we believe our evaluation of two functionally relevant polymorphisms (*T-786C* and *E298D*), two measures of cigarette smoking exposure (current smoking status and pack-year history), and two incident cardiovascular disease end points (CHD and ischemic stroke) in Caucasian and African-American individuals enrolled in the rigorously characterized ARIC study cohort provided a conservative mechanism to characterize this potential gene–environment interaction at the population level. Overall, we believe that the internal consistency of our results argue in favor of their validity, albeit at the cost of an increased number of statistical comparisons. In order to minimize the impact of the multiple tests completed in our analysis, we also assessed the FDR of our interaction test results. The estimated *q*-values for the interactions observed with *E298D* genotype and CHD risk in Caucasians ($q = 0.078$) and *T-786C* genotype and ischemic stroke risk in African-Americans ($q = 0.148$) enhance our confidence in the conclusions drawn from these findings. Moreover, previous studies have demonstrated that the *T-786C* and *E298D* polymorphisms are particularly associated with endothelial dysfunction in cigarette smokers [15−17], suggesting that existence of a causal interaction between genetic variation in *NOS3* and smoking in the pathogenesis of CHD and stroke is biologically plausible. Our observation of significant gene–smoking interactions in the absence of significant genotype–disease associations independent of the interaction (i.e. main effects), however, suggests that these findings should be interpreted with caution. Future studies in different populations will undoubtedly be required to validate our findings and improve our understanding of the complex relationship between *NOS3*, cigarette smoking, and ischemic cardiovascular disease.

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References

1. American Heart Association. Heart disease and stroke statistics-2004 update. Dallas, TX: 2003.
2. Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, et al. Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002;6:653–658. [PubMed: 12163423]
3. Targonski PV, Bonetti PO, Pumper GM, Higano ST, Holmes DR Jr, Lerman A. Coronary endothelial dysfunction is associated with an increased risk of cerebrovascular events. Circulation 2003;22:2805–2809. [PubMed: 12771004]

4. Ohashi Y, Kawashima S, Hirata K, Yamashita T, Ishida T, Inoue N, et al. Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. J Clin Invest 1998;12:2061–2071. [PubMed: 9854041]
5. De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA Jr, et al. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. J Clin Invest 1995;1:60–68. [PubMed: 7542286]
6. Knowles JW, Reddick RL, Jennette JC, Shesely EG, Smithies O, Maeda N. Enhanced atherosclerosis and kidney dysfunction in eNOS(−/−) Apoe(−/−) mice are ameliorated by enalapril treatment. J Clin Invest 2000;4:451–458. [PubMed: 10683374]
7. Kawashima S, Yokoyama M. Dysfunction of endothelial nitric oxide synthase and atherosclerosis. Arterioscler Thromb Vasc Biol 2004;6:998–1005. [PubMed: 15001455]
8. Ambrose JA, Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease: an update. J Am Coll Cardiol 2004;10:1731–1737. [PubMed: 15145091]
9. Barua RS, Ambrose JA, Eales-Reynolds LJ, DeVoe MC, Zervas JG, Saha DC. Dysfunctional endothelial nitric oxide biosynthesis in healthy smokers with impaired endothelium-dependent vasodilatation. Circulation 2001;16:1905–1910. [PubMed: 11602492]
10. Barua RS, Ambrose JA, Srivastava S, DeVoe MC, Eales-Reynolds LJ. Reactive oxygen species are involved in smoking-induced dysfunction of nitric oxide biosynthesis and upregulation of endothelial nitric oxide synthase: an *in vitro* demonstration in human coronary artery endothelial cells. Circulation 2003;18:2342–2347. [PubMed: 12707237]
11. Miyamoto Y, Saito Y, Nakayama M, Shimasaki Y, Yoshimura T, Yoshimura M, et al. Replication protein a1 reduces transcription of the endothelial nitric oxide synthase gene containing a – 786T → C mutation associated with coronary spastic angina. Hum Mol Genet 2000;18:2629–2637. [PubMed: 11063722]
12. Tesauro M, Thompson WC, Rogliani P, Qi L, Chaudhary PP, Moss J. Intracellular processing of endothelial nitric oxide synthase isoforms associated with differences in severity of cardiopulmonary diseases: Cleavage of proteins with aspartate versus Glutamate at position 298. Proc Natl Acad Sci USA 2000;6:2832–2835. [PubMed: 10717002]
13. Casas JP, Bautista LE, Humphries SE, Hingorani AD. Endothelial nitric oxide synthase genotype and ischemic heart disease: meta-analysis of 26 studies involving 23028 subjects. Circulation 2004;11:1359–1365. [PubMed: 15007011]
14. Wang J, Dudley D, Wang XL. Haplotype-specific effects on endothelial no synthase promoter efficiency: modifiable by cigarette smoking. Arterioscler Thromb Vasc Biol 2002;5:e1–e4. [PubMed: 12006409]
15. Nakayama M, Yoshimura M, Sakamoto T, Shimasaki Y, Nakamura S, Ito T, et al. Synergistic interaction of T-786 → C polymorphism in the endothelial nitric oxide synthase gene and smoking for an enhanced risk for coronary spasm. Pharmacogenetics 2003;11:683–688. [PubMed: 14583681]
16. Nasreen S, Nabika T, Shibata H, Moriyama H, Yamashita K, Masuda J, et al. T-786C polymorphism in endothelial no synthase gene affects cerebral circulation in smokers: possible gene–environmental interaction. Arterioscler Thromb Vasc Biol 2002;4:605–610. [PubMed: 11950698]
17. Leeson CP, Hingorani AD, Mullen MJ, Jeerooburkhan N, Kattenhorn M, Cole TJ, et al. Glu298Asp endothelial nitric oxide synthase gene polymorphism interacts with environmental and dietary factors to influence endothelial function. Circ Res 2002;11:1153–1158. [PubMed: 12065317]
18. The ARIC Investigators. The Atherosclerosis Risk in Communities (ARIC) study: design and objectives. Am J Epidemiol 1989;4:687–702.
19. White AD, Folsom AR, Chambless LE, Sharret AR, Yang K, Conwill D, et al. Community surveillance of coronary heart disease in the Atherosclerosis Risk in Communities (ARIC) study: methods and initial two years' experience. J Clin Epidemiol 1996;2:223–233. [PubMed: 8606324]
20. Rosamond WD, Folsom AR, Chambless LE, Wang CH, McGovern PG, Howard G, et al. Stroke incidence and survival among middle-aged adults: 9-year follow-up of the Atherosclerosis Risk in Communities (ARIC) cohort. Stroke 1999;4:736–743. [PubMed: 10187871]
21. The National Survey of Stroke. National Institute of Neurological and Communicative Disorders and Stroke. Stroke 1981;2(Pt 2 Suppl 1):I1–91.

22. Bray MS, Boerwinkle E, Doris PA. High-throughput multiplex SNP genotyping with MALDI-TOF mass spectrometry: practice, problems and promise. Hum Mutat 2001;4:296–304. [PubMed: 11295828]

23. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;2:263–265. [PubMed: 15297300]

24. Barlow WE, Ichikawa L, Rosner D, Izumi S. Analysis of case–cohort designs. J Clin Epidemiol 1999;12:1165–1172. [PubMed: 10580779]

25. Stephens M, Donnelly P. A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet 2003;5:1162–1169. [PubMed: 14574645]

26. Li R, Folsom AR, Sharrett AR, Couper D, Bray M, Tyroler HA. Interaction of the glutathione *S*-transferase genes and cigarette smoking on risk of lower extremity arterial disease: The Atherosclerosis Risk in Communities (ARIC) study. Atherosclerosis 2001;3:729–738. [PubMed: 11257276]

27. Hosmer, D.; Lemeshow, S. Applied logistic regression. John Wiley & Sons; New York: 1989.

28. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci USA 2003;16:9440–9445. [PubMed: 12883005]

29. Sofowora G, Dishy V, Xie HG, Imamura H, Nishimi Y, Morales CR, et al. *In-vivo* effects of Glu298Asp endothelial nitric oxide synthase polymorphism. Pharmacogenetics 2001;9:809–814. [PubMed: 11740345]

30. Wang XL, Sim AS, Badenhop RF, McCredie RM, Wilcken DE. A smoking-dependent risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene. Nat Med 1996;1:41–45. [PubMed: 8564837]

31. Howard G, Wagenknecht LE, Burke GL, Diez-Roux A, Evans GW, McGovern P, et al. Cigarette smoking and progression of atherosclerosis: The Atherosclerosis Risk in Communities (ARIC) study. JAMA 1998;2:119–124. [PubMed: 9440661]

Table 1

Baseline characteristics by incident case status

Characteristics[a]	CRS	CHD cases[b]	Stroke cases[c]
N	1065	1085	300
Sex (%male)	41.4	67.4	53.0
Race (%African-American)	27.2	23.4	45.0
Age (years)	53.8 ± 0.10	55.8 ± 0.17	56.7 ± 0.32
Current smoker (%yes)	24.8	36.1	36.8
Pack-years ≥ 20 (%yes)	29.0	48.6	41.4
Diabetes (%)	11.1	25.1	34.6
Hypertension (%)	29.8	49.4	66.0
SBP (mmHg)	120.4 ± 0.58	128.3 ± 0.63	134.8 ± 1.30
DBP (mmHg)	73.3 ± 0.37	76.2 ± 0.38	79.4 ± 0.81
BMI (kg/m^2)	27.7 ± 0.20	28.3 ± 0.15	29.0 ± 0.31
HDL cholesterol (mmol/l)	1.38 ± 0.02	1.12 ± 0.01	1.25 ± 0.02
LDL cholesterol (mmol/l)	3.46 ± 0.03	3.92 ± 0.03	3.75 ± 0.07
Total cholesterol (mmol/l)	5.48 ± 0.03	5.86 ± 0.03	5.76 ± 0.08
Triglycerides (mmol/l)	1.41 ± 0.03	1.87 ± 0.04	1.73 ± 0.07

CRS, cohort random sample; Pack-years, (cigarettes smoked per day/20) × (years smoked); SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CHD, coronary heart disease.

[a] Characteristics weighted according to the sampling fraction. Data presented as mean ± standard error of the mean.

[b] $P < 0.05$ for comparison of CHD cases to 980 non-cases in the CRS for all characteristics.

[c] $P < 0.05$ for comparison of stroke cases to 1036 non-cases in the CRS for all characteristics.

Table 2

T-786C and *E298D* genotype frequency by incident coronary heart disease (CHD) or stroke case status

Genotype[a]	Caucasian			African-American		
	Noncases	Incident cases	*P*-value	Noncases	Incident cases	*P*-value
CHD						
E298D						
G/G	279 (47.2%)	365 (46.7%)		242 (77.3%)	182 (80.2%)	
G/T + T/T	336 (52.8%)	416 (53.3%)	*P*=0.862	70 (22.7%)	45 (19.8%)	*P*=0.437
T-786C						
T/T	248 (42.3%)	276 (37.4%)		212 (68.4%)	155 (74.9%)	
T/C + C/C	362 (57.7%)	463 (62.7%)	*P*=0.079	91 (31.6%)	52 (25.1%)	*P*=0.126
Stroke						
E298D						
G/G	296 (47.0%)	70 (46.7%)		255 (78.5%)	86 (74.1%)	
G/T + T/T	364 (53.0%)	80 (53.3%)	*P*=0.939	68 (21.5%)	30 (25.9%)	*P*=0.362
T-786C						
T/T	268 (42.5%)	56 (39.2%)		223 (69.7%)	66 (60.6%)	
T/C + C/C	386 (57.5%)	87 (60.8%)	*P*=0.466	90 (30.3%)	43 (39.5%)	*P*=0.099

[a]Genotype data presented as absolute (percent) genotype frequency, and weighted according to sampling fraction.

Table 3

Hazard rate ratio between *T-786C* and *E298D* polymorphisms and risk of incident CHD or stroke

Polymorphism	Caucasian HRR	95% CI	*P*-value	African-American HRR	95% CI	*P*-value
CHD						
E298D	*G/T* + *T/T* versus *G/G*			*G/T* + *T/T* versus *G/G*		
Model 1[a]	0.94	0.75−1.17	*P*=0.586	0.87	0.57−1.34	*P*=0.526
Model 2[b]	1.03	0.80−1.33	*P*=0.819	0.66	0.40−1.09	*P*=0.105
T-786C	*T/C* + *C/C* versus *T/T*			*T/C* + *C/C* versus *T/T*		
Model 1[a]	1.13	0.90−1.42	*P*=0.210	0.80	0.52−1.20	*P*=0.279
Model 2[b]	1.24	0.96−1.62	*P*=0.106	0.83	0.53−1.31	*P*=0.421
Stroke						
E298D	*G/T* + *T/T* versus *G/G*			*G/T* + *T/T* versus *G/G*		
Model 1[a]	0.94	0.65−1.36	*P*=0.742	1.50	0.89−2.53	*P*=0.133
Model 2[c]	0.97	0.65−1.44	*P*=0.884	1.35	0.76−2.41	*P*=0.311
T-786C	*T/C* + *C/C* versus *T/T*			*T/C* + *C/C* versus *T/T*		
Model 1[a]	1.06	0.72−1.55	*P*=0.783	1.70	1.05−2.75	*P*=0.032
Model 2[c]	1.09	0.73−1.64	*P*=0.665	1.63	0.93−2.88	*P*=0.090

CHD, coronary heart disease; HRR, hazard rate ratio; CI, confidence interval.

[a] Adjusted for age, sex, and study center.

[b] Adjusted for age, sex, study center, current smoker, diabetes, hypertension, HDL cholesterol, total cholesterol, and body mass index.

[c] Adjusted for age, sex, study center, current smoker, diabetes, and hypertension.

Table 4

E298D genotype by smoking interaction and risk of incident CHD and stroke[a,b]

Smoking exposure	Caucasian *E298D* genotype		Interaction[c]	African-American *E298D* genotype		Interaction[c]
CHD[d]	*G/G n* = 365/279	*G/T* + *T/T n* = 416/335		*G/G n* = 182/241	*G/T* + *T/T n* = 44/70	
Current smoker						
No	1 (referent)	0.83 (0.61−1.13)	2.10 (1.17−3.76)	1 (referent)	0.63 (0.34−1.16)	1.17 (0.43−3.18)
Yes	1.19 (0.77−1.83)	2.07 (1.39−3.07)	*P*=0.013	1.90 (1.17−3.10)	1.40 (0.62−3.18)	*P*=0.762
Pack-years						
< 20 years	1 (referent)	0.84 (0.61−1.17)	1.50 (0.89−2.53)	1 (referent)	0.61 (0.35−1.06)	1.30 (0.43−3.90)
≥ 20 years	1.23 (0.83−1.83)	1.56 (1.08−2.24)	*P*=0.126	1.19 (0.73−1.93)	0.94 (0.35−2.50)	*P*=0.641
Stroke[e]	*G/G n* = 70/296	*G/T* + *T/T n* = 80/363		*G/G n* = 85/254	*G/T* + *T/T n* = 30/68	
Current smoker						
No	1 (referent)	0.81 (0.51−1.30)	1.70 (0.71−4.06)	1 (referent)	1.26 (0.57−2.77)	1.24 (0.37−4.19)
Yes	1.58 (0.83−3.00)	2.18 (1.19−3.99)	*P*=0.234	2.00 (1.08−3.69)	3.12 (1.22−7.99)	*P*=0.726
Pack-years						
< 20 years	1 (referent)	0.80 (0.46−1.38)	1.48 (0.66−3.31)	1 (referent)	1.36 (0.67−2.74)	1.00 (0.25−4.01)
≥ 20 years	1.49 (0.81−2.75)	1.77 (0.98−3.21)	*P*=0.337	1.39 (0.70−2.75)	1.88 (0.53−6.62)	*P*=0.999

CHD, coronary heart disease; HRR, hazard rate ratio; CI, confidence interval.

[a] Number of cases/noncases in each genotype cell.

[b] Data presented as HRR (95% CI).

[c] Multiplicative *E298D* by smoking interaction term HRR (95% CI), and *P*-value.

[d] Adjusted for age, sex, study center, diabetes, hypertension, HDL cholesterol, total cholesterol, and body mass index.

[e] Adjusted for age, sex, study center, diabetes, and hypertension.

Table 5

T-786C genotype by smoking interaction and risk of incident CHD and stroke[a,b]

Smoking exposure	Caucasian *T-786C* genotype		Interaction[c]	African-American *T-786C* genotype		Interaction[c]
CHD[d]	*T/T n* = 276/247	*T/C* + *C/C n* = 463/362		*T/T n* = 154/212	*T/C* + *C/C n* = 52/90	
Current smoker						
No	1 (referent)	1.33 (0.97−1.81)	0.83 (0.46−1.49)	1 (referent)	0.60 (0.34−1.07)	2.04 (0.79−5.31)
Yes	1.92 (1.21−3.05)	2.11 (1.41−3.17)	*P*=0.527	1.68 (1.00−2.83)	2.08 (0.95−4.54)	*P*=0.142
Pack-years						
< 20 years	1 (referent)	1.08 (0.77−1.50)	1.44 (0.85−2.44)	1 (referent)	0.66 (0.39−1.11)	1.88 (0.68−5.15)
≥ 20 years	1.22 (0.79−1.88)	1.89 (1.32−2.70)	*P*=0.179	1.07 (0.63−1.82)	1.32 (0.58−3.00)	*P*=0.222
Stroke[e]	*T/T n* = 56/267	*T/C* + *C/C n* = 87/386		*T/T n* = 65/223	*T/C* + *C/C n* = 43/89	
Current smoker						
No	1 (referent)	1.09 (0.66−1.79)	0.94 (0.39−2.28)	1 (referent)	1.34 (0.66−2.72)	1.49 (0.45−4.96)
Yes	2.17 (1.06−4.43)	2.22 (1.20−4.11)	*P*=0.892	1.86 (0.94−3.68)	3.72 (1.39−9.99)	*P*=0.515
Pack-years						
< 20 years	1 (referent)	0.94 (0.54−1.65)	1.33 (0.59−3.02)	1 (referent)	1.06 (0.55−2.06)	3.73 (1.08−12.9)
≥ 20 years	1.56 (0.80−3.04)	1.97 (1.09−3.57)	*P*=0.488	1.02 (0.49−2.13)	4.03 (1.54−10.6)	*P*=0.037

CHD, coronary heart disease; HRR, hazard rate ratio; CI, confidence interval.

[a] Number of cases/noncases in each genotype cell.

[b] Data presented as HRR (95% CI).

[c] Multiplicative *T-786C* by smoking interaction term HRR (95% CI), and *P*-value.

[d] Adjusted for age, sex, study center, diabetes, hypertension, HDL cholesterol, total cholesterol, and body mass index.

[e] Adjusted for age, sex, study center, diabetes, and hypertension.

Table 6

NOS3 haplotype frequency by incident coronary heart disease (CHD) or stroke case status

Haplotype[a]	CHD Noncase[b]	CHD Incident case[b]	CHD *P*-value[c]	Stroke Noncase[b]	Stroke Incident case[b]	Stroke *P*-value[c]
Caucasian[d]	*n* = 606	*n* = 735		*n* = 650	*n* = 142	
TG	54.4%	53.0%	*P*=0.494	54.2%	53.9%	*P*=0.935
CT	23.0%	23.0%	*P*=0.987	23.0%	22.5%	*P*=0.870
C*G*	13.1%	16.1%	*P*=0.034	13.2%	14.8%	*P*=0.496
*T****T***	9.6%	8.0%	*P*=0.160	9.7%	8.8%	*P*=0.648
African-American[d]	*n* = 303	*n* = 207		*n* = 313	*n* = 109	
TG	78.8%	79.2%	*P*=0.876	79.3%	72.9%	*P*=0.069
CT	6.5%	3.9%	*P*=0.076	6.0%	7.3%	*P*=0.519
CG	9.9%	10.1%	*P*=0.891	9.8%	13.8%	*P*=0.145
TT	4.9%	6.8%	*P*=0.230	4.8%	6.0%	*P*=0.536

[a] Haplotype frequencies weighted according to cohort random sample strata. Tagging polymorphisms are in bold.

[b] Estimated copy frequency of each haplotype.

[c] χ^2 *P*-value for the distribution of each haplotype by CHD or stroke case status.

[d] Haplotype includes the *T-786C* and *E298D* polymorphisms, respectively.

Table 7

NOS3 haplotype by smoking interaction and risk of incident CHD and stroke in Caucasians[a,b]

Smoking exposure	Haplotype		Interaction[c]
CHD[d]	*Non-CT n* = 430/350	*CT n* = 305/256	
Current smoker			
No	1 (referent)	1.00 (0.74−1.35)	1.50 (0.83−2.70)
Yes	1.46 (1.00−2.13)	2.18 (1.40−3.38)	*P*=0.179
Pack-years			
< 20 years	1 (referent)	0.95 (0.68−1.33)	1.39 (0.84−2.30)
≥ 20 years	1.32 (0.93−1.87)	1.74 (1.21−2.51)	*P*=0.206
Stroke[e]	*Non-CT n* = 84/374	*CT n* = 58/276	
Current smoker			
No	1 (referent)	0.87 (0.53−1.45)	1.59 (0.65−3.86)
Yes	1.67 (0.92−3.04)	2.32 (1.18−4.51)	*P*=0.309
Pack-years			
< 20 years	1 (referent)	0.92 (0.52−1.63)	1.24 (0.54−2.80)
≥ 20 years	1.70 (0.98−2.94)	1.93 (0.99−3.74)	*P*=0.610

CHD, coronary heart disease; HRR, hazard rate ratio; CI, confidence interval.

[a] Number of cases/noncases in each haplotype cell.

[b] Data presented as HRR (95% CI).

[c] Multiplicative haplotype by smoking interaction term HRR (95% CI), and *P*-value.

[d] Adjusted for age, sex, study center, diabetes, hypertension, HDL cholesterol, total cholesterol, and body mass index.

[e] Adjusted for age, sex, study center, diabetes, and hypertension.