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Letter to the Editor

No evidence for involvement of the human inducible nitric oxide synthase gene in susceptibility to coronary artery disease

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Nitric oxide (NO) has been implicated in various aspects of atherosclerosis and was shown to possess both protective and cytotoxic properties (1). While endothelial nitric oxide synthase (eNOS) is thought to preserve endothelial function (2), inducible nitric oxide synthase (iNOS) is involved in inflammation and in the defense against infectious organisms (3, 4). However, NO can also have damaging effects during acute and chronic inflammatory conditions (4, 5). NO reacts with several cellular oxidants to form highly reactive nitrating radicals, which can oxidize lipids and proteins (6). Hence, temporarily controlled iNOS expression is thought to play a role in the defense against infectious organisms, while chronic expression may be associated with inflammation (7). Consistent with the inflammatory process of atherosclerosis, iNOS expression was detected in human and rabbit atherosclerotic lesions and colocalized with signs of lipid oxidation, suggesting that iNOS may influence atherosclerosis (8–10). In contrast, increased endothelial iNOS expression may overcome the reduced eNOS expression of dysfunctional endothelium and hence may restore endothelial function (2). Hence, the influence of iNOS expression in different cell types in the artery wall on atherosclerosis is not yet clear.

Two potentially functional polymorphisms, a 4-bp insertion/deletion (+/−) in an AAAT/AAAAT repeat at position −756 to −716 and a pentanucleotide CCTTT repeat located 2.5 kbp upstream of the transcription start site, have been detected in the promoter region of the *iNOS* gene. Recent in vitro studies suggest that the +allele of the 4-bp repeat polymorphism has a 25-fold higher transcriptional activity than the – allele in certain cell types (11). Alleles with 14 repeats of the CCTTT pentameric polymorphism were also shown to respond with higher expression upon interleukin 1β cytokine stimulation compared to alleles with shorter pentameric repeats (12).

To study the potential role of *iNOS* in atherosclerosis, we investigated the association of these two *iNOS* polymorphisms with coronary artery disease (CAD) in a case-control study involving 498 Caucasians (342 men and 156 women). The study was approved by the local Ethics Committee and all participants gave written informed consent to participate. The sample consisted of 260 consecutive patients with angiographically documented CAD with >50% stenosis in at least one coronary artery. The control group consisted of 238 subjects without a history of CAD, stroke, or peripheral vascular disease and was recruited from angiographically negative individuals and the general population. Controls were slightly younger than cases (59.6±10.5 vs. 63.3±10.0 years) and included more females (42% vs. 21%). However, adjustment for age and sex did not alter the results of our analysis. All the major known risk factors that are not affected by CAD medication were associated with CAD. History of hypertension (46.7% vs. 28.8%), diabetes (20.7% vs. 3.5%) and smoking (73.6% vs. 47.6%) was found significantly more often in cases than in controls. The body mass index (27.7±4.6 vs. 25.9±4.1 kg/m^2) was higher in cases and high-density lipoprotein cholesterol was lower in cases than in controls (1.28±0.33 vs. 1.67±0.47 mmol/L). Due to the high proportion of cases on lipid-lowering drugs (81%), plasma total cholesterol (5.2±1.3 vs. 5.9±1.0 mmol/L) and low-density lipoprotein cholesterol (3.2±1.1 vs. 3.5±0.9 mmol/L) were lower in CAD patients than in controls.

For both polymorphisms, the genotype frequencies in controls were in agreement with those predicted by the Hardy-Weinberg equilibrium and were similar to other studies in Caucasians (12–15). The allele frequency for the minor +allele of the 4-bp insertion/deletion polymorphism was 14% in controls (Figure 1A). Allele or genotype distribution was not statistically different in the case group (p=0.74) and carriers of the +allele were not at higher risk for atherosclerosis than non-carriers [odds ratio (OR) 1.10, confidence interval (CI) 0.77–1.58].

A total of 11 alleles were detected for the CCTTT pentameric repeat polymorphism, ranging from 8 to 19 repeats (Figure 1B). Comparison of the genotype

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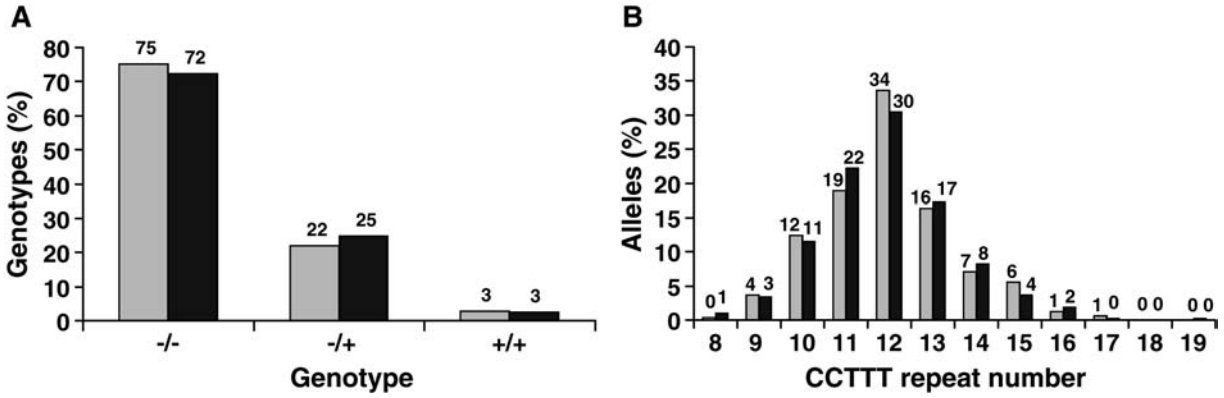

Figure 1 Allele and genotype frequencies of *iNOS* polymorphisms in the case-control study. (A) Genotype distribution of the *iNOS* +/− polymorphism in controls (gray) and in cases (black). (B) Allele distribution of the *iNOS* CCTTT repeat polymorphism in controls (gray) and in cases (black). To amplify the region of the *iNOS* CCTTT repeat polymorphism (accession number AF017634), primer U5553 ACCCCTGGAAGCCTACAACTGCAT and the fluorescent (Joe) labeled primer L5735 GCCACTGCACCCTAGCCTGTCTCA were used. To amplify the region encompassing the 4-bp insertion/deletion (+/−) polymorphism in *iNOS* (accession number D29675), the fluorescent (Joe)-labeled primer U299 CGCTCCAGTCTTGGTGACAGAATGA and L439 GCTTTGGTGGAATGGCAGGTAGGAT were used. The PCR products were then analyzed on an ABI Prism 310 Genetic Analyzer using GeneScan 500 ROX as internal standard and allele assignment was done with ABI Genotyper software (all from Applied Biosystems, Foster City, CA, USA).

distribution between controls and cases revealed no significant difference ($p=0.75$). Since the 14-repeat allele was previously associated with disease, we analyzed the association of this allele with CAD separately. No significant difference between the two groups was detected for the frequency of the 14-repeat allele ($p=0.54$), or when we grouped into alleles containing less than 14 repeats and alleles with 14 repeats and more ($p=0.88$, OR 1.02, CI 0.70–1.48).

Our data support earlier findings of a minor role of the +/− polymorphism in CAD. These studies investigated the association of the +/− polymorphism with the severity of CAD in an autopsy study and an angiographical study. Kunnas et al. (16) investigated the association of the +/− polymorphism in an autopsy study and found that the +/− genotype of *iNOS* was associated with higher stenosis than the −/− genotype. However, this was only true for a subgroup of men >55 years of age and only for the left anterior descending artery. No association of the +/− polymorphism was observed in the right coronary artery and in the left circumflex artery. Morris et al. (14) investigated the association of the +/− polymorphism with the severity of CAD and found no association, even when they tested for an interaction with smoking, which is thought to enhance the oxidative burden associated with increased iNOS expression. However, this investigation showed an association of the +/− polymorphism with angina pectoris. Male carriers of the + allele were more likely to have unstable angina, higher plasma glucose and waist/hip ratios. The + allele may therefore contribute to indices of insulin resistance and angina severity in male CAD patients, but does not play a major role in the pathogenesis of atherosclerosis.

While our data on the +/− *iNOS* polymorphism in CAD support recent findings, we extended the investigation to the CCTTT polymorphism, which was also shown to influence transcription of the *iNOS* gene (12). However, no association with CAD was detected in a logistic regression analysis for CCTTT alleles with >13 repeats, or when we analyzed an association of the 14-repeat allele alone, which was previously shown to have the highest transcriptional activity (12). Hence, the CCTTT polymorphism does not confer a higher risk for CAD.

Although the power of this study is limited, a major effect of the *iNOS* polymorphisms investigated on CAD could have been detected. This study had a power of 80% to detect an odds ratio of 1.6 or larger for the +/− polymorphism and an OR of 1.9 for alleles with ≥14 CCTTT repeats.

In summary, we show that the possibly functional 4-bp insertion/deletion (+/−) and the CCTTT repeat polymorphism in the *iNOS* promoter do not play a major role in the development of atherosclerosis.

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