A Common Variant of the Endothelial Nitric Oxide Synthase (Glu\textsuperscript{298}→Asp) Is a Major Risk Factor for Coronary Artery Disease in the UK

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Background—Endothelium-derived nitric oxide (NO) is synthesised from L-arginine by endothelial nitric oxide synthase (eNOS) encoded by the \textit{NOS 3} gene on chromosome 7. Because reduced NO synthesis has been implicated in the development of coronary atherosclerosis, which has a heritable component, we hypothesised that polymorphisms of \textit{NOS 3} might be associated with increased susceptibility to this disorder.

Methods and Results—Single-strand conformation polymorphism analysis of \textit{NOS 3} identified a G→T polymorphism in exon 7 of the gene which encodes a Glu→Asp amino acid substitution at residue 298 of eNOS. We investigated the relationship between this Glu\textsuperscript{298}→Asp variant and atherosclerotic coronary artery disease (CAD) using 2 independent case-controlled studies. In the first study (CHAOS), cases consisted of 298 unrelated patients with positive coronary angiograms and controls were 138 unrelated healthy individuals ascertained through a population health screen. In the second study (CHAOS II), the cases were 249 patients with recent myocardial infarction (MI), and a further 183 unrelated controls. There was an excess of homozygotes for the Asp298 variant among patients with angiographic CAD, and among patients with recent MI when compared with their respective controls (35.9% versus 10.2%, \textit{P}<0.0001 in CHAOS, and 18.1% versus 8.7%, \textit{P}<0.02 in CHAOS II). In comparison to Glu298 homozygotes, homozygosity for Asp298 was associated with an odds ratio of 4.2 (95% CI, 2.3 to 7.9) for angiographic CAD and 2.5 (95% CI, 1.3 to 4.2) for MI.

Conclusions—Homozygosity for a common \textit{NOS 3} polymorphism (894 G→T) which encodes a Glu\textsuperscript{298}→Asp amino acid substitution in eNOS is a risk factor for angiographic CAD and recent MI in this population. (\textit{Circulation}. 1999;100:1515-1520.)

Key Words: coronary disease ■ myocardial infarction ■ nitric oxide ■ genetics

Nitric oxide (NO) is synthesised from L-arginine and molecular oxygen\textsuperscript{1} by a family of 3 enzymes (the nitric oxide synthases [NOS]).\textsuperscript{2} In the endothelial cell, NO is synthesised by the endothelial (e) NOS encoded by a 26 exon gene (\textit{NOS 3}) located on chromosome 7q35 to 36.\textsuperscript{3} In addition to relaxing vascular smooth muscle cells, endothelium-derived NO inhibits platelet\textsuperscript{4} and leukocyte\textsuperscript{5} adhesion to vascular endothelium, inhibits vascular smooth muscle cell migration\textsuperscript{6} and growth,\textsuperscript{7} and limits the oxidation of atherogenic low-density lipoprotein.\textsuperscript{8} These actions suggest that endothelial NO may have an important atheroprotective role beyond its effect on vessel tone and blood pressure and that an alteration in the activity of the vascular NO system could contribute to the pathogenesis of atherosclerosis. Several observations support this view. In a rabbit model, long-term systemic inhibition of NO production with the NOS inhibitor L-NAME (at levels insufficient to increase blood pressure), enhances the formation of early atherosclerotic lesions.\textsuperscript{9,10} In humans, endothelium-dependent vasodilatory responses are defective in individuals with established atherosclerosis\textsuperscript{11} and in those with atherosclerotic risk factors.\textsuperscript{12} These changes are seen in coronary,\textsuperscript{13} brachial, and femoral vessels\textsuperscript{14} and may predate lesion formation, resulting in part from a reduction in bioavailable NO. Local\textsuperscript{14} or systemic\textsuperscript{15} administration of L-arginine ameliorates this endothelial dysfunction.

We hypothesised that functionally important variants of the \textit{NOS 3} could influence individual susceptibility to atheroscle-
rosis by altering the amount of NO generated by the endothelium. This disease has an inherited component, although many genes are likely involved. We undertook a search for polymorphisms within the promoter and coding regions of NOS 3 and have identified several common polymorphisms with the potential to alter eNOS protein expression or function. We have briefly reported a negative case-control study with one of these variants in an East Anglian cohort of essential hypertensive patients. We now describe our detailed findings following genotyping for this same variant in 2 well-characterized cohorts of patients with coronary atherosclerosis recruited from the Cambridge Heart AntiOxidant Studies (CHAOS and CHAOS II).

Methods

Coronary Artery Disease Subjects
The coronary artery disease (CAD) cases for the first study were drawn from the database of the Cambridge Heart Antioxidant Study (CHAOS). Details of the inclusion criteria and recruitment have been described previously. Briefly, 2002 patients with positive coronary angiograms were recruited during 1992 to 1994 at Papworth Hospital, Cambridge, for a randomized controlled clinical trial designed to test the hypothesis that high-dose vitamin E (α-tocopherol) would reduce the risk of myocardial infarction (MI) in individuals with CAD. Blood samples for DNA analysis were collected before randomization from most of the patients entered after the first year of the study. Genotyping was restricted to 298 unrelated white subjects aged ≥70 who had both angiographically proven CAD (>50% stenosis affecting at least 1 vessel) and a suitable blood sample for DNA extraction. Information on age, gender, blood pressure, cholesterol levels, drug therapy, smoking status, and coexisting disease were obtained by a specialist nurse on the day of angiography and stored on a computer database.

Myocardial Infarction Subjects
The first 249 consecutive white individuals with acute MI (defined as a history of chest pain associated with regional ST segment elevation of ≥1 mm in 2 or more adjacent limb leads or ≥2 mm in 2 or more adjacent precordial leads and/or a rise and fall in serum creatine kinase measured over 48 hours) were recruited via coronary care units of participating East Anglian hospitals to a second randomized controlled trial (CHAOS II) examining the effect of folate supplementation in the secondary prevention of MI. They provided a blood sample for genotyping of the NOS 3 gene. Information on age, gender, blood pressure, cholesterol levels, drug therapy, smoking status, and coexisting disease were obtained at the acute admission and stored on a computer database.

Healthy Controls
Thirty-two thousand healthy East Anglian subjects aged 40 to 69 years with no history of cardiovascular or other disease were identified between 1990 and 1994 from general practice registers; the subjects were invited for health screening at one of 43 health centers where information on diet, smoking habits, alcohol consumption, and family history of illness was obtained. Casual blood pressure (mean of 2 readings) was recorded, and a random cholesterol level was measured from a sample of finger-prick blood using a Reflotron analyser (Boehringer Mannheim UK). Blood was also collected for DNA analysis from subjects at the last 4 of the general practices.

Control Data Set for CHAOS
One hundred thirty-eight unrelated white control subjects who did not differ significantly in terms of blood pressure, cholesterol levels, or smoking status form the larger data set were genotyped and used as a control group for the CAD subjects in CHAOS. Because of the predominance of males and the high frequency of smokers among the CHAOS cases, the controls could not be matched completely for gender and smoking status.

Control Data Set for CHAOS II
Unrelated white control subjects from the original health screen who matched CHAOS II patients most closely for age and sex but from whom DNA was not originally collected were contacted and invited to provide a blood sample for DNA analysis. One hundred eighty-three individuals agreed to take part.
DNA Extraction and Genotyping

Genomic DNA was prepared from samples of whole blood by standard methods. Oligonucleotide primers for polymerase chain reaction (PCR) were designed using the published sequence of the human NOS 3 gene (GenBank/EMBL L10693-L10709).\(^1\) Polymorphism analysis of the promoter region and exons was undertaken in 64 chromosomes by PCR-single strand conformational polymorphism analysis and DNA sequencing. Two variants were identified in the promoter of the gene and one within the coding region; all were single base substitutions\(^2\) (Figure 1). The coding sequence variant was a G→T substitution in exon 7 (at position 894) in codon 298 which alters the amino acid at this residue from Glu to Asp. In CHAOS, genotyping of this polymorphism was performed by PCR amplification of exon 7 with the flanking intronic primers 5'-CATGAGGCTCAGCCCCAGAAC-3' (sense) and 5'-AGTCAATCCCTTTGTGTCTCAC-3' (antisense) followed by MboI restriction endonuclease digestion for 16 hours at 37°C and resolution by electrophoresis on a 2.5% agarose gel. The 206 bp PCR product is cleaved into 119 bp and 87 bp fragments in the presence of a T at nucleotide 894 (which corresponds to Asp\(^{298}\) but not in its absence (Figure 2). In CHAOS II, samples were also genotyped using allele-specific oligonucleotide probes on the ABI 7700 TaqMan (Perkin Elmer). The probes (5'-CCC CAG ATG A(G/T)C CCC CAG AAC TCT; wild type (G) FAM-labeled, mutant (T) TET-labeled) annealed to a 141 bp fragment of exon 7 generated by PCR using a second pair of primers, 5'-GAA ACG GTC GCT TCG ACG T (forward) and 5'-ATC CCA CCC AGT CAA TCC CT (reverse) according to the manufacturer’s instructions.

The CHAOS patients and their controls were also genotyped for a bi-allelic polymorphism in the promoter (−922 A/G) by mismatch PCR (with primers 5'-ACCTTATCATCTCACCTGTTTTCAG-3' and 5'-GCTGGGTTTGTAGTGTGCTGTG-3') followed by allele-specific endonuclease digestion with Cac 8I and agarose gel electrophoresis. We also genotyped a proportion of CHAOS patients (n=122) for the variable number tandem repeat (VNTR) polymorphism in intron 4, for which an association with CAD has been described.\(^3\)

Power Calculations and Statistical Analysis

The allele frequency of the exon 7 variant was ascertained initially in a pilot study of 50 cases with angiographically proven CAD and 100 healthy controls. The frequency of the Asp\(^{298}\) homozygotes and the Asp\(^{298}\) allele was found to be 36% and 47%, respectively, in cases versus 10% and 32%, respectively, in controls. This suggested that only 221 cases and controls would be needed to detect a 15% difference in the frequency of this allele with 90% power at P=0.01. The number of cases was, however, increased to permit analyses by gender, smoking status, and disease severity. Genotype and allele frequencies were compared between groups by \(\chi^2\) analysis and odds ratios, and 95% CIs were calculated. Continuous and non-normally distributed variables were analyzed by Student’s t test and the Mann-Whitney U test, respectively. Statistical analyses was performed using Minitab (Minitab Inc) and P<0.05 was regarded as significant.

Results

Case-Control Study of Angiographic CAD (CHAOS)

The frequency of the Glu\(^{298}\)→Asp variant was compared in a group of 298 patients with angiographically-proven CAD and 138 healthy control subjects from the same region of East Anglia. The baseline demographic characteristics of these 2 groups are shown in Table 1. There was an excess of males and smokers among the CAD cases compared with controls, in common with previous studies with this type of design. The distribution of genotypes in the control group did not differ significantly from that expected under Hardy-Weinberg equilibrium, but there was a significant deviation from Hardy-Weinberg proportions in the case group, with an excess of homozygotes for the Asp\(^{298}\) variant among the CHAOS patients with CAD. The proportion of Asp\(^{298}\) homozygotes in the patients with CAD was 35.9% compared with 10.2% in controls (P<0.0001, Table 2). In comparison to Glu\(^{298}\) homozygotes, the odds ratio for CAD was 4.2 (95% CI, 2.3 to 7.9) for Asp\(^{298}\) homozygotes and 0.7 (95% CI, 0.4 to 1.1) for heterozygotes. The proportion of Asp\(^{298}\) homozygotes among CAD cases was not significantly altered when subjects were subdivided by gender, smoking status, or the number of diseased vessels (data not shown). There were insufficient DNA samples available from patients suffering

| TABLE 1. Demographic Characteristics of CAD Cases and Healthy Controls in CHAOS |
|-----------------|-----------------|-----------------|
| Controls (n=138) | CAD Cases (n=298) | P |
| **Age, y**       | 58.1±0.5        | 58.9±0.3        | ... |
| Male/female      | 71/67           | 273/25          | <0.0001* |
| Current smokers  | 19              | 134             | <0.0001* |
| Cholesterol, mmol/L | 6.07±0.1     | 5.96±0.1        | ... |
| Systolic BP, mm Hg | 126.5±1.0  | 132.2±1.2       | 0.004 |
| Diastolic BP, mm Hg | 77.1±0.7  | 80.5±2.0        | ... |
| Diabetics        | 0               | 29              | ... |
| Previous MI      | 0               | 72              | ... |
| No. of diseased vessels | ... | ... | ... |

P value by Student’s t-test unless indicated. * indicates \(\chi^2\) analysis. BP indicates blood pressure. Values are mean±SEM.
MI or dying during follow-up to determine the predictive power of the Glu298→Asp variant for these endpoints.

The controls and a subset of 122 CHAOS patients were also genotyped for the previously described 420 and 393 bp alleles of the intron 4 VNTR. The respective frequencies of these alleles were 0.84 and 0.16 in controls versus 0.83 and 0.17 in the patients with CAD (P=NS). The frequencies of the A and G alleles of the −922 A/G promoter polymorphism were 0.63 and 0.37, respectively, in the CHAOS patients compared with 0.66 and 0.33 in the controls (P=NS).

Case-Control Study of Myocardial Infarction (CHAOS II)
In the second study, the first 249 unrelated CHAOS II trial recruits admitted to coronary care units in participating East Anglian hospitals with acute MI, who provided a blood sample for DNA analysis at admission, were genotyped for the Glu298→Asp polymorphism. An independent group of 183 healthy controls from the original health screen who most closely matched the MI cases for age, gender, and smoking status were also genotyped. There was a small excess of smokers (28%) among the cases (Table 3). In addition, the average cholesterol level was lower among the cases than controls in this study. Genotype frequencies were in Hardy-Weinberg proportions in both the case and control groups. Once again, there was an excess of individuals homozygous for Asp298 among MI cases (18.1%) compared with healthy controls (8.7%) (P<0.02, Table 4). In comparison to Glu298 homozygotes, the odds ratio for MI was 2.5 (95% Cl, 1.3 to 4.2) for Asp298 homozygotes and 1.2 (95% Cl, 0.8 to 1.8) for heterozygotes (Table 5).

**Discussion**
The major finding of this study was a strong association between a Glu298→Asp polymorphism of the eNOS and the risk of CAD. This increased risk was confined to individuals homozygous for the Asp298 variant and, in the first CHAOS study, amounted to a >4-fold risk of CAD compared with individuals homozygous for Glu298. In a second independent study (CHAOS II), a significant excess of the Asp298 homozygotes was also seen among individuals with recent acute MI when compared with healthy controls and the odds ratio for acute MI among Asp298 homozygotes was 2.5 times that of Glu298 homozygotes.

The cases for both studies were drawn from the same East Anglian population but were otherwise ascertained independently. In the first study, cases were recruited at the time of elective coronary angiography at the regional cardiac center, in the second study, cases were individuals with acute MI recruited at the time of admission to the coronary care units of participating hospitals in the East Anglian region. The difference between the 2 trials in terms of the selection criteria for CAD may explain the apparently smaller excess of Asp298 homozygotes in CHAOS II versus the original CHAOS study, although confidence intervals for the odd ratios for the 2 studies overlap (Table 5). Recent reports from Japan also support a role for the Glu298→Asp polymorphism in the development of coronary atherosclerosis and its complications, and suggest, as does our study, that the excess risk is confined to Asp homozygotes.

Because NO is considered to be atheroprotective, the excess risk of CAD or MI among Asp298 homozygotes may reflect a reduction in the amount or activity of endothelial nitric oxide synthase among such subjects. It is not clear from this association study whether the Glu298→Asp polymorphism is a functional genetic variant or a marker for another functional variant within this or an adjacent gene. Despite the apparently conservative nature of a Glu→Asp amino acid substitution there is ample evidence that such a substitution can substantially alter protein function. Comparison with the recently solved crystal structure of murine inducible nitric oxide synthase (iNOS), suggests that residue 298 of human eNOS (homologous with residue 308 of murine iNOS) is proximal to residues critical for substrate binding (residues Glu571 in murine iNOS and Glu561 in human eNOS). Thus, if the Asp298 variant of eNOS is associated with altered NO synthesis, the mechanism is unlikely to have a direct effect on substrate binding. One possibility is that this variant might influence subcellular targeting or interaction with other regulatory proteins such as caveolin-1.

If the Asp298 variant of eNOS leads to an altered NO synthesis, this could provide a mechanism for its increased prevalence among CAD patients in the CHAOS study and might also explain the unexpectedly large benefit of α-tocopherol in preventing MI. α-Tocopherol presumably acts to prevent the accelerated NO destruction caused by free oxygen radicals in the atherosclerotic vessel wall. Although a reduction in endothelial production of NO may influence progres-

### Table 2. Genotype and Allele Frequencies of the Glu298→Asp Polymorphism in Angiographically Defined CAD Cases (CHAOS) and Controls

<table>
<thead>
<tr>
<th></th>
<th>Glu/Glu</th>
<th>Glu/Asp</th>
<th>Asp/Asp</th>
<th>Total</th>
<th>Glu</th>
<th>Asp</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD cases</td>
<td>120 (40.3)</td>
<td>71 (23.8)</td>
<td>107 (35.9)</td>
<td>298</td>
<td>311 (52.2)</td>
<td>285 (47.8)</td>
<td>596</td>
</tr>
<tr>
<td>Controls</td>
<td>66 (47.8)</td>
<td>58 (42.0)</td>
<td>14 (10.2)</td>
<td>138</td>
<td>190 (68.8)</td>
<td>86 (31.2)</td>
<td>276</td>
</tr>
</tbody>
</table>

χ²=34.4 (2df), P<0.0001 for genotype; χ²=21.4 (1df), P<0.0001 for allele frequencies.

### Table 3. Demographic Characteristics of Cases with Acute MI from the CHAOS II Study and a Further Set of Healthy Controls

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=183)</th>
<th>MI cases (n=249)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>59.0±0.6</td>
<td>61.8±0.7</td>
<td>0.004</td>
</tr>
<tr>
<td>Male/female</td>
<td>138/45</td>
<td>187/62</td>
<td>...</td>
</tr>
<tr>
<td>Current smokers</td>
<td>41</td>
<td>69</td>
<td>0.02*</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.66±0.1</td>
<td>4.55±0.2</td>
<td>&lt;10⁻⁶</td>
</tr>
<tr>
<td>Diabetics</td>
<td>0</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

*P by Student’s t test unless indicated. * indicates χ² analysis. Values are mean±SEM.
sion of atherosclerosis in the coronary artery wall, it may be less important for overall plaque stability and therefore rupture. This may reflect the lower prevalence of homozygosity for Asp\textsuperscript{298} among patients with acute MI in CHAOS II compared with patients in the original CHAOS study. However, another possible factor relates to the significantly lower cholesterol levels in patients recruited into CHAOS II compared with the population controls used. This presumably reflects widespread use of cholesterol-lowering agents, especially HMG-CoA reductase inhibitors, whose increased use succeeded the first and preceded the second of our case-controlled studies.

We have found previously that the Glu\textsuperscript{298}→Asp polymorphism is not associated with essential hypertension\textsuperscript{19} in keeping with 2 negative linkage studies of the NOS 3 gene in hypertensive sibling pairs.\textsuperscript{26,27} This does not exclude the Asp\textsuperscript{298} variant from encoding a hypofunctional protein, because the levels of NOS inhibition required to accelerate atherosclerosis in animal models do not alter systemic blood pressure.\textsuperscript{9,10} It is also possible that the Glu\textsuperscript{298}→Asp polymorphism has effects on eNOS activity only when the enzyme is oxidatively stressed and/or the tetrahydrobipterin cofactor is rate-limiting, as may be the case in atherosclerotic vessels.\textsuperscript{28} These hypotheses are currently under examination in cells transfected with eNOS cDNAs carrying the 2 codon 298 variants.

The odds ratio for the Asp\textsuperscript{298} variant in the first study is substantially greater than that reported previously for other CAD candidate gene polymorphisms. Nevertheless, any findings from genetic association studies should be interpreted with some caution. Although the design provides a powerful tool for detecting the effects of a disease-modifying gene (because of the positive association which localizes the disease locus to within a few hundred kilobases compared with several megabases for a locus identified by linkage), such studies are sensitive to the effects of undetected confounding or bias that may arise during the selection of either case or control subjects. The former was reduced by recruiting cases and controls from a single semirural region, where the predominantly white population enabled recruitment to be restricted to a single ethnic group. Despite this, the case group in the initial study were not in Hardy-Weinberg equilibrium. This can arise because of a strong association between an allele and disease state, undetected population stratification, or genetic mistyping. The potential for population stratification cannot be excluded completely, but the possibility of a false positive association is substantially reduced by the results of our second study (CHAOS II), involving independently ascertained cases (albeit with a different atherosclerosis phenotype) and controls from the same geographical region. Further, in none of a 10% sample from CHAOS, and in only 8 (out of 249) samples from CHAOS II was there a discrepancy between the genotypes assigned by the PCR/RFLP and allelic discrimination methods. This suggested a maximum error rate of 3% for the PCR/RFLP method of genotyping and could not explain the observed excess of Asp\textsuperscript{298} homozygotes. Reanalysis of the data in Table 4 to reflect these changes still gave a significant excess of Asp\textsuperscript{298}/Asp\textsuperscript{298} homozygotes in the CHAOS II sample (P=0.035).

There was the expected clustering of CAD risk factors among cases, and our original control group could not be entirely matched for smoking and sex. We did not detect, however, any association between the Glu\textsuperscript{298}→Asp variant and any of these possible confounders. Importantly, the frequency of the Asp\textsuperscript{298} allele and corresponding homozygotes among our controls was almost identical to that predicted from our previous study in East Anglian hypertensive patients and to that reported here in the new group of controls.\textsuperscript{19}

A role for another NOS 3 polymorphism in CAD was also reported recently by Wang et al, who showed that the risk of CAD was increased 1.3-fold in smokers homozygous for the rare 393 bp VNTR allele in intron 4 of the NOS 3 gene.\textsuperscript{21} This variant is unlikely to be functional, however, but could be in linkage disequilibrium with another variant lying elsewhere in the gene. Its relationship to the Glu\textsuperscript{298}→Asp polymorphism remains to be to be studied. We did not detect an increase in the frequency of the 393 bp allele among cases from the first CHAOS study in comparison to controls.

In summary, we have identified several polymorphisms in the NOS 3 gene and one of these polymorphisms, Glu\textsuperscript{298}→Asp, was found to be a major risk factor for CAD in our white population from the East Anglian region. This finding is potentially important but requires further confirmation in other populations.

### Table 4. Genotype and Allele Frequencies of the Glu\textsuperscript{298}→Asp Polymorphism in Cases with Acute MI (CHAOS II) and Corresponding Controls

<table>
<thead>
<tr>
<th></th>
<th>MI cases</th>
<th>Controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu/Glu</td>
<td>97 (38.9)</td>
<td>86 (47.0)</td>
<td>183</td>
</tr>
<tr>
<td>Glu/Asp</td>
<td>107 (43.0)</td>
<td>81 (44.3)</td>
<td>188</td>
</tr>
<tr>
<td>Asp/Asp</td>
<td>45 (18.1)</td>
<td>16 (8.7)</td>
<td>61</td>
</tr>
</tbody>
</table>

χ²=8.15, (2df), P<0.02 for genotype; χ²=6.91 (1df), P<0.01 for allele frequencies.

### Table 5. Comparison of Odds Ratios for CAD (CHAOS I) and MI (CHAOS II) Among Individuals Heterozygous or Homozygous for the Glu\textsuperscript{298}→Asp Polymorphism

<table>
<thead>
<tr>
<th></th>
<th>CHAOS</th>
<th>CHAOS II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu/Asp</td>
<td>0.7 (0.4–1.1)</td>
<td>1.2 (0.8–1.8)</td>
</tr>
<tr>
<td>Asp/Asp</td>
<td>4.2 (2.2–7.9)</td>
<td>2.5 (1.3–4.2)</td>
</tr>
</tbody>
</table>

*Odds ratios are calculated with respect to the Glu/Glu wild type.

### Acknowledgments

We would like to thank Mathias Chiano of the MRC Biostatistics Unit, Addenbrooke’s Hospital, Cambridge, for helpful statistical advice. This work was supported by a Project Grant from the British
Heart Foundation and a Medical Research Council Clinical Training Fellowship (to A.D.H.).

References


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_Circulation_. 1999;100:1515-1520
doi: 10.1161/01.CIR.100.14.1515

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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