Fibrillin-1 Genotype Is Associated With Aortic Stiffness and Disease Severity in Patients With Coronary Artery Disease

Tanya L. Medley, BAppSc (Hons); Timothy J. Cole, PhD; Christoph D. Gatzka, MD; William Y.S. Wang, MMBS; Anthony M. Dart, FRCP, DPhil; Bronwyn A. Kingwell, PhD

Background—Elevated pulse pressure is associated strongly with adverse cardiovascular outcome; however, the genetic basis of this condition is unknown. This study examined whether genotypic variation in the extracellular matrix protein fibrillin-1, the Marfan gene, was associated with aortic stiffening and therefore could contribute to cardiovascular risk associated with pulse pressure elevation in coronary disease.

Methods and Results—Patients (n=145; 113 men), 62±9 years of age (mean±SD), with angiographically confirmed coronary disease, were studied. Carotid applanation tonometry was used to assess central blood pressures, and in conjunction with Doppler velocimetry, to assess aortic input and characteristic impedance. Fibrillin-1 genotype was characterized by a variable nucleotide tandem repeat and 2 single-nucleotide polymorphisms. The variable nucleotide tandem repeat was a good predictor of underlying haplotypes with 3 genotypes (2-2, 2-4, and 2-3) accounting for 86% of the population. The 2-3 genotype had higher input impedance (P=0.002), characteristic impedance (P=0.005), and carotid pulse pressure (P=0.002) compared with the 2-2 and 2-4 genotypes. Disease severity assessed by previous angioplasties and the number of patients with a stenosis >90% was also greater in the 2-3 genotype. Furthermore, in a multivariate analysis, fibrillin-1 genotype and central pulse pressure were independent of conventional risk factors in determining coronary disease severity. There was no difference in age, sex ratio, body mass index, smoking status, cholesterol level, or medication among the 3 genotypes.

Conclusions—Although a causative link has not been shown, these data are consistent with an important role for fibrillin-1 genotype in cardiovascular risk associated with large-artery stiffening and pulse pressure elevation in individuals with coronary disease. (Circulation. 2002;105:810-815.)

Key Words: mechanics ■ coronary disease ■ arteries ■ blood pressure ■ aorta

Pulse pressure has been strongly and independently related to adverse cardiovascular outcome,1–3 whereas its principal determinant, large-artery stiffness, recently has been recognized as an independent predictor of all-cause and cardiovascular death.4 These studies have provoked a substantial interest in the structural and genetic basis of large-artery stiffening. Although there has been a great deal of focus on genes associated with the renin-angiotensin system5–7 and relatively rare congenital disorders,8,9 the current study sought to examine genetic variation in an extracellular matrix protein, fibrillin-1 in patients with coronary artery disease (CAD). Fibrillin-1 is the principal constituent of 10-nm microfibrils and functions as a skeleton for the deposition of tropoelastin, providing both load-bearing and anchoring functions within the arterial wall.10 In addition, fibrillin-1 is the gene affected in Marfan syndrome, in which large-artery stiffening and elevated pulse pressure are the main determinants of aortic dilation.9,11 More recently, a variable nucleotide tandem repeat (VNTR) polymorphism in the fibrillin-1 gene has been associated with brachial systolic and pulse pressure in a healthy middle-aged male population.12 The causative link between large-artery stiffness and pulse pressure suggests that fibrillin-1 genotype may contribute significantly to arterial stiffness in the general population. Such genetic predisposition to arterial stiffening and pulse pressure elevation may be an antecedent to atherosclerosis.13,14 Furthermore, arterial stiffness has recently been shown to contribute independently of coronary disease severity and other major risk factors to reduction in ischemic threshold on exertion in patients with CAD.15 The aim of the current study was thus to determine whether fibrillin-1 genetic variation was associated with large-artery stiffness and disease severity in patients with CAD.

Methods

Subjects and Study Design

Patients (n=145; 113 men), 62±9 years of age (mean±SD), with angiographically confirmed CAD, gave informed consent for partic-
ipation in the study, which was approved by the Ethics Committee of the Alfred Healthcare Group. Patients with previous bypass surgery were excluded because of the possible confounding effects of surgery on large-artery stiffness. Angiographically identified stenoses >50% in the major coronary vessels at the time of the study were used to classify patients as having single-vessel (89 patients), double-vessel (39 patients), or triple-vessel (17 patients) disease.

All patients attended the laboratory after an overnight fast. β-Blocking agents were discontinued for at least 24 hours and nitrates for at least 4 hours before all measurements were taken. Blood was drawn for fasting lipids and lymphocyte extraction. Patients then rested in a quiet, temperature-controlled room (22°C) for at least 15 minutes and until blood pressure had stabilized. Aortic input and characteristic impedance were then assessed noninvasively.

**Resting Hemodynamics**

Resting brachial arterial blood pressure and heart rate were measured 3 times at 3-minute intervals with a Dinamap vital signs monitor (1846 SX, Critikon) after 10 minutes of supine rest. The mean of 3 values was taken to represent resting levels.

**Aortic Impedance**

Aortic flow velocity was measured with a continuous-wave zero-crossing Doppler velocimeter (Multi-Dopplex MD1, Huntleigh Technology), and right carotid pressure was measured simultaneously byplanation tonometry (SPT-301, Millar Instruments). Ensemble average time series of both flow and pressure (10 cardiac cycles aligned to the foot of the pressure waveform) were used to calculate aortic input impedance. Moduli where either flow or pressure modulus was <2.5% of the respective modulus of the first harmonic were discarded. Linear interpolation was performed to calculate mean impedance spectra, as shown in Figure 2. For statistical comparison, input impedance at both the first harmonic (1.0±0.1 Hz for all genotypes) and 1.75 Hz were reported as a measure of large-artery stiffness at frequencies incorporating pressure wave reflection. Characteristic impedance in the absence of wave reflection was calculated as the arithmetic mean of moduli >2 Hz. All impedance values are reported in arbitrary units of velocity.

**Genotyping**

Genomic DNA was prepared from whole blood lymphocytes with the use of routine procedures. The VNTR (TAAAAs in intron 28) and 2 single-nucleotide polymorphisms (SNP2 in exon 15, SNP5 in intron 27) of the fibrillin-1 gene were amplified by means of specific PCR conditions and primers: VNTR Forward: 5′-FAM-ATC TCA GAG TAC ATA GAG TGT TGT TTG AG 3′ and Reverse: 5′-GCC TGC AAC CCT GGC TAC CAT TCA AC 3′; SNP2 Forward: 5′-TAA TAA GTG CCT TCT TCT GCC AC 3′ and Reverse: 5′-GTT AGC CAT GAT GGT TTT TCA TCA A A 3′; SNP5 Forward: 5′-TTG CCA AAG TTG TTG GCT TAT GT and Reverse: 5′-CTT ACC AAC AAA TAG CCT ATC 3′. Genotypes for VNTR were determined with the use of an ABI Prism Genescan 337. Each allele was verified by sequencing. All SNP-amplified fragments were purified and sequenced both forward and reverse.

**Biochemical Analyses**

LDL, HDL, and total cholesterol and triglycerides were determined enzymatically with a Cobas-BIO centrifugal analyzer (Roche Diagnostic Systems).

**Statistical Analyses**

ANOVA and ANCOVA were used to compare data stratified by genotype, with the least significant difference used to compare individual means. χ² analysis was used to compare categoric variables. All data were analyzed by means of SPSS for Windows Version 9.0.1. (SPSS Inc). Unless otherwise stated, group results are presented as mean±SD, and statistical significance was deemed to have been achieved at a level of P<0.05.

**Results**

Five alleles, identified as 1, 2, 3, 4, and 5 according to the number of TAAAA repeats, were recognized. These corresponded to 7 genotypes, with the 2-2, 2-3, and 2-4 genotypes accounting for 86% of the population. The major genotypes were used for subsequent analysis, and relative genotype frequencies and number of patients in each group are shown in Table 1. Genotype frequencies were not significantly different from an age-matched (61±9 years), healthy group with no evidence of CAD on history or examination drawn from the same locality (healthy group, n=170, 2-2, 62.9%; 2-4, 16.5%; 2-3, 9.4%). Age, sex ratio, body mass index (BMI), blood lipids, heart rate, smoking status, previous history of infarction, diabetes (Table 1), or medication (Table 2) were not different among the 3 genotypes.

**CAD Severity and Fibrillin-1 Genotype**

Although there was no difference in the number of coronary vessels with a stenosis >50% among genotypes, there was evidence for greater CAD severity in the 2-3 genotype compared with 2-2 or 2-4. Compared with the other genotypes, a greater proportion of the 2-3 group had undergone previous angioplasty (P=0.05). In addition, the magnitude of the maximum coronary stenosis was greater in the 2-3 compared with the 2-4 genotype (P=0.05 in pairwise analysis), although in ANOVA incorporating all 3 genotypes, the probability value was 0.14 (Table 1). Because maximum stenosis alone would underestimate disease severity in those who had undergone angioplasty previously, patients were classified categorically as having either a maximum stenosis >90% or previous angioplasty. In this analysis, disease severity was clearly greatest in the 2-3 genotype and least in the 2-4 genotype (Table 1). Furthermore, in a multivariate analysis incorporating conventional risk factors (age, sex,
mean pressure, LDL cholesterol, and triglycerides), only fibrillin-1 genotype \((r=0.21; P=0.03)\) and carotid pulse pressure were significant predictors of disease severity \((r=0.28; P=0.03)\). Similar results were obtained if carotid pulse pressure was substituted with carotid systolic pressure.

**Blood Pressure and Fibrillin-1 Genotype**
The 2-3 genotype was associated with higher mean arterial pressure \((P=0.04)\) as well as higher brachial \((P=0.03)\) and carotid systolic \((P=0.003)\) and pulse pressure \((P=0.002, \text{Figure } 2)\). The relation between fibrillin-1 genotype and both input \((P=0.02)\) and characteristic \((P=0.03)\) impedance remained significant when age, sex, LDL cholesterol, triglycerides, BMI, smoking status, medication profile, and, importantly, mean arterial pressure were entered into the analysis as covariates. The other significant covariates were mean pressure and BMI.

**Large-Artery Stiffness and Fibrillin-1 Genotype**
Input and characteristic impedance were higher in the 2-3 genotype compared with 2-2 and 2-4, which were similar (Table 2, Figure 2). The relation between fibrillin-1 genotype and both input \((P=0.02)\) and characteristic \((P=0.03)\) impedance remained significant when age, sex, LDL cholesterol, triglycerides, BMI, smoking status, medication profile, and, importantly, mean arterial pressure were entered into the analysis as covariates. The other significant covariates were mean pressure and BMI.

**SNP2 and SNP5**
The allele frequency for SNP2 was 0.9 for the T variant and 0.1 for the C variant (Table 3). For SNP5, the frequency of the G allele was 0.9 and the A allele 0.1 (Table 3). Genotypes for both were in Hardy-Weinberg equilibrium. Variants in SNP2, SNP5, and the VNTR all showed strong frequency-matched linkage disequilibrium \((P<0.001)\); SNP2 versus SNP5 \([D'=94\%]\), SNP2 versus VNTR \([D'=79\%]\), SNP5 versus VNTR \([D'=84\%]\); Table 3). The SNP analysis indicates that patients carrying the 3 allele for the VNTR (intron 28) predominantly carry the T allele for SNP2 (exon 15) and the G allele for SNP5 (intron 27). The same SNP alleles are also linked to the 2 allele of the VNTR. The pattern of linkage disequilibrium noted suggests that the VNTR is a good predictor of the underlying haplotypes of the gene and was therefore used in subsequent discussion.

**Discussion**
The current data indicate that within a moderate-severity CAD population, genetic variation in fibrillin-1, the Marfan gene, modulates large-artery stiffness and pulse pressure. Patients with the 2-3 genotype had stiffer large arteries, higher pulse pressure, and more severe CAD than other genotypes. Furthermore, both fibrillin-1 genotype and pulse pressure were independent predictors of coronary disease severity. There is substantial evidence linking pulse pressure to adverse cardiovascular outcome,\(^1\)\(^–\)\(^3\) and a recent study has established large-artery stiffness as a predictor of total and cardiovascular death,\(^4\) although precise mechanisms have not been established. The current study suggests that genetic variation in fibrillin-1 is an important factor contributing to risk associated with pulse pressure and large-artery stiffening in patients with CAD.

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<th>TABLE 1. Patient Characteristics Stratified by Genotype</th>
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<td>Genotype</td>
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<td>Frequency, %</td>
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<td>Current, Exsmokers/Nonsmokers</td>
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<td>Diabetes, n (%)</td>
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<td>Previous angioplasty or maximum stenosis &gt;90%, n (%)</td>
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<td>Aortic area, cm²</td>
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<td>Input impedance (at 1st harmonic), (mm Hg · s · cm⁻¹)</td>
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<td>Input impedance (at 1.75 Hz), (mm Hg · s · cm⁻¹)</td>
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<tr>
<td>Characteristic impedance, (mm Hg · s · cm⁻¹)</td>
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All data are mean±SD. M indicates male; and F, female.

*P<0.05.
Genetic variation in the proteins constituting the aortic wall and regulating the turnover of the extracellular matrix are likely to influence elastic properties. As a major constituent of extensible microfibrils, fibrillin-1 is a strong candidate. These microfibrils are associated with the elastic fibers that separate the medial vascular smooth muscle cells and constitute the concentric elastic lamellae.\(^\text{10}\) By directing the orientation of elastin fibers, fibrillin-1 microfibrils play a vital role in load bearing.\(^\text{10}\) Fibrillin-1 contains a large number of calcium-binding, epidermal growth factor repeats, in which mutations have been associated with both Marfan syndrome\(^\text{22,23}\) and abdominal aortic aneurysms.\(^\text{24}\) The mechanism linking the VNTR polymorphism examined in the current study with a functional correlate has not been determined; however, there are many precedents for linkage between intron polymorphisms and effects on gene expression.\(^\text{25–27}\) A 17-bp polymorphic repeat, associated with neurological affective disorders, situated in intron 2 of the human serotonin transporter gene, when tested in transgenic mice had allelic-dependent, enhancer-like activity, indicating that repeat variants may differentially regulate transcription.\(^\text{27}\) Furthermore, the fibrillin-1 polymorphism examined in the current study is only 15 bp downstream of the 3' splice boundary with exon 28\(^\text{28}\) and may therefore influence RNA splicing. A good example of such an effect has recently been reported in the human CFTR gene, in which variants of a TG repeat and T repeat situated in intron 8 have been shown to cause exon skipping.\(^\text{29}\)

Patients with CAD were examined in this study because large-artery stiffness appears to be an important risk marker in this group.\(^\text{15,30–32}\) There was no difference between genotype frequencies in the CAD group compared with age-matched, healthy individuals. There was, however, a trend for the 2-3 genotype to be more prevalent (control subjects, 9.4%; patients with CAD, 13.1%) and the 2-2 genotype less prevalent in the patients with CAD compared with control subjects (control subjects, 62.9%; patients with CAD, 57.9%). The study was not sufficiently powered to detect differences of this magnitude, but the trends are supportive of a causative role of the 2-3 genotype in relation to coronary disease. In comparison to previously published work, the major genotypes (2-2, 2-3, and 2-4) made up a significantly smaller percentage of the population (86% versus 95%, \(P<0.001\)),\(^\text{12}\) indicating a higher percentage of the rarer genotypes in our CAD cohort. Among the major genotypes, however, the frequency distribution was not different. The higher systolic and pulse pressures associated with the 2-3 genotype were also consistent with those previously reported for normal, healthy, middle-aged men.\(^\text{12}\)

CAD patients with a genetic predisposition for stiffer large arteries are likely to be at increased coronary risk. Pulse pressure elevation as a result of large-artery stiffening may mediate increased risk through exacerbating coronary disease severity\(^\text{14}\) and through reduction in ischemic threshold.\(^\text{15}\) When previous angioplasty or a maximum stenosis \(>90\%\) was used to classify disease severity, the 2-3 genotype was clearly associated with more severe disease. Furthermore, both fibrillin-1 genotype and carotid pulse pressure were independent of each other and conventional risk factors in predicting CAD severity. Thus, fibrillin-1 genotype may influence CAD severity through both pulse pressure–dependent and pulse pressure–independent mechanisms. The precise mechanisms that link elevated pulse pressure to adverse cardiovascular outcome\(^\text{1–3}\) have not been established but may include pulse pressure–induced endothelial dysfunction\(^\text{15,14}\), as an antecedent to atherosclerosis. From a functional perspective, we have also shown that CAD patients with stiffer large vessels have reduced time to ischemia on treadmill testing, independent of their disease severity.\(^\text{15}\) This latter finding provides clinical data to support experimental studies that have demonstrated both increased cardiac work and re-
duced coronary perfusion associated with elevated pulse pressure induced by aortic bandaging or directing aortic flow through a stiff bypass.33–35 Through this mechanism, patients with stiffer vessels may be more symptomatic with respect to angina independent of the magnitude of their maximum stenosis. This would certainly be consistent with the greater prevalence of angiplasty in the 2-3 genotype. A genetic predisposition to stiffer large arteries may thus increase ischemic risk both directly through effects on afterload and coronary perfusion, in addition to contributing to initiation of the atherosclerotic process.

The relation between aortic input impedance and genotype probably is a result of intrinsic large-vessel stiffness rather than wave reflection, because the magnitude of the difference in intrinsic and characteristic impedance among genotypes was similar. Furthermore, these associations were independent of age, sex, LDL cholesterol, triglycerides, BMI, smoking status, medication regimen, and, importantly, mean arterial pressure. Although higher mean pressure would cause a passive increase in large-artery stiffness, the magnitude of the difference between the 2-3 and other genotypes was small and likely to have had negligible effects. Furthermore, the relation between genotype and impedance was independent of mean arterial pressure in covariate analysis, suggesting that the 2-3 genotype is associated with vascular structural differences and not just functional changes associated with higher mean distending pressure. The higher pulse pressure associated with the 2-3 genotype would arise as a consequence of higher impedance. Pulse pressure differences were more marked centrally than peripherally, perhaps reflecting greater importance of fibrillin-1 containing elastic fibers in the proximal aorta compared with peripheral arteries such as the brachial.

Conclusions

Although a causative link has not been shown, these data are consistent with an important role for fibrillin-1 genotype in relation to cardiovascular risk associated with large-artery stiffening and pulse pressure elevation in individuals with coronary disease. This finding has potential implications for risk stratification and therapeutic targeting not only in patients with existing disease but also in the general population.

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