Matrix Metalloproteinase-3 Genotype Contributes to Age-Related Aortic Stiffening Through Modulation of Gene and Protein Expression

Tanya L. Medley, Bronwyn A. Kingwell, Christoph D. Gatzka, Prakash Pillay, Timothy J. Cole

Abstract—Matrix metalloproteinases (MMPs) include most major constituents of the arterial wall as substrates. A common promotor polymorphism (5A/6A) is associated with differences in MMP-3 (stromelysin-1) activity, and associations with certain forms of vascular disease have been shown. This study investigated whether the MMP-3 5A/6A promoter polymorphism contributes to age-related large artery stiffening. MMP-3 5A/6A genotype was determined in 203 (135 male) low cardiovascular risk, unmedicated individuals who were divided prospectively into two groups (30 to 60 years, n=126; ≥61 years, n=77). Noninvasive large artery stiffness was measured as ascending aortic input impedance from brachial blood pressure, carotid tonometry, and Doppler ascending aortic blood flow. In the older group, homozygotes had higher aortic input ($P<0.01$) and characteristic ($P<0.01$) impedance, ie, higher stiffness, than heterozygotes after correction for the effects of age, gender, and mean arterial pressure. There was no such difference in the younger group. Gene expression was subsequently investigated in dermal biopsies in randomly selected older men from the same cohort with real-time PCR (n=40). In 5A homozygotes, gene expression was 4-fold higher ($P<0.05$), and in 6A homozygotes, 2-fold lower ($P<0.05$) compared with the heterozygotes. Differences in gene expression were associated with corresponding significant changes in MMP-3 protein levels. Concordance between dermal and aortic gene and protein expression was shown in a separate cohort of postmortem aortic samples (n=7). We conclude that MMP-3 genotype may be an important determinant of vascular remodeling and age-related arterial stiffening, with the heterozygote having the optimal balance between matrix accumulation and deposition. (Circ Res 2003;92:1254-1261.)

Key Words: large artery stiffness ▪ aging ▪ stromelysin-1 ▪ matrix metalloproteinases ▪ pulse pressure

Large artery stiffness is a principal determinant of pulse pressure and both are related to cardiovascular and coronary mortality independent of other major risk factors in hypertensive patients.1–3 However, in the general population, arterial stiffness has only been shown to predict mortality in the elderly.4 These studies have provoked a substantial interest in the structural and genetic basis of large artery stiffening. The renin-angiotensin-aldosterone system is an important modulator of both blood pressure and large artery stiffness and associated genes have been shown to determine large artery stiffness.5–8 In addition, the genetic basis of congenital disorders involving matrix proteins and associated with arterial stiffening has now been determined.9–11 The matrix metalloproteinases (MMPs) are another set of potential candidate proteins in the general population given their important role in matrix homeostasis and vascular remodeling. MMP-3 (stromelysin-1) may be particularly significant to arterial wall remodeling. This is as a result of its broad substrate spectrum, which includes most major constituents of the arterial wall, such as fibronectin, type IV, V, IX, and X collagens, gelatin, laminins, elastin, and proteoglycan proteins. In addition, MMP-3 activates other MMPs including fibroblast collagenase (MMP-1) and gelatinase B (MMP-9).12,13

A naturally occurring and common polymorphism affects the MMP-3 promoter, where either 5 or 6 consecutive adenines (5A/6A) alter transcription factor binding and affect MMP-3 promoter activity. The 5A allele has greater promoter activity in cell culture experiments,14 but this has not yet been confirmed in human tissue. Interestingly, at the clinical level both alleles have been linked with adverse cardiovascular outcomes. The 5A allele has been associated with acute coronary events15 and aortic aneurysms,16 both of which may reflect increased matrix degradation. In contrast, the 6A allele has been associated with carotid intima-media thickening17–19 and progression of coronary artery disease (CAD) in post–bypass patients,20 suggesting that a lower expression may result in matrix accumulation and plaque progression.21 Although these studies suggest a profound influence of MMP-3 genotype on matrix composition, the potential influ-
ence of this polymorphism on the elastic properties of the large arteries has not been examined. Given the known associations between large artery stiffness and cardiovascular risk,1–3 and particularly myocardial ischemic risk,22,23 it would be useful from a risk stratification perspective to understand how MMP-3 genotype affects large artery stiffness. The present study thus examined whether the MMP-3 promoter polymorphism (5A/6A) modulates age-related large artery stiffening in healthy humans. Because arterial stiffness is strongly related to age as it is a predictor of mortality mostly in the elderly,4 as well as gender and blood pressure, stratification occurred by these variables. We also examined genotype, gene expression and protein expression in skin biopsies from the same cohort. The results of the latter were confirmed in postmortem aortic samples from a different population.

Materials and Methods

Subjects and Design

Two hundred and three (135 male) individuals aged 55±11 years (mean±SD; range 30 to 80) gave informed consent to participate in the study that was approved by the Ethics Committee of the Alfred Healthcare Group. Only unmedicated nonsmokers (for at least 10 years) with an alcohol intake of <2 standard drinks per day, blood pressure ≤145/95 mm Hg, fasting total cholesterol ≤6.0 mmol/L, fasting triglycerides ≤2 mmol/L, fasting plasma glucose ≤7.0 mmol/L, and no family history of premature cardiovascular disease were included. In addition, participants must have had no angina at rest or on exertion, a normal ECG, no evidence of valvular heart disease and no signs or symptoms of congestive heart failure. To examine the effects of age-related large artery stiffening, subjects were prospectively divided into two age groups (30 to 60 years, n=126; ≥61 years, n=77).

Resting Blood Pressure

Resting brachial arterial blood pressure and heart rate were measured 3 times, at 3-minute intervals, using a Dinamap vital signs monitor (1846 SX, Critikon) after subjects rested undisturbed in a quiet temperature-controlled room (22°C) for ≥10 minutes and until blood pressure had stabilized.

Ascending Aortic Input Impedance

Aortic flow velocity was measured using a Doppler velocimeter (3.5Hz Multi-Dopplex MD1, Huntleigh Technology), and right carotid pressure was measured simultaneously by applanation tonometry (SPT-301, Millar Instruments) as previously described.24 This involves calibration of the carotid blood pressure amplitude assuming equality of carotid and brachial mean and diastolic pressure, with differences attributable to pressure amplification modifying systolic pressure and contour alone. Although it is possible to obtain tonometric carotid artery pulse pressure in almost perfect agreement with invasive aortic waveforms,25 we believe that overall error is reduced by calibration against brachial pressure as previously described and extensively discussed by others.26 From the recordings of simultaneous flow, I(t), and pressure, P(t), a single operator (T.L.M.) selected 10 cardiac cycles using custom-written software. The start of each cycle was defined as the ‘foot’ [the maximum of P(t)2]. P(t) was linearly detrended assuming equality at the start and end of each cycle and calibrated as described above. P(t) and I(t) were averaged for all selected cycles truncated to the length of the shortest cycle and transferred to Excel 97 SR-2 (Microsoft Corp). Using Matlab for Windows software version 6.1.0.450 (The Mathworks, Inc), P(t) and I(t) were Fourier-transformed, using only those coefficients of both P and I, which contributed at least 2.5% of the amplitude of the fundamental frequency. For statistical comparison, ascending aortic input impedance (Za) at both the first harmonic (1.0±0.1 Hz for all genotypes) and at 1.75 Hz (linear interpolation between the first and second harmonic) were calculated as a measure of large artery stiffness at frequencies incorporating pressure wave reflection. Characteristic impedance (Zc) was calculated as the average of coefficients above 2 Hz.

Biochemical Analyses

Fasting venous blood was drawn for lipid analysis and DNA extraction. Total and low-density lipoprotein (LDL) cholesterol and triglycerides were determined enzymatically with a Cobas-BIO centrifugal analyzer (Roche Diagnostic Systems).

Genotyping

Genomic DNA was prepared from whole blood using routine procedures. The MMP-3 promoter polymorphism was amplified from 100 ng of genomic DNA in a 15-μL PCR reaction using the following primers: forward, 5′-GATTACAGACATGGGTGACG-3′; and reverse, 5′-GAATTCACATCAGTGGCCAC-3′. PCR fragments were sized using an ABI Prism Genescan 337 (PE Biosystems) and verified by DNA sequencing.

Gene Expression

A 3-mm diameter skin punch biopsy (∼10 mg) was taken from the iliac crest from 40 randomly selected subjects in the older group under local anesthesia (1% lignocaine, all subjects male for cosmetic reasons due to the potential for scarring, 5A/5A n=10; 5A/6A n=20; 6A/6A n=10). All samples were immediately snap frozen in liquid nitrogen. Total RNA was extracted using the Trizol reagent method (Life Technologies Inc), treated with DNase I (Ambion Inc) and stored at −70°C. cDNA was synthesized using PE Biosystems reverse transcriptase kit with random hexamer primers. Real-time PCR amplifications were performed in triplicate using the following primers and probe: forward primer, 5′-TCTCTGTAGTTGGTCTACATCCA-3′; reverse primer, 5′-CCTGTATGTAAGGTGGGTTTTCCT-3′; and probe, 5′-CCTTCTGTGCATTCCGAAGTGG-3′. MMP-3 gene expression was normalized to 18S RNA as an endogenous control (ABI Prism 7700, Applied Biosystems).

Protein Expression

Total protein was extracted from skin biopsies (n=25) using the Trizol reagent method (Life Technologies, Inc). Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). Protein expression was determined by Western blot. Exactly 8 μg of total protein was separated by SDS-PAGE on 7.5% gels, blotted onto nitrocellulose membranes and immunostained with a human MMP-3 primary antibody (1:1000 1.5 hour, Sigma). After addition of a secondary antibody, blots were exposed to X-ray film. Membranes were then stripped and reprobed with β-actin antibody (1:10,000 1 hour, Sigma) to confirm expression levels of MMP-3. Protein expression was quantitated by densitometry using the Quantity One software (Bio-Rad) relative to β-actin as a loading control. Protein expression was compared between genotypes using ANCOVA to adjust for age and gender effects.
TABLE 1. Subject Characteristics in the Younger Group (30 to 60 years)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>5A/5A</th>
<th>5A/6A</th>
<th>6A/6A</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>34</td>
<td>51</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Frequency, %</td>
<td>27</td>
<td>41</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>M/F</td>
<td>25/9</td>
<td>31/20</td>
<td>27/14</td>
<td>0.80</td>
</tr>
<tr>
<td>Age, y</td>
<td>48±7</td>
<td>49±7</td>
<td>48±7</td>
<td>0.80</td>
</tr>
<tr>
<td>BMI, kg · m⁻²</td>
<td>26.5±3.8</td>
<td>25.9±3.4</td>
<td>26.8±4.6</td>
<td>0.51</td>
</tr>
<tr>
<td>Resting HR, bpm</td>
<td>60±9</td>
<td>59±9</td>
<td>62±9</td>
<td>0.52</td>
</tr>
<tr>
<td>Aortic root area, cm²</td>
<td>3.6±0.6</td>
<td>3.4±0.8</td>
<td>3.5±0.8</td>
<td>0.45</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.9±0.7</td>
<td>4.9±0.7</td>
<td>4.9±0.7</td>
<td>0.95</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.9±0.7</td>
<td>2.8±0.9</td>
<td>2.9±0.8</td>
<td>0.48</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.0±0.3</td>
<td>1.0±0.4</td>
<td>1.1±0.4</td>
<td>0.53</td>
</tr>
<tr>
<td>Plasma glucose, mmol/L</td>
<td>5.5±0.6</td>
<td>5.4±0.6</td>
<td>5.5±0.7</td>
<td>0.99</td>
</tr>
<tr>
<td>Brachial SBP, mm Hg</td>
<td>115±11</td>
<td>116±11</td>
<td>119±12</td>
<td>0.49</td>
</tr>
<tr>
<td>Brachial DBP, mm Hg</td>
<td>71±6</td>
<td>73±7</td>
<td>74±8</td>
<td>0.24</td>
</tr>
<tr>
<td>Brachial MAP, mm Hg</td>
<td>88±8</td>
<td>89±8</td>
<td>90±9</td>
<td>0.20</td>
</tr>
<tr>
<td>Ascending aortic input impedance (at 1st harmonic), mm Hg · s · cm⁻¹</td>
<td>1.49±0.48</td>
<td>1.55±0.52</td>
<td>1.37±0.41</td>
<td>0.19</td>
</tr>
<tr>
<td>Ascending aortic input impedance (at 1.75 Hz), mm Hg · s · cm⁻¹</td>
<td>1.43±0.84</td>
<td>1.26±0.44</td>
<td>1.13±0.35</td>
<td>0.16</td>
</tr>
<tr>
<td>Characteristic impedance, mm Hg · s · cm⁻¹</td>
<td>1.16±0.43</td>
<td>1.13±0.41</td>
<td>1.09±0.40</td>
<td>0.81</td>
</tr>
</tbody>
</table>

M indicates male; F, female; BMI, body mass index; LDL, low-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; and HR, heart rate.

All data are mean±SD.
was 2-fold lower and 5A/5A gene expression 4-fold higher compared with 5A/6A heterozygotes. These calculations were determined using the ABI Prism 7700 PCR protocol, where one delta cycle threshold \((\Delta C_T)\) equals a 2-fold difference in template concentration after samples are normalized to 18S rRNA (Figure 2). All results remained significant after known covariates of arterial stiffness were entered in the analysis as covariates (age and mean arterial pressure). Skin biopsies were not studied in females due to the risk of scarring. The relationship between MMP-3 genotype and arterial stiffness was, however, unaffected by gender, and thus there is no reason to believe that this relationship would be any different for females.

The level of MMP-3 gene expression in the 7 aortic samples (from a different population) was similar to that measured in skin (Figure 2).

### Table 2. Subject Characteristics in the Older Group (≥61 years)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Frequency, %</th>
<th>M/F</th>
<th>Age, y</th>
<th>BMI, kg (\cdot) m(^{-2})</th>
<th>Resting HR, bpm</th>
<th>Aortic root area, cm(^2)</th>
<th>Total cholesterol, mmol/L</th>
<th>Triglycerides, mmol/L</th>
<th>Plasma glucose, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>5A/5A</td>
<td>16</td>
<td>21</td>
<td>12/4</td>
<td>67±6</td>
<td>3.3±0.4</td>
<td>5.0±0.5</td>
<td>2.64±0.25</td>
<td>26.4±2.5</td>
<td>1.1±0.4</td>
<td>5.6±0.6</td>
</tr>
<tr>
<td>5A/6A</td>
<td>35</td>
<td>45</td>
<td>23/12</td>
<td>66.5</td>
<td>3.1±0.5</td>
<td>3.1±0.5</td>
<td>25.2±3.4</td>
<td>25.2±3.4</td>
<td>1.0±0.4</td>
<td>5.4±0.6</td>
</tr>
<tr>
<td>6A/6A</td>
<td>26</td>
<td>34</td>
<td>17/9</td>
<td>67±4</td>
<td>3.2±0.7</td>
<td>4.8±0.6</td>
<td>25.6±2.3</td>
<td>25.6±2.3</td>
<td>1.1±0.4</td>
<td>5.6±0.7</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.42</td>
<td>0.42</td>
<td>0.74</td>
<td>0.50</td>
</tr>
</tbody>
</table>

### Table 3. Ascending Aortic Input Impedance in the Younger and Older Groups

<table>
<thead>
<tr>
<th>Age Group, y</th>
<th>5A/5A</th>
<th>5A/6A</th>
<th>6A/6A</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascending aortic input impedance (at 1st harmonic), mm Hg (\cdot) s (\cdot) cm(^{-1})</td>
<td>30–60</td>
<td>1.49±0.48</td>
<td>1.55±0.52</td>
<td>1.37±0.41</td>
</tr>
<tr>
<td>≥61</td>
<td>2.32±1.11*</td>
<td>1.72±0.55</td>
<td>2.28±0.87</td>
<td>0.01*</td>
</tr>
<tr>
<td>Characteristic impedance, mm Hg (\cdot) s (\cdot) cm(^{-1})</td>
<td>30–60</td>
<td>1.16±0.43</td>
<td>1.13±0.41</td>
<td>1.09±0.40</td>
</tr>
<tr>
<td>≥61</td>
<td>1.58±0.79*</td>
<td>1.19±0.45</td>
<td>1.60±0.65*</td>
<td>1.60±0.65*</td>
</tr>
</tbody>
</table>

*P<0.05 of an unpaired t test between the two age groups within each genotype corrected for multiple testing.
Protein Expression
Consistent with MMP-3 mRNA expression data, heterozygotes produced an intermediate MMP-3 protein expression in the skin biopsies compared with homozygotes (P<0.03; Figure 3). When this was entered as a covariate (with age and mean arterial pressure), differences in gene expression between genotypes remained significant. This trend was consistent in aortic samples although not significant due to low numbers (data not shown).

Discussion
MMP-3 plays a pivotal role in matrix homeostasis in the large arteries, but genetic variation in MMPs has not previously been examined in the context of large artery stiffness. Given the associations between large artery stiffness and coronary events\(^2\) and cardiovascular mortality\(^1,4\), understanding the genetic contribution to large artery stiffness may have potential value in risk stratification and therapeutic targeting. In this study MMP-3 genotype assessed by the 5A/6A promoter polymorphism was associated with large artery stiffness in older (> 60 years) but not young individuals at low cardiovascular risk. Specifically, the 5A/6A genotype was associated with more elastic large arteries and a level of gene and protein expression intermediate between the two homozygotes. Furthermore, all associations were independent of potential confounding factors. Because large artery stiffness is the primary cause of isolated systolic hypertension\(^28\), the clinical implications of this study include a predisposition to this condition in homozygotes for the MMP-3 promoter polymorphism.

MMP-3 Genotype, Gene Expression, and Protein Expression
Although gene expression and protein levels were determined in skin samples, it is important to note that the 7 aortic samples from a separate population yielded quantitatively similar results. This association was consistent at both the mRNA and the protein level, suggesting that the skin data were relevant to MMP-3 expression within the aorta. This is the first time that the MMP-3 genotype has been shown to affect both gene and protein expression in human tissue. MMP-3 protein levels do not however, necessarily correspond to activity. It was not possible to measure MMP-3 activity in the small dermal samples collected in the present study. However, previous studies have shown a good association between protein expression and activity.\(^29\)

MMP-3 Genotype and Vascular Remodeling
There is a fine balance between matrix degradation and deposition to maintain optimum vessel wall structure in the face of prevailing mechanical stresses. The importance of optimal MMP activity throughout life is illustrated by studies in transgenic mice. Studies using both MMP and tissue inhibitor of metalloproteinase (TIMP) knockout mice suggest that MMPs may be antiatherogenic but promote aneurysm
and plaque instability. Such findings suggest that either high or low MMP activity is detrimental. Our findings indicate that an intermediate level of MMP-3 expression, and therefore the 5A/6A heterozygote, may also be optimal with regard to large artery stiffness. This is well supported by the observation that low expression of MMPs promotes matrix accumulation and progression of atherosclerosis and specifically CAD. In MMP-3–deficient mice fed a high cholesterol diet, aortic atherosclerotic lesions were more severe than control mice expressing MMP-3. Data in humans are consistent with the low activity MMP-3 6A/6A genotype being associated with CAD progression and intima-media thickening. Because coronary atherosclerosis has been strongly associated with large vessel stiffening, the relationship between low MMP-3 expression and large vessel stiffening is plausible.

In addition, there is evidence that high expression of MMPs may promote vessel stiffening. Elevated MMP expression and the 5A allele of the MMP-3 polymorphism in particular, are associated with large vessel wall thinning and aneurysmal disease. This has also been shown experimentally in TIMP-1–deficient mice, where MMP activity is high and aneurysms in the thoracic and abdominal aorta more common. In Marfan syndrome, wall thinning, aortic dilation, and aneurysmal disease are associated with stiffening of the large arteries and elevated MMP expression. Because arterial elastic properties are conveyed primarily by the matrix, it is also plausible that excessive degradation of the elastic matrix will result in vessel wall stiffening. Thus, aortic dilation and wall thickening are likely adaptations to a stiff aorta such as occurs with aging. The intermediate gene and protein expression and more elastic large arteries in the MMP-3 5A/6A genotype suggest an optimal balance between matrix breakdown and accumulation.

Age-Related Large Artery Stiffening and MMP-3 Genotype

Age and atherosclerosis influence large artery stiffening by differing mechanisms, and MMPs are important in both. This population is uncomplicated by disease, suggesting that large artery stiffening is primarily a product of alterations in maintenance remodeling. As expected, we found higher input and characteristic impedance in the older group. Age-dependent stiffening is due largely to fatigue and fragmentation of extracellular matrix components as a result of cyclic stress. MMPs and particularly MMP-3 with its broad substrate spectrum, modulate these processes. Although it is acknowledged that numerous polymorphisms are likely to contribute to large artery stiffening, our results demonstrate that genetic variation in MMP-3 is associated with altered gene and protein expression, and may differentially regulate extracellular matrix composition and thereby age-related large artery stiffening. The stiffening effects of both homozygotes may result from the direct actions of MMP-3 on its known substrates or from activation of other MMPs. Because elastin fibers are relatively inert and have a long half life, it is possible that years of exposure to genotype-related differences is necessary before fibers actually fatigue and fracture (5A/5A) or exces-
sive accumulation of matrix (6A/6A) occurs. These facts may explain the presentation of arterial stiffening associated with the homozygote genotypes later in life. Furthermore, our data are consistent with the observation by Franklin that pulse pressure continues to rise after age 60 years in the face of stable mean pressure, suggesting that arterial stiffening secondary to structural deterioration is more pronounced after this age.42

**Therapeutic Implications**

The elevated large artery stiffness associated with the two homozygotes identifies these individuals as potential candidates for therapy aimed at reducing the risk of isolated systolic hypertension. In older populations, where isolated systolic hypertension is more prevalent, long-term antihypertensive treatment with ACE inhibitors and calcium antagonists has shown some pressure-independent decreases in arterial stiffness (see review). A more targeted approach are therapies including advanced glycation end product (AGE) breakers, which affect the large arteries more directly.43

**Conclusion**

The MMP-3 promoter polymorphism (5A/6A) modulates age-related large artery stiffening. Specifically, the 5A/6A genotype is associated with delayed large artery stiffening compared with the two homozygotes. The current data show that the 5A/6A heterozygote results in an intermediate level of gene and protein expression resulting in more elastic large arteries. Genetic variation at many loci are likely to be most useful in risk prediction and therapeutic targeting and may have relevance to isolated systolic hypertension.

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