A Role of Matrix Metalloproteinase-8 in Atherosclerosis

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Rationale: Atherosclerotic lesions express matrix metalloproteinase (MMP)8, which possesses proteolytic activity on matrix proteins particularly fibrillar collagens and on nonmatrix proteins such as angiotensin (Ang) I. Objective: We studied whether MMP8 plays a role in atherogenesis.

Methods and Results: In atherosclerosis-prone apolipoprotein E–deficient mice, inactivating MMP8 resulted in a substantial reduction in atherosclerotic lesion formation. Immunohistochemical examinations showed that atherosclerotic lesions in MMP8-deficient mice had significantly fewer macrophages but increased collagen content. In line with results of in vitro assays showing that Ang I cleavage by MMP8 generated Ang II, MMP8 knockout mice had lower Ang II levels and lower blood pressure. In addition, we found that products of Ang I cleavage by MMP8 increased vascular cell adhesion molecule (VCAM)-1 expression and that MMP8-deficient mice had reduced VCAM-1 expression in atherosclerotic lesions. Intravital microscopy analysis showed that leukocyte rolling and adhesion on vascular endothelium was reduced in MMP8 knockout mice. Furthermore, we detected an association between MMP8 gene variation and extent of coronary atherosclerosis in patients with coronary artery disease. A relationship among MMP8 gene variation, plasma VCAM-1 level, and atherosclerosis progression was also observed in a population-based, prospective study.

Conclusions: These results indicate that MMP8 is an important player in atherosclerosis. (Circ Res. 2009;105:921-929.)

Key Words: atherosclerosis ■ matrix metalloproteinase ■ gene

Chronic inflammation plays an important role in the pathogenesis of atherosclerosis, which, in turn, is the main underlying cause of the majority of coronary heart disease and stroke.1 One of the earliest steps in atherogenesis is the attachment of circulating leukocytes to the vascular endothelium consequent to the expression of adhesion molecules. Following adhesion to endothelial cells, leukocytes penetrate the endothelium into the arterial intima, differentiate into macrophages, uptake modified lipoproteins, and become lipid-laden foam cells which represent a major component of atherosclerotic lesions.1 In addition, vascular smooth muscle cells and extracellular matrix proteins, particularly types I and III collagens and proteoglycans, are also major constituents of atherosclerotic plaques.1

The matrix metalloproteinases (MMPs) are zinc-dependent proteases that can degrade extracellular matrix proteins.2 Some MMPs can also cleave nonmatrix proteins such as cytokines and growth factors.2 There is substantial evidence indicating that MMPs participate in a number of diseases with matrix remodeling and inflammation. Several MMPs have been demonstrated to be involved in atherosclerosis in which these different proteases appear to exert divergent effects.3–14 MMP8 (also known as collagenase-2) has also been suggested to be implicated in atherosclerosis.15–17 MMP8 possesses proteolytic activity on several matrix proteins particularly type I collagen and on some nonmatrix proteins such as angiotensin (Ang) I.18 It has been shown that endothelial cells, macrophages, and smooth muscle cells in atherosclerotic lesions express MMP8,15 that its expression is increased in rapidly progressing lesions,16 and that elevated serum MMP8 concentrations are associated with the presence of atherosclerosis and with unfavorable cardiovascular outcome.17 However, it has remained unknown whether MMP8 plays a causal role in atherogenesis or is simply a bystander marker. To address this issue, we studied whether inactivating the MMP8 gene would have an effect on atherogenesis in atherosclerosis-prone apolipoprotein (apo)E-deficient mice. We found that inactivating MMP8 resulted in a significant decrease in atherosclerotic lesion formation.
reduction in the extent of atherosclerosis, with a decrease in macrophages but an increase in collagen content in the atherosclerotic lesions. In addition, we found that MMP8 knockout mice had lower levels of Ang II, lower blood pressure, decreased vascular cell adhesion molecular (VCAM)-1 expression and reduced vascular leukocyte recruitment. Furthermore, we observed that in humans, MMP8 gene variation was associated with VCAM-1 level and severity and progression of atherosclerosis.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animals
All experiments were conducted according to the Animals (Scientific Procedures) Act of 1986 (United Kingdom) and the institutional guidelines. MMP8+/−/apoE−/− mice19 were crossed with MMPB+−/apoE−/− mice to generate MMP8+/−/apoE−/− double heterozygous mice. MMP8+−/apoE−/− double heterozygous mice were bred to produce MMP8+/−/apoE−/− double knockout mice and MMP8−/−/apoE−/− controls (littermates of MMP8−/−/apoE−/− double knockout mice). Six-week-old males of both types of mouse were fed a Western diet for 12 weeks.

Characterization of Atherosclerotic Lesions
The extent of aortic atherosclerotic lesions in MMP8−/−/apoE−/− double knockout mice and controls was analyzed by en face staining of aortas with oil red O, as previously described.20 The contents of macrophages, smooth muscle cells, collagen type I, Ang II, and VCAM-1 in aortic atherosclerotic lesions were examined by immunohistochemical analyses.

Real-Time RT-PCR
MMP2, MMP9, MMP13, and MMP14 expression levels in MMP8−/−/apoE−/− mice and control mice were quantified by real-time RT-PCR.

Measurements of Cholesterol and Triglycerides
Plasma cholesterol and triglyceride levels in the MMP8−/−/apoE−/− double knockout mice and controls were determined with the use of commercially available assay kits.

Angiotensin I Cleavage by MMP8 and Mass Spectrometry
Ang I was incubated with purified human MMP8. An uncleaved Ang I control was generated by incubating Ang I in the assay buffer without MMP8. Ang I and cleavage products in the above solutions (from the cleavage reaction and the control, respectively) were extracted and analyzed by liquid chromatography–electrospray tandem mass spectrometry. The mass spectral data were processed into peak lists and searched against the Swiss Prot database. The relative intensity of the angiotensin peptides was calculated using the total ion count of each peptide.

Enzyme-Linked Immunosorbent Assay
Commercially available ELISA kits were used to measure plasma Ang I and Ang II. Concentrations of VCAM-1 in plasma samples were also measured by ELISA kits.

Blood Pressure Measurement
Blood pressure was measured in conscious mice using a volume pressure recording sensor and occlusion tail cuff.

Analyses of Other Ang II–Forming Enzymes
Tissue extracts were prepared from aortas from MMP8+/−/apoE−/− or MMP8−/−/apoE−/− mice and assayed for other Ang II–forming enzymes including angiotensin-converting enzyme (ACE), chymases, and cathepsins.

Flow Cytometry
Mouse endothelial cells (C166) were incubated with Ang I that had been subjected to cleavage by MMP8 or uncleaved Ang I, in the absence or presence of the Ang II type 1 receptor (AT1R) antagonist losartan and the Ang II type 2 receptor (AT2R) antagonist PD123319, and then subjected to flow cytometric analysis of VCAM-1 expression.

Intravalvular Microscopy
Mesentery wire ends were superfused with bicarbonate-buffered solution at 37°C at a rate of 2 mL/min. Cell rolling was determined by counting the number of leukocytes rolling per minute in each vessel. Cell adhesion was quantified by counting, for each vessel, the number of adherent leukocytes in a 100 μm length.

Human Subjects
We studied MMP8 gene variation in relation to extent of atherosclerosis in a group (n=2000) of patients with coronary artery disease (CAD) documented angiographically as having >50% diameter stenosis in at least 1 coronary artery.21 We also examined MMP8 gene variation in relation to progression of atherosclerosis in subjects of the Bruneck study, a population-based, prospective study of atherosclerosis.22–25

Functional Analysis of MMP8 Gene rs1940475 SNP
MMP8zymogens with Glu87 or Lys87 were synthesized and subjected to activation by 4-aminophenylmercuric acetate, followed by Western blot analysis.

Statistical Analyses
The statistical analyses performed in this study are described in the Online Data Supplement.

Results
Reduced Atherosclerosis in MMP8-Deficient Mouse
Extensive aortic atherosclerotic lesions were observed in MMP8−/−/apoE−/− mice (controls) fed a Western diet (Figure 1A). In contrast, MMP8+/−/apoE−/− double knockout mice had substantially less aortic atherosclerosis (36% reduction compared with controls, P=0.007; Figure 1A). Compared with the lesions in the controls, those in the double knockout mice had significantly fewer macrophages (70% reduction, P=0.002; Figure 1B). In addition, there was a trend toward lower smooth muscle cell content in the lesions.
in the double knockout mice than lesions in the controls ($P=0.057$; Figure 1C). On the other hand, collagen content was higher in the lesions in the double knockouts than lesions in the controls (differing by 27%, $P=0.034$; Figure 1D). There was no significant difference in plasma cholesterol and triglyceride levels between the 2 groups ($P=0.887$ for cholesterol and $P=0.117$ for triglyceride). The levels of expression of the other mouse collagenases including MMP2, MMP3, MMP13, and MMP14 did not differ between the 2 groups ($P=0.108$, $P=0.611$, $P=0.509$, and $P=0.318$, respectively; Online Figure II).

Reduced Ang II Level and VCAM-1 Expression in MMP8 Knockout Mouse
MMP8 has been shown to cleave Ang I–producing Ang II,26 a well-established atherogenic factor,27–29 and Ang1–7. To verify this finding, we incubated Ang I with MMP8 and then performed liquid chromatography–electrospray tandem mass spectrometric analysis. The analysis showed cleavage of Ang I by MMP8 generated Ang II and Ang1–7, with the former being the major product (Figure 2 and Online Table V).

We then examined whether there was a difference in Ang II level between MMP8–/-apoE–/- double knockout mice and controls. Indeed, we found that Ang II levels in atherosclerotic lesions and blood were lower in double knockout mice than in controls ($P=0.002$ and $P=0.026$, respectively; Figure 3A and 3B). In contrast, plasma levels of Ang I, the processor of Ang II, were higher in the double knockout group than in the control group ($P=0.018$; Figure 3C), which might be explained by reduced Ang I cleavage in double knockout mice. Consistent with the finding of reduced Ang II levels in double knockout mice, we found that blood pressure was lower in double knockout mice than in controls ($P=0.017$ for systolic and $P=0.023$ for diastolic blood pressure; Figure 4). We found no difference between MMP8–/-/apoE–/- double knockout mice and controls in the levels of the other Ang II–forming enzymes ACE, chymases, and cathepsins (Online Figure III).

Previous studies demonstrated that Ang II induced the expression of adhesion molecules,28,29 such as VCAM-1,30–34 which plays an important role in atherogenesis.35 In agreement, our experiments showed that Ang II in a wide range of concentration increased VCAM-1 expression in endothelial cells (Online Figure IV). In addition, we found that the products of Ang I cleavage by MMP8 increased VCAM-1 expression. As shown
in Figure 5A and 5B, flow cytometric analysis showed that cultured endothelial cells treated with products of Ang I cleavage by MMP8 expressed more VCAM-1 than endothelial cells treated with uncleaved Ang I (P=0.002). This effect was abolished in the presence of the AT1R antagonist losartan and the AT2R antagonist PD123319 (Figure 5C and 5D).

We also examined whether there was a difference in VCAM-1 expression level between MMP8−/−/apoE−/− double knockout mice and controls. We observed that VCAM-1 expression in endothelial and other cells in atherosclerotic lesions was substantially reduced in the double knockout mice compared with controls (Figure 3).
6A through 6C). Plasma levels of soluble VCAM-1 were also lower in double knockout mice than in controls (Figure 6D).

Reduced Leukocyte Recruitment in MMP8 Knockout Mice

Intravital microscopy examination showed that leukocyte rolling and adhesion on vascular endothelium was attenuated (by \( \geq 50\% \)) in double knockout mice compared with controls \((P=0.049 \text{ and } P=0.009, \text{ respectively; Figure } 7)\), which might be explained, in part, by the reduction of Ang II and VCAM-1 levels in the double knockout mice.

Association of MMP8 Gene Variation With VCAM-1 Level and Atherosclerosis in Humans

In addition to the animal and in vitro studies described above, we investigated whether in humans, there was a relationship between MMP8 gene variation and interindividual differences in atherosclerosis. To address this question, we ascertained whether MMP8 gene variation was associated with extent of atherosclerosis in patients \((n=2000)\) with CAD. In addition, we examined whether MMP8 gene variation was associated with atherosclerosis progression in a population-based, prospective study (the Bruneck study, \(n=782\)).

In the study of CAD patients, we initially genotyped 1000 subjects for a panel of 16 single nucleotide polymorphisms (SNPs) consisting of all common SNPs (with minor allele frequency of \( \geq 0.05 \)) identified by resequencing the MMP8 gene proximal promoter (2 kb) and coding regions and tagging SNPs selected from the HapMap database to capture \((r^2 \geq 0.8)\) common SNPs (with minor allele frequency of \( \geq 0.05 \)) in the introns, 5' upstream sequence and 3'-untranslated region. An association was detected between the extent of coronary atherosclerosis and SNP rs1940475 (Single Nucleotide Polymorphism database identification number) \((P=0.01)\). We then genotyped 1000 additional CAD patients for this SNP and analyzed the data for this SNP in the entire sample \((n=2000)\). The analysis showed a highly significant association between the SNP and extent of coronary atherosclerosis \((P=0.0008)\), with the T allele having a lower frequency in patients who had \( > 50\% \) stenosis in 2 or 3 coronary arteries than in patients with \( > 50\% \) stenosis in 1 coronary artery (odds ratio=0.80, 95% confidence inter-

![Figure 4. Reduced blood pressure in MMP8+/-/apoE-/- mice.](chart)

![Figure 5. Products of Ang I cleavage by MMP8 increase VCAM-1 expression in endothelial cells. A and B, Representative plot (A) and mean fluorescence intensity (B) in flow cytometric analyses of VCAM-1 expression on endothelial cells incubated with uncleaved Ang I or with products of Ang I cleavage by MMP8. C and D, Representative plot (C) and mean fluorescence intensity (D) in flow cytometric analyses of VCAM-1 expression on endothelial cells incubated with uncleaved Ang I or with products of Ang I cleavage by MMP8, in the presence of the AT,R antagonist losartan and the AT,R antagonist PD123319. Error bars in the column charts are SEM.)](chart)
val = 0.70 to 0.91; Table). The association remained significant after taking into account the number of SNPs tested in this study, as the probability value (0.0008) for the association observed was smaller than the significance threshold (0.005) calculated using the SNPSpD method to take into consideration the number of SNPs tested and the degree of linkage disequilibrium between the SNPs.

In line with the finding in the above study of CAD patients, the population-based, prospective study (the Bruneck study) showed that the T allele of SNP rs1940475 was associated with a protective effect against carotid atherosclerosis progression in a 10-year follow-up (odds ratio, 0.77 [95% confidence interval 0.60 to 0.98] for atherosclerosis progression between 1990 and 1995; odds ratio 0.74 [95% confidence interval 0.56 to 0.99] for atherosclerosis progression between 1995 and 2000) (Online Table VI). In addition, we found that plasma levels of soluble VCAM-1 were lower in individuals who carried the T allele (P = 0.014, Online Table VII).

MMP8 is secreted from cells as a latent form, and activation is achieved by removing the propeptide domain of the zymogen.18 The rs1940475 is a nonsynonymous substitution resulting in a change from glutamic acid (Glu) to lysine (Lys) at amino acid residue 87 in the propeptide domain of MMP8. We carried out in vitro analysis to investigate whether this SNP could have an effect on MMP8 activation. The analysis indicated that MMP8 zymogen with Lys87 (produced by the T allele) is less amenable to activation than the zymogen with Glu87 (produced by the C allele) (Online Figure V).

**Discussion**

Our study shows that inactivating MMP8 causes a substantial reduction in the extent of atherosclerosis in apoE-deficient mice fed a Western diet. The atherosclerotic lesions in the MMP8 knockout mice have fewer macrophages but higher collagen content. Compared with controls, MMP8-deficient mice have higher Ang I and lower Ang II levels, likely reflecting the ability of MMP8 to cleave Ang I and generate Ang II. Consistently, we found that blood pressure was lower in MMP8 knockout mice than in controls. In agreement with the finding that Ang II induces adhesion molecule expression,28–34 we found that products of Ang I cleavage by MMP8 increases VCAM-1 expression in cultured endothelial cells. In addition, we observed reduced expression of VCAM-1 in MMP8 knockout mice than in controls. In addition to the
findings from the mouse study, we detected an association between MMP8 gene variation and extent of coronary atherosclerosis in CAD patients and an association of the MMP8 gene variation with plasma VCAM-1 level and progression of atherosclerosis in a population-based, prospective cohort study (the Bruneck study). Functional analysis indicated that the MMP8 genetic variant associated with reduced atherosclerosis had an effect on MMP8 zymogen activation. Taken together, these results indicate that MMP8 is an important player in atherogenesis.

Classically, MMPs are implicated in matrix remodeling through their matrix protein–degrading activity. However, relatively recent studies have revealed that MMPs have other important biological functions arising from their proteolytic activity on nonmatrix proteins. In particular, an increasing number of cytokines, chemokines, and growth factors have been shown to be processed by MMPs, and there is growing evidence indicating that the actions of MMPs on these nonmatrix proteins play important roles in tumorigenesis and a number of inflammatory diseases. Although currently the most appreciated roles of MMPs in atherosclerosis are mainly related to matrix protein degradation that facilitates cell migration and undermines atherosclerotic plaque stability, it is conceivable that actions of MMPs on nonmatrix proteins also play a part in the development and progression of atherosclerosis. The findings that MMP8 can cleave Ang I generating Ang II and that MMP8 knockout mice have reduced Ang II levels seem to support this notion. It is plausible that the role of MMP8 in atherogenesis is partly related to its proteolytic activity on matrix proteins particularly type I collagen, as well as its ability to cleave Ang I generating Ang II. Thus, the reduction in atherosclerotic lesion formation in MMP8 knockout mice could be partly attributable to reduced blood pressure and reduced VCAM-1 expression as a result of lower Ang II levels.

The best known Ang I convertor is ACE, but other enzymes (such as chymases, cathepsins, and MMP8) have also been shown to be able to cleave Ang I to generate Ang II. ACE is a zinc-dependent metalloproteinase and belongs to the metalloproteinase superfamily that also includes MMPs. The catalytic sites of ACE and collagenses share similarities in amino acid sequence and structure. Interestingly, it has been shown that the ACE inhibitors captopril, lisinopril, quinapril, and enalapril also inhibit MMP activity through interacting with the MMP active site, analogous to their interaction with the active site of ACE. Although ACE is likely to be the main convertor of Ang I, it is probable that other enzymes such as MMP8, chymases, and cathepsins also contribute to Ang I conversion, particularly at locations where ACE levels are low.

Previous studies have examined the effects of several MMPs on atherogenesis in apoE-deficient mice or LDL receptor deficient mice. These studies have suggested divergent effects of these enzymes in atherosclerotic lesion formation and stability. It was shown that expressing human MMP1 in macrophages in apoE-deficient mice resulted in smaller aortic atherosclerotic lesions with reduced fibrillar collagen content. Similarly, inactivating MMP3 increased aortic atherosclerotic lesion sizes and collagen content. Inactivating MMP13 also increased, whereas overexpressing MMP14 decreased, collagen accumulation in aortic atherosclerotic lesions without an apparent effect on lesion size. In contrast, inactivating MMP2 resulted in decreased aortic atherosclerotic lesions with reduced numbers of smooth muscle cells. Inactivating MMP9 also led to smaller aortic atherosclerotic lesions with reduced numbers of smooth muscle cells but also with fewer macrophages and lower collagen content. Moreover, it was shown that overexpressing active MMP9 in macrophages in apoE-deficient mice induced aortic atherosclerotic plaque disruption. MMP12 has also been shown to exert an effect on atherosclerotic lesion stability. In contrast, inactivating MMP7 had no effect in these characteristics. Thus, the findings from these studies suggest that these MMPs exert divergent effects, some impacting on lesion size, some affecting collagen content, and some being required for macrophage and/or smooth muscle cell accumulation. As described earlier, our study showed that MMP8 knockout resulted in reduced atherosclerotic lesions with fewer macrophages and increased collagen content. Thus, in apoE-deficient mice, MMP8 has a different profile of activities from the other MMPs mentioned above. However, similar to MMP-1, -3, -13, and -14,

<table>
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<th>rs1940475 SNP</th>
<th>Subjects With Significant Atherosclerosis* in One Coronary Artery</th>
<th>Subjects With Significant Atherosclerosis* in Two or Three Coronary Arteries</th>
<th>Odds Ratio (95% Confidence Interval)† for Significant Atherosclerosis* in Two or Three Coronary Arteries</th>
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<td>C/C</td>
<td>170 (22.2)</td>
<td>342 (27.7)</td>
<td>Reference</td>
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<tr>
<td>C/T</td>
<td>398 (52.0)</td>
<td>632 (51.2)</td>
<td>0.77 (0.62–0.97); P=0.028</td>
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<tr>
<td>T/T</td>
<td>198 (25.8)</td>
<td>260 (21.1)</td>
<td>0.63 (0.49–0.83); P=0.0008</td>
</tr>
<tr>
<td>Total</td>
<td>766 (100.0)</td>
<td>1234 (100.0)</td>
<td></td>
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*Significant atherosclerosis was defined by ≥50% stenosis. †Calculated by logistic regression analysis with adjustment for age, gender, smoking, hypercholesterolemia, hypertension, and diabetes.
MMP8 reduced collagen accumulation in atherosclerotic lesions, which could potentially have implications on atherosclerotic lesion stability.

The present study has some limitations. The study only assessed atherosclerosis in the aorta following a Western diet for 12 weeks. Further work is warranted to study the effect of MMP8 deficiency on atherosclerosis in other vascular sites, in shorter and longer durations of Western diet, and in chow diet. For the CAD patients and the Bruneck cohort, no Ang II level data were available, and therefore we were unable to determine whether there was an association between Ang II level and MMP8 genotype. This would also warrant investigating in future work. It would also be interesting to investigate the relative contributions of the reduction in Ang II generation and the increase in collagen content to atherosclerotic lesion development in MMP8 knockout mice in future studies.

In summary, our study indicates that MMP8 is an important player in atherogenesis. Taken together with the aforementioned studies of other MMPs, this work provides further evidence of involvement of MMPs in atherosclerosis and further supports the notion that different MMPs have divergent effects in the atherogenic process.

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Disclosures
None.

References


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A Role of Matrix Metalloproteinase-8 in Atherosclerosis: Correction

In the article that appears on page 921 of the October 23, 2009, issue, the authors omitted the following text from the Sources of Funding section.

The work in the article by Laxton et al. forms part of the research themes contributing to the translational research portfolio of Barts and the London Cardiovascular Biomedical Research Unit, which is supported and funded by the National Institute of Health Research.

The authors regret this error. This error has been noted in the online version of the article, which is available at http://circres.ahajournals.org/cgi/content/full/105/9/921

Reference


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Online Supplement

Methods

Animals
MMP8+/apoE+ mice (C57BL/6 background, Jackson Laboratory, stock number 005514, strain name B6;129X1-Mmp8tm1Lotn/J) were crossbred with MMP8+/apoE− mice (C57BL/6 background, Jackson Laboratory, stock number 002052, strain name B6.129P2-Apoetm1Unc/J) to generate MMP8+/apoE− double heterozygous mice. MMP8+/apoE+ double heterozygous mice were bred to produce MMP8+/apoE− double knockout mice and MMP8+/apoE− controls (littermates of MMP8+/apoE− double knockout mice). The breeding was performed by the Jackson Laboratory (Bar Harbor, USA). Supplemental Figure 1 (Online Supplemental Material) shows the presence of the MMP8 gene in the MMP8+/apoE+ mice (controls) and absence of the MMP8 gene in the MMP8+/apoE− knockout mice. Six week old males of both types of mouse were fed a Western diet (Research Diet, D12108, containing 21% fat, 1.25% cholesterol and 0% cholate) for 12 weeks.

Characterization of atherosclerotic lesions
The extent of aortic atherosclerotic lesions in MMP8+/apoE− double knockout mice and controls was analyzed by en face staining of aortas with oil red O, as previously described. The stained aortas were photographed using a digital camera, and the oil red O-stained areas analyzed by computer-assisted quantification using the Image-Pro software.

The contents of macrophages, smooth muscle cells, collagen type I, Ang II and VCAM-1 in atherosclerotic lesions were examined using the immunohistochemical method. In brief, the hearts with aortic roots were dissected from the animals and frozen sections of the aortic roots were subjected to immunostaining with the use of antibodies for the macrophage marker Mac-1 (BD Biosciences Pharmingen), the smooth muscle cell marker α-actin (Sigma-Aldrich), collagen I (Abcam) and VCAM-1 (BD Biosciences Pharmingen), respectively. Images were captured by a digital imaging system, and areas of positive stains were measured using the Image-Pro software.

For Ang II immunostaining, formalin fixed paraffin embedded sections mounted on slides were deparaffinized with xylene and ethanol, followed by antigen retrieval by incubating the sections with 10mM citrate buffer (pH 6.0) for 20min at 100°C. The sections were then washed three times with phosphate buffered solution (PBS) and soaked in absolute methanol containing 3% hydrogen peroxide for 15min at room temperature to remove endogenous peroxidase activity. To block nonspecific binding, the sections were incubated with 10% non-immune swine serum for 20min. The sections were then incubated with rabbit anti-angiotensin II antibody (Phoenix Pharmaceuticals, 1:200 dilution) for 60min at room temperature. The slides were washed with PBS again and incubated with biotin-conjugated swine anti-rabbit IgG antibody for 30min. They were washed again with PBS and then incubated with avidin-biotin-peroxidase complex (Vectorstain Elite ABC kit, Vector Laboratories) for 30min. After another wash with PBS, the sections were incubated with 0.03% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine. The slides were then washed in running tap water, counterstained with hematoxylin. Non-immunized rabbit IgG was used as a negative control. The relative intensity of Ang II staining in atherosclerotic lesions was defined by the ratio of Ang II staining intensity over the total luminance, subtracted by that of the IgG negative control.

Real-time reverse-transcriptase-PCR (real-time RT-PCR)
RNA was extracted from aortas from MMP8+/apoE+ or MMP8+/apoE− mice and reversed transcribed to cDNA. Real-time PCR of MMP2, MMP9, MMP13 and MMP14 was performed in duplicate in an ABI Prism 7900HT Sequence Detection System. The PCR primer sequences are described in Online Supplement Table 1. Real-time RT-PCR data of MMP2, MMP9, MMP13 and MMP14 respectively were analysed using the ∆∆CT method, standardized against GAPDH reference house-keeping gene values.
Measurements of cholesterol and triglycerides
Plasma cholesterol and triglyceride levels in the MMP8−/−/apoE−/− double knockout mice and controls were determined with the use of cholesterol quantitation kit (Calbiochem) and triglyceride measurement kit (Sigma-Aldrich).

Angiotensin I cleavage by MMP8 and mass spectrometry
Ang I cleavage by MMP8 was performed as in a previous study. In brief, Ang I (Phoenix Pharmaceuticals) was incubated with purified human MMP8 (BioMol International), at a substrate concentration of 25μmol/L and an enzyme/substrate ratio of 1:100, at 37°C for 20h, in assay buffer (50mmol/L Tris, 10mmol/L CaCl₂, 150mmol/L NaCl, 0.05% Brij 35, pH 7.5) in the presence of 0.5mmol/L 4-aminophenylmercuric acetate (APMA) to activate proMMP8. An uncleaved Ang I control was generated by incubating Ang I in the assay buffer in the presence of APMA but without MMP8, at 37°C for 20h.

Ang I and cleavage products in the above solutions (from the cleavage reaction and the control, respectively) were extracted with the use of C18 ziptip (Millipore), and eluted in 80% acetonitrile and 0.1% trifluoroacetic acid. The eluates were dried in a SpeedVac, and each sample resuspended in 50mmol/L ammonium bicarbonate. A 5μl aliquot of each sample was analysed by liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) using a CapLC system (Waters Corporation). Peptides were resolved by reversed phase chromatography on a 75μm C18 PepMap column. A gradient of acetonitrile in 0.05% formic acid was delivered to elute the peptides at a flow rate of 200nl/min. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the m/z and the charge state of the peptide. The mass spectral data was processed into peak lists (MS/MS data) and searched against the Swiss Prot database using the MASCOT search algorithm (www.matrixscience.com). Angiotensin peptides identified with a significant score of >30 were validated by manual interpretation which confirmed the agreement between the spectra and the peptide sequences. The relative intensity of the angiotensin peptides was calculated using the total ion count of each peptide.

Enzyme-linked immuno-sorbent assay (ELISA)
Commercially available ELISA kits were used to measure Ang I (Phoenix Pharmaceuticals) and Ang II (Phoenix Pharmaceuticals). Concentrations of VCAM-1 in plasma samples were also measured by ELISA kits (R&D systems).

Blood pressure measurement
Blood pressure was measured in conscious mice using a volume pressure recording sensor and occlusion tail cuff (Coda2, Kent Scientific, USA).

Analyses of other angiotensin II forming enzymes
Tissue extracts were prepared by a previously described method with modifications. In brief, aortas from MMP8+/+ /ApoE−/− or MMP8−/−/ApoE−/− mice were cut into small pieces and immediately placed into ice cold 20mmol/L Na-phosphate buffer in Precellys CK14 bead tubes, and lysed at 4°C in a Precelllys24 homogeniser. The lysate from each aorta was transferred into a microfuge tube and centrifuged at 25,000g for 30min at 4°C. The pellet was resuspended in 10mmol/L Na-phosphate buffer containing 2mol/L KCl and 0.1% Nonidet P-40 (pH 7.4), then divided into two microfuge tubes and centrifuged again as above. The pellet in one tube was resuspended in 10mmol/L Na-phosphate buffer containing 2mol/L KCl and 0.6% Nonidet P-40 and stored at 4°C for 18 hours; thereafter the sample suspension was centrifuged and the supernatant was subjected to ACE activity assay. The pellet in the other tube was resuspended in 10mmol/L Na-phosphate buffer containing 2mol/L KCl and 0.01% Nonidet P-40 (pH 7.4) and stored at 4°C for 18 hours; subsequently the sample suspension was subjected to analyses of chymase and cathepsin activity. Protein concentration was measured using the Bradford method.

ACE activity was determined by incubating the aorta extract with fluorogenic peptide substrate V
(R&D systems) in assay buffer containing 50mmol/L 2-(N-morpholino)ethanesulfonic acid (pH 6.5) and chymostatin, with or without the ACE inhibitor captopril (100μmol/L). The reaction mixture was incubated at 37°C for 90min, and fluorescence measured using a spectrofluorometer (Wallac Victor II). The assays for each sample were performed in duplicate. For each sample, ACE activity was calculated by subtracting fluorescence reading in the assay with captopril from the fluorescence reading in the assay without captopril, and standardized against the protein concentration in sample.

The activity of chymases and cathepsins was analysed by incubating the aorta extract with a colorimetric substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide, Chymase Assay Kit, Sigma-Aldrich) of these enzymes, with or without the inhibitor chymostatin which inhibits chymases and cathepsins. The reaction mixture was incubated at 37°C for 30min, and absorbance at 405nm was measured using a spectrophotometer (Dynex MRX Revelation). The assays for each sample were carried out in duplicate. For each sample, enzymatic activity was calculated by subtracting absorbance reading in the assay with chymostatin from the absorbance reading in the assay without chymostatin, and standardized against the protein concentration in sample.

**Flow Cytometry**

Mouse endothelial cells (C166) were obtained from American Type Culture Collection and cultivated in Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum (10%), penicillin and streptomycin, at 37°C in a humidified atmosphere of 5% CO₂ in air. To determine the range of concentration of Ang II that induced VCAM-1 expression in the endothelial cells, the cells were incubated with Ang II at the concentrations of 10⁻⁶, 10⁻⁷, 10⁻⁸ and 10⁻⁹mol/L respectively for 18 hours, followed by flow cytometric analyses. In subsequent experiments, cells were incubated with Ang I (10⁻⁷mol/L) that had been subjected to cleavage by MMP8 (the cleavage reaction performed as described above) or uncleaved Ang I (10⁻⁷mol/L, the uncleaved Ang I control which contained the cleavage reaction reagents except MMP8 as described above), in the absence or presence of the Ang II type 1 receptor (AT1R) antagonist losartan (10⁻⁴mol/L) and the Ang II type 2 receptor (AT2R) antagonist PD123319 (10⁻⁴mol/L). For flow cytometric analyses, the cells were then harvested with Accutase (Sigma-Aldrich), washed and suspended in PBS containing 0.2% BSA at 10⁶ cell per ml. The cell suspensions were incubated for 40min on ice with a rat anti-mouse VCAM-1 monoclonal antibody (5μg/ml) (BioLegend) or an isotype-matched control antibody (5μg/ml) (BioLegend). Subsequently, the cells were washed and incubated for 40min on ice with a fluorescein-5-isothiocyanate conjugated goat anti-rat monoclonal antibody (5μg/ml) (BioLegend), and then analyzed by flow cytometry.

**Intravital microscopy**

Intravital microscopy was carried out as previously described⁶. In brief, mice were anesthetized with diazepam and hypnorn, and the mesenteric vascular bed was prepared for viewing by intravital microscopy. Mesenteries were superfused with bicarbonate-buffered solution [132mmol/L NaCl, 4.7mmol/L KCl, 1.2mmol/L MgSO₄, 17.9mmol/L NaHCO₃, and 2.0mmol/L CaCl₂ (pH 7.4)], gassed with 5% CO₂ and 95% N₂) at 37°C at a rate of 2ml/min. The temperature of the stage was maintained at 37°C. Cell rolling was determined by counting the number of leukocytes rolling per minute in each vessel. Cell adhesion was quantified by counting, for each vessel, the number of adherent leukocytes in a 100μm length. A minimum of three postcapillary venules (diameter between 20 and 40μm; length of at least 100µm) were observed for each mouse. All experiments were conducted according to the Animals (Scientific Procedures) Act of 1986 (United Kingdom) and the institutional guidelines.

**Human subjects**

We studied MMP8 gene variation in relation to extent of atherosclerosis in a group (n=2000) of patients with CAD documented angiographically as having >50% diameter stenosis in at least one coronary artery⁷. The patients were all British Europeans and were recruited from the Southampton General Hospital. Guidelines for coronary angiography from the American College of Cardiology and the American Heart Association were followed⁸. Demographic and clinical characteristics of the subjects are shown in Online Supplement Table 2.
We also examined MMP8 gene variation in relation to progression of atherosclerosis in subjects of the Bruneck Study, a population-based, prospective study of atherosclerosis. At the outset of the Bruneck Study in 1990, a sex- and age-stratified random sample (n=1000, consisting of 125 women and 125 men in each of the fifth to eighth decade) was drawn from all inhabitants of Bruneck, Italy. A total of 936 individuals took part in the study, and data collection was completed for 919 subjects. The demographic and clinical characteristics of these subjects were very similar to those of the entire cohort. A follow-up took place in 1995 with 826 available subjects and a second follow-up was carried out in 2000. Genotyping was successfully performed for 782 participants who formed the current study population. Carotid artery atherosclerosis was measured by ultrasound scanning and monitored over the 10-year period between 1990 and 2000 as described in detail previously. Blood samples were taken after an overnight fast and 12 hours of abstinence from smoking. Plasma levels of inflammation markers were measured by standard methods as described previously. Demographic and clinical characteristics of the subjects are shown in Online Supplement Table 3.

The above studies were approved by the respective local research ethics committee, and all subjects gave informed consent.

**MMP8 re-sequencing and genotyping**

We sequenced the 2kb proximal promoter region, all exons and intron-exon junctions of the MMP8 gene in 30 unrelated individuals of European ancestry to identify common sequence variants in these regions. We then genotyped 1000 CAD patients recruited from Southampton General Hospital for a panel of 16 SNPs consisting of all common SNPs (with minor allele frequency ≥ 0.05) identified by the re-sequencing described above and tagging SNPs selected from the HapMap database to capture (r² ≥ 0.8) common SNPs (with minor allele frequency ≥ 0.05) in the introns, 5’ upstream sequence and 3’ untranslated region. The SNPs are detailed in Online Supplement Table 4. Following the initial analysis which showed that the rs1940475 SNP was associated with extent of coronary atherosclerosis and the association remained significant after taking into account the other SNPs examined, we genotyped 1000 additional CAD patients recruited from Southampton General Hospital for the rs1940475 SNP. We also genotyped the Bruneck Study subjects for this SNP. Genotyping was carried out with the use of the 5’ nuclease assay method or the KASPar system.

**Functional analysis of MMP8 gene rs1940475 SNP**

Full-length MMP8 cDNA for the C (Glu87) allele was obtained from OriGene (Rockville, MD) and cDNA for the T (Lys87) allele was created by site-directed in vitro mutagenesis. The cDNAs for the Glu87 and Lys87 alleles respectively were cloned into a TOPO expression vector (Invitrogen). The MMP8 zymogens with Glu87 or Lys87 were synthesized with the use of the TNT quick coupled transcription/translation system (Promega). The zymogens were incubated in the presence or absence of the activator APMA (1.5mM) at 37°C for 60min and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blot analysis with the use of a monoclonal anti-MMP8 antibody (R&D Systems) that detects both the latent and active forms of MMP8. The intensities of the bands of the latent form (60kDa and 70kDa) and active form (40kDa and 45kDa) were quantified with the use of Image-pro software.

**Statistical analyses**

t-Test was used to ascertain differences between MMP8+/apoE−/− double knockout and MMP8+/−/apoE−/− control mice in extent of atherosclerotic lesions and contents of macrophage, smooth muscle cell, collagen, Ang II and VCAM-1, plasma levels of Ang I, Ang II, soluble VCAM-1, cholesterol and triglycerides, blood pressure, and leukocyte rolling and adhesion on vascular endothelium. t-Test was performed to compare mean fluorescence intensity in flow cytometric analysis.

Logistical regression analysis and linear regression analysis were performed to ascertain a relationship between MMP8 SNPs and the number of coronary arteries with significant atherosclerosis (>50% stenosis) in the CAD patients and a relationship between the MMP8 rs1940475 SNP and progression of carotid atherosclerosis in the Bruneck Study cohort. Analysis of variance was used to test a
difference in plasma VCAM-1 level between rs1940475 genotypes in the Bruneck Study cohort. The SNPSpD method\textsuperscript{13,14} was used to calculate the significance threshold required to keep type I error rate at 5% accounting for the 16 MMP8 SNPs examined and linkage disequilibrium between them, which gave a significance threshold of 0.005. This threshold was used in the study of the CAD patients in which the 16 MMP8 SNPs were tested. In the Bruneck study, only one SNP (rs1940475) was tested and therefore the significance threshold of 0.05 was used.
Online Supplemental Figure 1

Agarose gel image showing that the MMP8 gene is present in the MMP8+/+/ApoE-/- mice (controls) and absent in the MMP8-/-/ApoE-/- mice.

PCR was performed on genomic DNA samples from the MMP8+/+/ApoE-/- or MMP8-/-/ApoE-/- mice, using the primers flanking exon 3 of the mouse MMP8 gene. The sequences of the PCR primers are 5'-GCAAACCATTCTTACTCTTCTC-3' and 5'-CTTTGCCCAATGTTTCAGGT-3', respectively. The expected PCR amplicon is 234 bp in length. Lane 1, DNA size marker (1kb DNA ladder, Invitrogen Cat 15615-016). Lanes 2-11, PCR products from different mice.
Results of real-time RT-PCR showing that the other mouse collagenases MMP2, MMP9, MMP13 and MMP14 are not altered in MMP8 knockout mice. RNA was extracted from aortas from MMP8<sup>+/−</sup>/ApoE<sup>−/−</sup> or MMP8<sup>−/−</sup>/ApoE<sup>−/−</sup> mice and subjected to real-time RT-PCR analysis of MMP2, MMP9, MMP13 and MMP14 and GAPDH. Real-time RT-PCR data were analysed using the ΔΔCT method, standardized against GAPDH reference house-keeping gene values. Data shown in chart are mean MMP CT values after being subtracted by GAPDH CT values. Error bars represent standard error of mean.
Online Supplement Figure 3

Charts showing that there is no significant difference in the activity of other angiotensin II forming enzymes (ACE, chymases and cathepsins) between MMP8^{+/+}/ApoE^{-/-} and MMP8^{-/-}/ApoE^{-/-} mice. Data shown are mean ± standard error of mean.
Online Supplement Figure 4

Chart showing that Ang II in a wide range of concentration increases VCAM-1 expression in cultured endothelial cells. Shown in chart are mean fluorescence intensities (and standard error of mean) in flow cytometric analysis. p-values shown are for comparisons between Ang II treated cells and untreated cells.
**Online Supplement Figure 5**

Western blot analysis showing that MMP8 zymogen with Lys87 is less amenable to activation than MMP8 zymogen with Glu87

(A) Image of Western blot analysis. MMP8 zymogens with Glu87 or Lys87 were incubated in the presence or absence of the activator 4-aminophenylmercuric acetate (APMA) and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blot analysis with the use of a monoclonal anti-MMP8 antibody that recognizes both active and latent MMP8.

(B) and (C) Curves represent intensities of the bands of latent (60kDa, and 70kDa likely representing a glycosylation form) and active (40kDa, and 45kDa likely representing a glycosylation form) MMP8 with Glu87 (red lines) or Lys87 (blue lines). (B) shows that in the absence of the activator APMA, the intensities of the 40kDa and 45kDa bands (active MMP8) are similar between MMP8 with Glu87 (red line, representing intensities of bands in lane 1 in Western blot image) and MMP8 Lys87 (blue line, representing intensities of bands in lane 2 in Western blot image). (C) shows that in the presence of APMA, the intensity of the 40kDa and 45kDa bands (active MMP8) produced by MMP8 with Lys87 (blue line, representing intensities of bands in lane 4 in Western blot image) are lower than those produced by MMP8 with Glu87 (red line, representing intensities of bands in lane 4 in Western blot image), indicating that MMP8 zymogen with Lys87 is less amenable to activation than the zymogen with Glu87.
**Online Supplement Table 1.**

**Real-time RT-PCR primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2</td>
<td>5’-GTCGCCCTAAACAGACAA-3’</td>
<td>5’-GGTCTCGATGGTGTTCTGGT-3’</td>
</tr>
<tr>
<td>MMP9</td>
<td>5’-CGTCGTGATCCCCACTTACT-3’</td>
<td>5’-AACACACAGGGTTTGCCTTC-3’</td>
</tr>
<tr>
<td>MMP13</td>
<td>5’-GCCCTGATGTTCCCATCTA-3’</td>
<td>5’-TTTTGGGATGCTTAGGGTTG-3’</td>
</tr>
<tr>
<td>MMP14</td>
<td>5’-GGATACCCAATGCCCATTGGCCA-3’</td>
<td>5’-CCATTGGGCATCCAGAAGAGAGC-3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-TGACGTGCCGCTGGAGAAAC-3’</td>
<td>5’-CCGGCATCGAAGGTGGAAGAGT-3’</td>
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</tbody>
</table>
### Online Supplement Table 2.
**Demographic and clinical characteristics of the Southampton CAD patients (n=2000)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>means (SD) or %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age - year</td>
<td>63.5 (10.0)</td>
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<tr>
<td>Male gender</td>
<td>74.4%</td>
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<tr>
<td>Body mass index</td>
<td>27.4 (4.3)</td>
</tr>
<tr>
<td>Smoking</td>
<td>72.8%</td>
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<tr>
<td>Hypertension</td>
<td>42.4%</td>
</tr>
<tr>
<td>Diabetes</td>
<td>12.7%</td>
</tr>
</tbody>
</table>

SD, standard deviation

### Online Supplement Table 3.
**Demographic and clinical characteristics of the Bruneck study subjects (n=782)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>means (SD) or %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age – year</td>
<td>62.8 (11.1)</td>
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<tr>
<td>Male gender</td>
<td>49.4%</td>
</tr>
<tr>
<td>Body mass index</td>
<td>25.6 (3.9)</td>
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<tr>
<td>Smoking</td>
<td>19.3%</td>
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<tr>
<td>Hypertension</td>
<td>68.1%</td>
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<tr>
<td>Diabetes</td>
<td>10.0%</td>
</tr>
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</table>

SD, standard deviation.
### Online Supplement Table 4.

**Human MMP8 gene SNPs examined**

<table>
<thead>
<tr>
<th>dbSNP ID</th>
<th>Major allele</th>
<th>Minor allele</th>
<th>Minor allele frequency in this study</th>
<th>Minor allele frequency in HapMap</th>
<th>SNP location</th>
<th>Comment</th>
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<tbody>
<tr>
<td>rs10895353</td>
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<td>tagging SNP</td>
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<td>rs11225395</td>
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<td>0.48</td>
<td>Promoter, position -799</td>
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<td>rs1320632</td>
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<td>G</td>
<td>0.09</td>
<td>0.11</td>
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<tr>
<td>rs2155052</td>
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<td>G</td>
<td>0.10</td>
<td>0.10</td>
<td>Promoter, position -17</td>
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<tr>
<td>rs17099450</td>
<td>G</td>
<td>C</td>
<td>0.05</td>
<td>0.04</td>
<td>Exon 1</td>
<td>non-synonymous (Cys3Ser)</td>
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<tr>
<td>rs3765620</td>
<td>A</td>
<td>G</td>
<td>0.43</td>
<td>0.48</td>
<td>Exon 1</td>
<td>non-synonymous (Ile32Thr)</td>
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<tr>
<td>rs12803000</td>
<td>T</td>
<td>G</td>
<td>0.05</td>
<td>0.04</td>
<td>Exon 2</td>
<td>synonymous</td>
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<tr>
<td>rs1940475</td>
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<td>T</td>
<td>0.49</td>
<td>0.48</td>
<td>Exon 2</td>
<td>tagging SNP, non-synonymous (Glu87Lys),</td>
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<tr>
<td>rs1892886</td>
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<td>A</td>
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<td>tagging SNP</td>
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<tr>
<td>rs17099436</td>
<td>T</td>
<td>A</td>
<td>0.05</td>
<td>0.06</td>
<td>Intron 4</td>
<td>tagging SNP</td>
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<tr>
<td>rs3740938</td>
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<td>A</td>
<td>0.06</td>
<td>0.06</td>
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<tr>
<td>rs2508383</td>
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<td>A</td>
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<td>tagging SNP</td>
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<td>rs1276282</td>
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<td>0.09</td>
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<td>3’-untranslated region</td>
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<td>0.38</td>
<td>3’-untranslated region</td>
<td>tagging SNP</td>
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</table>
Online Supplement Table 5.

Mass spectrometry results

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Matched peptides</th>
<th>Peptide m/z value</th>
<th>Peptide mass (Da)</th>
<th>Mascot Score</th>
<th>Relative % of total ion count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Measured</td>
<td>Predicted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncleaved Ang I</td>
<td>DRVYIHPFHL (Ang I)</td>
<td>432.84 3+</td>
<td>1295.64</td>
<td>49</td>
<td>100.0%</td>
</tr>
<tr>
<td>Products of Ang I cleavage by MMP8</td>
<td>DRVYIHPFHL (Ang I)</td>
<td>432.91 3+</td>
<td>1295.72</td>
<td>47</td>
<td>35.9%</td>
</tr>
<tr>
<td></td>
<td>DRVYIHPF (Ang II)</td>
<td>523.79 2+</td>
<td>1045.57</td>
<td>37</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td>DRVYIHP (Ang1-7)</td>
<td>450.25 2+</td>
<td>898.50</td>
<td>44</td>
<td>16.4%</td>
</tr>
</tbody>
</table>
Online Supplement Table 6.

Association between MMP8 gene variation and carotid atherosclerosis progression in the Bruneck study cohort (n=782)

<table>
<thead>
<tr>
<th>Carotid atherosclerosis progression*</th>
<th>Odds ratio (95% confidence interval) ‡</th>
<th>Odds ratio (95% confidence interval)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990-1995</td>
<td>0.77 (0.60-0.98), p=0.032</td>
<td>0.73 (0.57-0.95), p=0.018</td>
</tr>
<tr>
<td>1995-2000</td>
<td>0.74 (0.56-0.99), p=0.040</td>
<td>0.78 (0.58-1.04), p=0.090</td>
</tr>
</tbody>
</table>

Change in carotid atherosclerosis score†

| Change in carotid atherosclerosis score† | Regression coefficient (95% confidence interval)|| | Regression coefficient (95% confidence interval)# |
|-----------------------------------------|---------------------------------------------------|----------------------------------------|---------------------------------------------------|
| 1990-1995                               | -0.022 (-0.004 to -0.040), p=0.011               | -0.025 (-0.042 to -0.008), p=0.003   |
| 1995-2000                               | -0.022 (-0.002 to -0.042), p=0.025               | -0.022 (-0.042 to -0.002), p=0.032   |

*Carotid atherosclerosis progression was defined by the occurrence of atherosclerotic lesions in segments previously free of atherosclerosis or enlargement of non-stenotic lesions by a relative increase in the plaque diameter exceeding twice the measurement error of the carotid ultrasound examination method (for details see references9-12).

†Carotid atherosclerosis score, an indicator of atherosclerosis severity, was calculated by summing the maximum diameter of atherosclerotic plaques at 8 well-defined segments of the common and internal carotid arteries (for details see references9-12).

‡Odds ratio for carotid atherosclerosis progression related to each copy of the T allele, calculated by logistic regression analysis with adjustment for age, gender and baseline carotid atherosclerosis score.

§Odds ratio for carotid atherosclerosis progression related to each copy of the T allele, calculated by multivariate logistic regression analysis with adjustment for age, gender, baseline carotid atherosclerosis score, smoking, alcohol consumption, hypertension, diabetes, body mass index, waist-to-hip ratio, LDL and HDL levels, lipoprotein a, fibrinogen, the factor V Leiden mutation, C-reactive protein, ferritin and urinary albumin. Additional adjustment for soluble VCAM-1 yielded virtually identical results.

||Regression coefficient calculated by linear regression analysis of log_{e}-transformed carotid atherosclerosis score in relation to the T allele, with adjustment for age, gender and baseline carotid atherosclerosis score.

#Regression coefficient calculated by linear regression analysis of log_{e}-transformed carotid atherosclerosis score in relation to the T allele, with adjustment for age, gender and baseline carotid atherosclerosis score and other variables as in footnote* above.
Online Supplement Table 7.

Association between MMP8 genotype and plasma VCAM-1 level in the Bruneck study cohort (n=782)

<table>
<thead>
<tr>
<th>MMP8 Genotype*</th>
<th>VCAM-1 (ng/mL)†</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>661.2</td>
<td>0.014</td>
</tr>
<tr>
<td>C/T</td>
<td>624.5</td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>603.7</td>
<td></td>
</tr>
</tbody>
</table>

*Genotype of the rs1940475 SNP;
†Data shown are age- and sex-adjusted geometric means.
References


