Plasma Concentrations and Genetic Variation of Matrix Metalloproteinase 9 and Prognosis of Patients With Cardiovascular Disease

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Background—Matrix metalloproteinase (MMP)-9 secretion by macrophages and other inflammatory cells accelerates atherosclerotic progression and destabilizes vulnerable plaque in animal models. However, epidemiological data evaluating the prognostic impact of circulating concentrations and functional genetic variations of MMP-9 are lacking.

Methods and Results—In a prospective study of 1127 patients with documented coronary artery disease, we measured baseline plasma MMP-9 levels and determined the MMP-9/C-1562T and MMP-9/R279Q genotypes. During the follow-up period (mean of 4.1 years), 97 patients died from cardiovascular (CV) causes. Median concentrations of MMP-9 were significantly higher among patients who experienced a fatal CV event than among those who did not (62.2 versus 47.8 ng/mL; $P<0.0001$). The crude hazard risk ratio of CV death associated with increasing quartiles of MMP-9 was 1.4 (95% CI, 1.2 to 1.8; $P<0.0001$), and after adjustment for clinical and therapeutic confounders, it was 1.3 (95% CI, 1.1 to 1.6; $P=0.005$). Additional adjustment for highly sensitive CRP, interleukin-6, fibrinogen, and interleukin-18 revealed a hazard risk ratio to 1.2 (95% CI, 0.9 to 1.6; $P=0.15$). The T allele of the C-1562T polymorphism was associated with increased MMP-9 levels in a fairly codominant fashion ($P=0.004$). Although none of the polymorphisms was significantly related with future CV death, there was a significant association ($P=0.02$) between the R279Q polymorphism and CV events in patients with stable angina.

Conclusions—Plasma MMP-9 concentration was identified as a novel predictor of CV mortality in patients with coronary artery disease. Whether it provides independent prognostic information compared with other inflammatory markers will have to be additionally assessed. **(*Circulation*. 2003;107:1579-1585.)**

Key Words: metalloproteinases ■ inflammation ■ prognosis ■ coronary disease

Matrix metalloproteinases (MMPs) form a family of zinc-dependent enzymes with proteolytic activity against connective tissue proteins such as collagen, proteoglycans, and elastin.[1] Increased expression and activity of MMPs have been identified in various pathological processes, such as general inflammation, tumor metastasis, respiratory diseases, myocardial injury, vascular aneurysms, and remodeling.[2] Because of their major significance in vascular remodeling, MMPs are suspected to play an important role in the pathogenesis of cardiovascular (CV) diseases, such as atherosclerosis and restenosis.[3] MMPs have been identified in human atherosclerotic plaque shoulders and regions of foam cell accumulation and may thus contribute to plaque vulnerability[4,5] as well as de novo atherosclerotic remodeling.[6] Moreover, human monocyte-derived macrophages, which harbor MMPs, have been shown to induce collagen breakdown in fibrous caps,[7] and recently collagen breakdown and increased plaque vulnerability have been attributed to increased MMP-8 activity.[8]

MMP-9, also known as gelatinase B or 92-kDa type IV collagenase, is one of the MMPs found to be highly expressed in the vulnerable regions of atherosclerotic plaques, and for this reason it has been suggested to be causally involved in the remodeling processes associated with atherogenesis and plaque rupture.[3–5,9] The hypothesis of a causal role of MMP-9 in CV diseases is supported by genetic studies showing that functional promoter variations of the MMP-9 gene were related to presence and severity of CV diseases.[10–12] On the other hand, little is known about the clinical significance of circulating MMP-9 in CV diseases. Elevated levels of MMP-9 have been reported in patients with unstable angina.[13] However, prospective data on the impact of MMP-9 plasma levels on future CV prognosis are lacking.

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The aim of the present study was to investigate whether plasma MMP-9 concentrations and genetic variations of the MMP-9 gene might constitute risk markers for future CV death in a large cohort of patients with angiographically proven coronary artery disease (CAD). We focused the genetic study on two polymorphisms of special interest: the MMP-9/C-1562T promoter polymorphism, which influences the transcriptional activity, and the exonic MMP-9/R279Q polymorphism, which leads to an amino acid exchange in the catalytic domain of the MMP-9 enzyme.[14]

Methods

Study Population

Between November 1996 and June 2000, 1127 patients with stable (n=795) or unstable (n=332) angina who presented at the Department of Medicine II of the Johannes Gutenberg-University Mainz or the Bundeswehrzentralkrankenhaus Koblenz with at least 1 stenosis >30% diagnosed in a major coronary artery were enrolled in a CV registry entitled the Athero*Gene* Study. The study has been described in detail elsewhere.[15]

Among the 1127 patients, 1122 (99.6%) were followed for a median of 4.1 (maximum, 5.2) years. Follow-up information was obtained about death from CV causes (n=97), death from causes not related to heart disease (n=26), and nonfatal myocardial infarction (MI) (n=41). Information about the cause of death or clinical events was obtained from hospital or general practitioner charts.

Study participants were of German nationality. The study was approved by the ethics committee of the University of Mainz. Participation was voluntary, and each study subject gave written informed consent.

Laboratory Methods

Blood was drawn under standardized conditions before coronary angiography and stored at −80°C. Plasma MMP-9 concentrations were measured using a commercially available enzyme immunoassay (EIA) (Fuji Chemicals, Co, Tokyo, Japan). Coefficients of variations ranged from 3% to 12%, and repeat determinations on the same plasma sample were highly correlated (r=0.98). C-reactive protein was determined by a highly sensitive (hs), latex particle–enhanced immunoassay (detection range of 0 to 20 mg/L, Roche Diagnostics), and interleukin (IL)-6 (EASIA, Biosource Europe) and IL-18 (MBL Co, Ltd) were determined by commercially EIA. Fibrinogen was determined by derived method, and troponin I by an immunoassay (DADE Behring, Germany). Lipid serum levels were measured immediately. Genomic DNA was extracted from peripheral blood leukocytes. Primers and polymerase chain reaction conditions for genotyping are described elsewhere.[14]

Statistical Methods

Mean levels of variables were compared across quartiles of MMP-9 levels by ANOVA for continuous variables and χ^2 test for categorical variables. Variables with a skewed distribution, including MMP-9 level, were log-transformed. The association between MMP-9 genotype and plasma MMP-9 concentrations was investigated by ANOVA adjusted for relevant covariables. The cumulative survival plots by MMP-9 quartile were estimated by the Kaplan-Meier method and compared using the log-rank test. In all survival analyses, the end point was CV death, and data on patients who died from other causes were censored at the time of death. Hazard risk ratios (HRRs) for future CV death according to MMP-9 quartiles or MMP-9 genotype were estimated by Cox regression models adjusted for potential confounders. A secondary combined end point was also considered, including CV death and nonfatal MI. In analyses comparing different inflammatory markers, each marker was considered in quartiles and analyzed as an ordinal variable. $P<0.05$ was considered to be significant. All computations were carried out with SPSS, version 10.07.

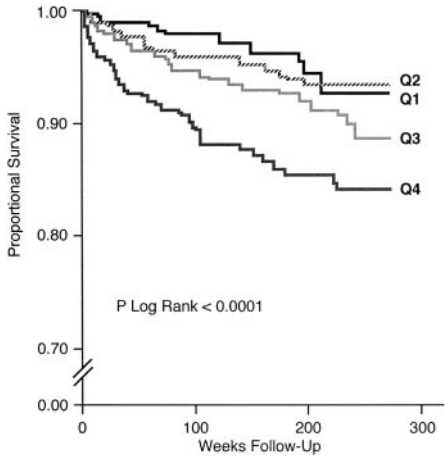

Figure 1. Kaplan-Meier curves for survival according to quartiles of MMP-9 (the numbers of CV deaths were 13, 17, 26, and 41 in Q1, Q2, Q3, and Q4, respectively).

Results

Baseline Characteristics of the Study Population According to MMP-9 Quartiles

Table 1 demonstrates patients' characteristics according to quartiles of MMP-9 levels. The median MMP-9 level was 49.2 ng/mL (interquartile interval of 33.4 to 71.6 ng/mL). History of smoking and previous MI were strongly associated with elevated levels of MMP-9, whereas statin intake was related to lower MMP-9 levels (median, 42.5 versus 52.4 ng/mL; $P<0.0001$). MMP-9 did not correlate with lipid concentrations except modestly with HDL cholesterol ($r=-0.15$). It also correlated with acute-phase reactants ($r=0.25$ for IL-6, $r=0.29$ for hs-CRP, and $r=0.26$ for fibrinogen). By contrast, only a weak correlation was observed between MMP-9 and IL-18 levels ($r=0.08$).

Plasma MMP-9 Concentrations and Future Cardiovascular Death

Median plasma concentrations of MMP-9 at baseline were significantly higher among patients who subsequently experienced a fatal CV event during follow-up compared with those who did not (62.2 versus 47.8 ng/mL; $P<0.0001$). Patients in the highest quartile of MMP-9 concentration had the highest probability of CV death during the follow-up period (Figure 1). After adjustment for most potential clinical and therapeutic variables, MMP-9 remained independently associated with future CV death. Additional adjustment on ejection fraction (EF) hardly modified the association, even though the significance of the test was lower because of missing EF values in 158 patients. Exclusion of the 331 patients with acute coronary syndrome did not alter the relationship between MMP-9 levels and CV mortality (Table 2).

We additionally evaluated the predictive value of MMP-9 levels in the context of other inflammatory predictors, including hs-CRP, fibrinogen, IL-6, and IL-18 (Figure 2). When considered separately, all inflammatory markers, except hs-CRP, were significantly associated with CV mortality. In a multivariate analysis including all 5 markers simultaneously, MMP-9 remained significantly associated with outcome

TABLE 1. Baseline Characteristics According to Quartiles of Plasma MMP-9 Levels (n=1127)

Variable	Quartile 1 (<33.4 ng/mL)	Quartile 2 (33.4 to 49.2 ng/mL)	Quartile 3 (>49.2 to 71.6 ng/mL)	Quartile 4 (>71.6 ng/mL)	*P*
Age, y	62.0±10.0	61.7±9.0	61.5±10.6	62.5±10.3	0.7
Sex, % male	79.9	75.4	74.9	76.4	0.5
BMI, kg/m^2	27.3 ±3.7	27.0±3.7	26.8±3.6	26.9±3.7	0.5
Ever smoker, %	60.9	51.1	67.0	68.2	0.004
History of					
Diabetes, %	17.6	17.1	14.8	17.5	0.8
Hypertension, %	73.9	73.9	73.1	65.7	0.03
Family history of CAD, %	38.4	37.1	45.9	40.4	0.2
Acute coronary syndrome, %	36.3	26.8	26.1	28.6	0.03
Multivessel disease (>2), %	47.2	42.1	42.8	47.9	0.5
History of MI, %	40.5	46.4	55.8	52.9	0.001
Revascularization, %*	62.7	61.9	58.7	63.8	0.6
LVEF, %	63.2±14.5	63.1±15.1	63.3±14.5	62.5±15.0	0.09
β-Blocker medication, %	55.6	60.0	57.6	53.9	0.6
Statin medication, %	38.4	41.1	30.4	23.2	<0.0001
ACE inhibitors, %	43.0	51.8	50.5	50.7	0.1
Antiplatelet therapy, %	87.3	91.8	89.4	86.1	0.2
Calcium antagonists, %	13.7	18.9	18.4	20.4	0.2
Troponin I, μg/L†	1.6±4.7	1.3±4.2	0.9±2.0	2.1±3.8	0.3

Data presented are percentage of patients or mean±SD.

*Invasive treatment denotes coronary artery bypass surgery or percutaneous transluminal coronary angioplasty during follow-up.

†Troponin I was determined in 332 patiens with unstable angina.

(P=0.04), together with fibrinogen (P=0.03) and IL-18 (P<0.0001). When additionally adjusting for all potential confounders and inflammatory markers, the HRR of MMP-9 was 1.22 (95% CI, 0.93 to 1.59; P=0.15), the only remaining significant predictor being IL-18 (HRR 1.79; 95% CI, 1.34 to 2.38; P<0.0001). However, because MMP-9, hs-CRP, fibrinogen, and IL-6 exhibited relatively strong correlations with one another, simultaneous inclusion of these 4 markers in the same model might mask their role by inflating the parameter variances. Therefore, we investigated the role of MMP-9 after controlling for IL-18 alone. Figure 3 shows the proportion of future CV deaths in patients classified according to the medians of MMP-9 and IL-18. Whatever the level of IL-18 (below or above the median), the proportion of CV events was approximately doubled in patients above the median of MMP-9 compared with those below, indicating a strong effect of MMP-9 independent of IL-18.

Patients who experienced a nonfatal MI during follow-up had elevated MMP-9 levels at baseline compared with patients free of event (median, 54.4 versus 47.5 ng/mL), but this difference did not reach significance, probably because of the low number of nonfatal MIs (n=41). When considering the combined end point including CV deaths and nonfatal MI, the unadjusted HRR associated with increasing MMP-9 quartiles was estimated as 1.30 (95% CI, 1.11 to 1.52; P<0.002).

Association Between MMP-9 Genotypes and Plasma MMP-9 Concentrations

Genotype distribution for the C-1562T and the R279Q polymorphisms were compatible with Hardy-Weinberg expectations, and allele frequencies were estimated as 0.13±0.01 for the −1562T allele and 0.35±0.01 for the 279Q allele. These frequencies were similar to those previously reported in a population from the United Kingdom.[14] Both polymorphisms were in strong linkage disequilibrium (D'=+0.9, P<0.0001), the −1562T and 279Q allele being preferentially associated. The two polymorphisms were associated with MMP-9 plasma levels in a fairly codominant fashion, the rare allele of each polymorphism being associated with increased MMP-9 levels (Table 3). Exclusion of patients with unstable angina even strengthened the association. Because of the strong linkage disequilibrium between the two polymorphisms, we tested whether each of them had an independent effect on phenotype. This analysis revealed that only the C-1562T polymorphism had an effect on MMP-9 levels, the association with the R279Q polymorphism being a consequence of its disequilibrium with the promoter polymorphism.

MMP-9 Genotype and Future Cardiovascular Event

Neither of the two MMP-9 polymorphisms was significantly associated with future CV mortality. However, when considering the combined end point including CV death and nonfatal MI, the association of the R279Q polymorphism with this combined end point reached significance (P=0.02) in the subgroup of patients with stable angina, patients carrying the 279Q allele having a higher risk than patients homozygous for the 279R allele (Table 4).

TABLE 2. HRR of Future CV Mortality According to Baseline MMP-9 Levels

	MMP-9 Quartile, range, ng/mL				
	1 (<33.4)	2 (33.4 to 49.2)	3 (>49.2 to 71.6)	4 (>71.6)	*P* for Trend
All patients (n=1122)					
No. of patients per quartile	284	278	281	279	...
No. of CV deaths	13	17	26	41	...
CV mortality, %	4.6	6.1	9.3	14.7	<0.0001
Age- and gender-adjusted					
HRR	1.0	1.05	1.52	2.66	<0.0001
95% CI	...	0.50 to 2.19	0.77 to 2.99	1.42 to 4.99	...
P	...	0.9	0.2	0.002	...
Model 1*					
HRR	1	1.24	1.45	2.41	0.005
95% CI	...	0.58 to 2.65	0.71 to 2.94	1.26 to 4.60	...
P	...	0.6	0.3	0.008	...
Model 2*					
HRR	1	1.32	1.75	2.23	0.03
95% CI	...	0.57 to 3.04	0.78 to 3.95	1.04 to 4.77	...
P	...	0.5	0.2	0.04	...
Stable angina (n=791)					
No. of patients per quartile	181	203	208	199	...
No. of CV deaths	8	12	16	33	...
CV mortality, %	4.4	5.9	7.7	16.6	<0.0001
Age- and gender-adjusted					
HRR	1.0	1.11	1.34	3.34	<0.0001
95% CI	...	0.44 to 2.78	0.57 to 3.17	1.54 to 7.25	...
P	...	0.8	0.5	0.002	...
Model 1*					
HRR	1.0	1.25	1.73	3.25	0.002
95% CI	...	0.49 to 3.22	0.67 to 4.45	1.47 to 7.21	...
P	...	0.6	0.3	0.004	...
Model 2*					
HRR	1.0	1.09	1.54	2.88	0.01
95% CI	...	0.38 to 3.08	0.57 to 4.18	1.19 to 6.95	...
P	...	0.9	0.4	0.02	...

*Model 1 further adjusted for history of hypertension, diabetes, ever smoking, HDL cholesterol, triglycerides (log transformed), extent of vessel disease, acute coronary syndrome, history of MI, interventional therapy, β-blocker, and statin therapies. Model 2 further adjusted for EF (n=964, because of 158 missing EF determinations).

Discussion

This study evaluated prospectively for the first time the predictive value of circulating levels and genetic variation of MMP-9 on future CV mortality in a large cohort of patients with CAD. Besides identifying the main correlates of MMP-9 levels like statin therapy and smoking, we demonstrated a strong association between baseline MMP-9 levels and future risk of CV death. This association was present in all subgroups evaluated across the entire spectrum of patients with CAD and persisted after adjustment for main clinical and therapeutic confounders.

A growing number of new inflammatory biomarkers of atherosclerosis has been identified in the past few years, including hs-CRP,[16,17] soluble adhesion molecules,[18] IL-6,[19] tumor necrosis factor-α,[20] and IL-18.[21] The present study suggests that MMP-9 might constitute a novel prognostic biomarker for characterizing individuals at higher CV risk. We also showed that MMP-9 correlated with acute-phase reactants, and for this reason it was difficult to disentangle the role of each marker when including all of the markers in the same model. However, this does not preclude that MMP-9 could have its own pathophysiological significance, as strongly suggested by experimental studies.[3–5,9] In the context of all these new inflammatory markers emerging as potential clinical tools, some of them being highly correlated and reflecting

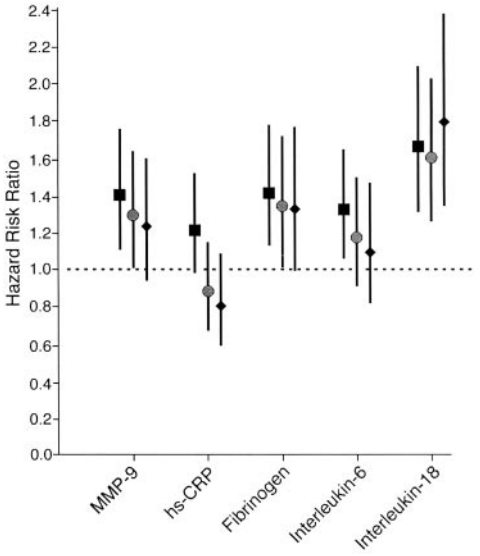

Figure 2. HRRs for CV death associated with different inflammatory markers. Each marker was considered in quartiles and analyzed as an ordinal variable, so that the HRR represents the increase of risk between 2 consecutive quartiles. Squares (model 1) indicate unadjusted HRR; circles (model 2) indicate HRRs for each marker in a multivariate analysis including all 5 inflammatory markers simultaneously; diamonds (model 3) indicate HRRs additionally adjusted for age, sex, body mass index, prevalence of diabetes, hypertension, smoking status, HDL cholesterol and triglyceride levels, unstable angina, extent of vessel disease, history of myocardial infarction, statin and β-blocker intake, invasive therapy, and ejection fraction. The models were fitted on a sub-sample of 894 subjects having all 5 inflammatory markers measured, except model 3, which was fitted in 771 subjects for whom ejection fraction was available.

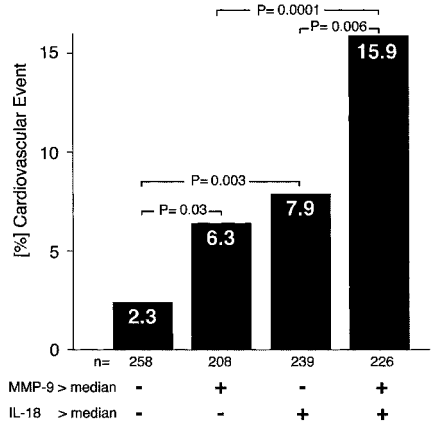

Figure 3. Percentage of CV deaths according to the median of baseline MMP-9 and IL-18 levels.

common biological pathways, it would be of greatest interest to evaluate which are likely to provide the most useful prognostic information. Additional large prospective studies established in various clinical settings are required to perform such a comparative analysis.

In contrast with acute-phase reactants, MMP-9 levels only weakly correlated with IL-18 levels, and the predictive value of MMP-9 was shown to be independent of that of IL-18, one of the strongest inflammatory predictors of CV risk identified so far. However, combined determination of MMP-9 and IL-18 identifies patients being at very high risk. This is in accordance with experimental work that demonstrates that IL-18, in synergy with IL-12, induces expression of MMP-1, MMP-13, and mainly MMP-9 on endothelial and smooth muscle cells as well as macrophages.[22] Evaluating both parts of the pathway seems superior to the determination of one single biomarker alone.

The predominant sources of MMP-9 detected in the circulation are unknown. MMP-9 is expressed in atherosclerotic plaques at multiple sites within the vascular tree, and circulating concentrations may reflect vessel wall expression. Because the inflammatory process is not confined to a single vulnerable plaque but rather appears more widespread in the coronary vessel tree,[23] circulating levels of MMP-9 are unlikely to derive from one special vulnerable plaque area. Second, MMP-9 might be released by circulating neutrophils and monocytes as a consequence of a general proinflammatory state. Third, the role of MMPs in myocardial matrix remodeling suggests that MMP-9 might in part derive from the myocardium itself.[2]

An elegant way to examine whether an association might be causal is to evaluate the impact of functional variations in

TABLE 3. Plasma Levels of MMP-9 According to the MMP-9 Polymorphisms in Patients With CAD

	All Patients			Stable Patients		
Polymorphism/Genotype	n	Geometric Mean (95% CI)	*P**	n	Geometric Mean (95% CI)	*P**
R279Q						
RR	445	45.8 (43.3 to 48.4)		318	46.2 (43.3 to 49.2)	
RQ	493	50.6 (48.0 to 53.4)	0.03	356	52.4 (49.3 to 55.7)	0.005
QQ	129	50.4 (45.4 to 55.9)		86	54.1 (47.8 to 61.2)	
C-1562T						
CC	776	46.8 (44.8 to 48.8)		546	47.7 (45.4 to 50.1	
CT	221	53.4 (49.3 to 57.9)	0.004	164	55.7 (50.9 to 60.9)	0.001
TT	22	55.3 (42.9 to 71.3)		14	65.6 (48.3 to 89.0)	

*Test for trend performed on log-transformed MMP-9 and adjusted for age, sex, history of smoking, and statin therapy.

TABLE 4. Future Cardiovascular Event According to MMP-9/R279Q Genotype

	All Patients				Stable Patients			
Genotype	CV Deaths (n=70)	*P*	CV Events* (n=110)	*P*	CV Deaths (n=56)	*P*	CV Events* (n=84)	*P*
RR, n=442	5.4		7.9		5.1		7.3	
RQ, n=491	7.1	0.37	12.2	0.09	8.7	0.10	14.1	0.02
QQ, n=129	8.5		11.6		10.5		12.8	

*CV events include CV death and nonfatal myocardial infarction.

the gene encoding the candidate protein. Several polymorphisms have been detected in the MMP-9 gene,[14] among which the C-1562T polymorphism was shown to influence gene expression.[10] In accordance with the increased activity of the −1562T allele, we found that this allele was associated with elevated MMP-9 plasma concentrations. By contrast, the R279Q polymorphism had no direct effect on plasma levels but was associated with future CV event in patients with stable angina. The R279Q polymorphism is located in the catalytic domain of the MMP-9 enzyme encoding the sequence required for binding of the enzyme to its substrate elastin.[24] An amino acid exchange in this region of the gene might affect the binding capacities of the protein to its substrate and have functional consequences on the process of vascular remodeling and plaque destabilization. MMP-9 might therefore act both as a circulating biomarker reflecting a proinflammatory state associated with a poorer survival and as a causative agent having a local effect on plaque destabilization.

Some limitations of our study merit consideration. First, measurements of MMP-9 were performed only at one time, hence changes during follow-up were not measured. Second, measurement of MMP-9 concentrations was performed on samples that were stored at −80°C. We therefore cannot exclude the possibility of protein degradation. However, because all samples were handled identically, this should not affect the difference between cases and controls.

Conclusions

In conclusion, plasma MMP-9 concentration was identified as a novel risk marker of future CV mortality in a large cohort of patients with CAD independently of main clinical and therapeutic confounders. Whether MMP-9 might provide additional information over other newly identified inflammatory markers will have to be assessed in additional studies. Furthermore, the MMP-9/R279Q polymorphism was related to future CV event in patients with stable angina, suggesting that MMP-9 may be causally involved in the atherogenic process.

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