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Functional polymorphisms in the promoter of the matrix metalloproteinase-9 (*MMP-9*) gene are not linked with significant plasma MMP-9 variations in healthy subjects

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Abstract

Background: Matrix metalloproteinase-9 (MMP-9) is involved in the degradation of the extracellular matrix during physiological and pathological processes. Two functional polymorphisms [$C^{-1562}T$ and microsatellite $(CA)_{13-25}$] in the promoter region of the *MMP-9* gene have been associated with several diseases. The aim of this study was to examine whether these *MMP-9* polymorphisms and haplotypes are linked with plasma MMP-9 variations in healthy subjects.
Methods: We studied 177 healthy male white volunteers (age range 20–55 years) who were non-smokers and not taking any medication. Genomic DNA was extracted from whole blood and genotypes for the $C^{-1562}T$ and the microsatellite $(CA)_n$ polymorphisms were determined. MMP-9 levels were measured in plasma samples by gelatin zymography.
Results: The frequency of the alleles C and T for the $C^{-1562}T$ polymorphism were 90% and 10%, respectively. The frequency of the alleles with less than 21 CA repeats (L) and with 21 repeats or higher (H) were 47% and 53%, respectively. We found no differences in plasma MMP-9 levels among the genotype groups or among different haplotypes (all $p>0.05$).
Conclusions: These findings suggest that functional polymorphisms in the promoter of the *MMP-9* gene are not linked with significant plasma MMP-9 variations in healthy subjects.
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Introduction

Matrix metalloproteinase-9 (MMP-9; gelatinase B; EC 3.4.24.35) is a zinc-dependent enzyme involved in the degradation of many components of the extracellular matrix during both physiological and pathological processes (1). Its activity is regulated by different mechanisms, including modulation of transcription, activation of latent MMP (pro-enzyme) and inhibition by tissue inhibitors of metalloproteinase. In addition, the MMP-9 transcriptional activity can be modulated by genetic polymorphisms in the promoter region of the *MMP-9* gene. For example, in vitro studies have shown that the C to T substitution at –1562 position ($C^{-1562}T$; rs 3918242) results in the loss of binding of a nuclear repressor protein, thus leading to increased MMP-9 expression (2). This polymorphism has been associated with increased susceptibility to cancer (3, 4), arterial aneurysms (5, 6), coronary atherosclerosis (2) and other cardiovascular diseases (7, 8).

Another important functional polymorphism in the promoter region of the *MMP-9* gene is the microsatellite $(CA)_n$ near the –90 position (rs 3222264), which corresponds to a sequence of variable numbers of cytosine-adenine repeats (13–27) and has a bimodal distribution of allele frequencies. The first and the second peaks occur, respectively, at $(CA)_{14}$ and $(CA)_{21-23}$ in many populations, although the most common alleles were $(CA)_{\geq 21}$ in the Japanese population (1). In vitro studies have shown a 50% reduction in the MMP-9 promoter activity with $(CA)_{14}$ compared with $(CA)_{21}$ (9). These findings may explain the association between a number of CA repeats ≥ 22 and carotid atherosclerosis (10). Moreover, patients with carotid atherosclerosis and carrying more than 20 repeats in one allele showed faster intima-media thickening growth and stenosis progressions of plaques (11). In addition, a case control study showed an association between the $(CA)_{23}$ and the occurrence of intracranial aneurysm (7).

While these studies indicate an important role for these two functional *MMP-9* gene polymorphisms [$C^{-1562}T$ and $(CA)_n$] in the susceptibility to different diseases (1), it is reasonable to hypothesize that these polymorphisms would affect pro-MMP-9 levels in plasma. Although we have previously shown that the $C^{-1562}T$ polymorphism alone does not affect pro-MMP-9 levels in plasma from healthy volunteers (12), it has been widely acknowledged that haplotype

(combination of genetic markers within a specific chromosome cluster location) analysis can provide much more useful information than the information derived from single polymorphisms analysis (13, 14).

In the present study, we examined whether there is an association between *MMP-9* haplotypes involving the $(CA)_n$ and the $C^{-1562}T$ polymorphisms and the plasma pro-MMP-9 levels in 177 white healthy males, who were non-smokers and not taking any medication. Examining how *MMP-9* gene haplotypes affect pro-MMP-9 levels could help to link functional *MMP-9* gene polymorphisms with corresponding biochemical variations. Finally, we also examined whether the circulating levels of pro-MMP-9 correlate with the plasma levels of other soluble markers of atherosclerosis and inflammation in healthy subjects.

Materials and methods

Subjects

The present study was carried out in accordance with the Declaration of Helsinki ethical guidelines. Approval for use of human subjects was obtained from the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil. Healthy male white volunteers (n = 177, age range 20–55 years), who were non-smokers and not taking any medication, were recruited from the local population to give blood after informed consent had been obtained. Arterial blood pressure and heart rate were measured three times after at least 15 min of rest. Venous blood samples were collected into tubes containing EDTA after overnight (>12 h) fasting and plasma was separated to measure plasma total cholesterol, triglycerides and MMP activity. Plasma samples were stored at −70°C until assayed for MMP activity. Genomic DNA was extracted from the cellular component of 1 mL of whole blood by a salting-out method and stored at −20°C until analyzed.

Genotyping

Genotypes for the $C^{-1562}T$ polymorphism were determined by polymerase chain reaction (PCR) amplification using the primers: 5′-GCC TGG CAC ATA GTA GGC CC-3′ (sense) and 5′-CTT CCT AGC CAG CCG GCA TC-3′ (antisense), and PCR conditions as previously described (2, 12). The amplified products were digested with *Sph*I (New England Biolabs, Ipswich, MA, USA) overnight at 37°C, producing fragments of 247 bp and 188 bp in the case of a polymorphic variant (allele T) or an undigested 435 bp band in the case of a wild type allele (allele C). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized by silver staining (Figure 1A). Genotyping results by PCR/restriction were controlled by sequencing some of the DNA samples for the $C^{-1562}T$ polymorphism.

To determine the genotypes for the $(CA)_n$ polymorphism, PCR was carried out using the primers: 5′-GAC TTG GCA GTG GAG ACT GCG GGC A-3′ (sense) and 5′-GAC CCC ACC CCT CCT TGA CAG GCA A-3′ (antisense), as previously described (15). PCR was performed in a 25 μL mixture with

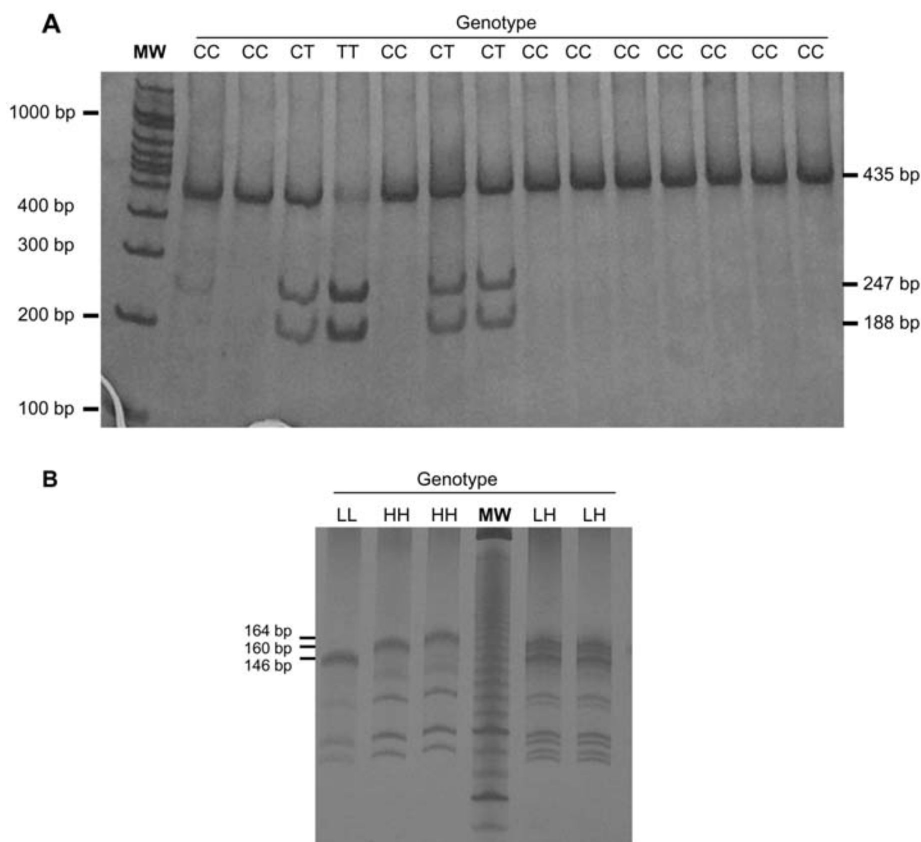

Figure 1 Polymorphism genotyping.
(A) Genotyping for the $C^{-1562}T$ polymorphism in the promoter region of the *MMP-9* gene. The PCR products were digested with restriction enzyme producing different fragments leading to specific genotypes. (B) Genotyping for the $(CA)_n$ polymorphism. The first three lanes are from sequenced samples used as standard: 14/14, 21/21 and 23/23, respectively.

100 ng DNA, 20 pmol of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1.0 U Taq polymerase and 2.5 μL of 10× PCR buffer. After an initial denaturation at 95°C for 3 min, the samples were subjected to 30 cycles of amplification, consisting of denaturation for 1 min at 95°C, annealing for 1 min at 69°C and extension for 1 min at 72°C, followed by a final extension for 10 min. The amplified products were separated in a 7% polyacrylamide-urea gel and visualized by silver staining (Figure 1B). Differences in molecular weight (or number of bases), from 144 bp (CA 13 repeats) to 168 bp (CA 25 repeats), were determined by comparison with migration of a 10-bp DNA ladder (Invitrogen, Carlsbad, CA, USA) and with some samples from homozygotes that were sequenced (Figure 1B). The DNA sequencing of the homozygotes samples was carried out by the method of Sanger et al. according to the protocol of a cycle sequencing ready reaction kit (Big Dye Terminator, Applied Biosystems, Foster City, CA, USA), with some modifications (16). The samples were classified as lower (L) for less than 21 CA repeats or higher (H) for samples with 21 or more CA repeats.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gelatin zymography of MMP-9

Gelatin zymography of MMP-9 from plasma was performed as previously described (17–21). Briefly, plasma samples were subjected to electrophoresis on 7% SDS-PAGE co-polymerized with gelatin (1%) as the substrate. After electrophoresis was complete, the gel was incubated for 1 h at room temperature in a 2% Triton X-100 solution and incubated at 37°C for 16 h in Tris-HCl buffer, pH 7.4, containing 10 mM $CaCl_2$. The gels were stained with 0.05% Coomassie Brilliant Blue G-250 and then destained with 30% methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. Enzyme activity was assayed by densitometry using a Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Kodak, Rochester, NY, USA). The proforms of MMP-2 and MMP-9 were identified as bands at 72 kDa and 92 kDa, respectively, by the relation of log M_r to the relative mobility of Sigma SDS-PAGE LMW marker proteins (Sigma, St. Louis, MO, USA).

Enzyme immunoassays of soluble vascular adhesion molecule-1, soluble intercellular adhesion molecule-1, soluble P-selectin and monocyte chemoattractant protein-1

To examine whether the circulating levels of pro-MMP-9 correlate with the plasma levels of other soluble markers of atherosclerosis and inflammation, we measured the plasma concentrations of soluble vascular adhesion molecule (sVCAM)-1, soluble intercellular adhesion molecule (sICAM)-1, soluble P (sP)-selectin and monocyte chemoattractant protein (MCP)-1 in 60 healthy subjects. The plasma concentrations of sVCAM-1, sICAM-1, sP-selectin and MCP-1 were measured with commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Inc., Minneapolis, MN, USA) according to manufacturer's instructions (22).

Haplotype inference

Haplotypes were inferred using the Bayesian statistically based program PHASE version 2.1 (http://www.stat.washington.edu/stephens/software.html) (23) to estimate the haplotype frequencies in the population and the two haplotypes for each subject (Table 1). These results were used to evaluate a possible relationship between haplotypes and plasma pro-MMP-9 levels. The possible haplotypes including genetic variants for two *MMP-9* polymorphisms studied (H or L variants for CA repeats and C or T variants for the $C^{-1562}T$) were: H1 (HC), H2 (HT), H3 (LC) and H4 (LT).

Table 1 Estimated haplotype frequency in the studied population.

	Haplotypes		Frequency, %
	$(CA)_n$	$C^{-1562}T$	
H1	H	C	44.5
H2	H	T	8.1
H3	L	C	46.0
H4	L	T	1.4

Statistical analysis

The results are expressed as means ± SD. The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy-Weinberg equilibrium by using the χ^2 test (StatView, Cary, NC, USA). To assess potential relationships between each polymorphism or haplotypes and plasma pro-MMP-9, we used the Kruskal-Wallis test followed by the Dunn multiple comparison test. Because of the relatively low frequency of the TT genotype, we combined both TT and CT genotypes together (CT+TT group) and compared these with the CC genotype group. The genotype groups were evaluated for differences in demographic data and plasma MMPs levels by the Mann-Whitney test.

The Spearman correlation (r) was calculated for associations between the circulating levels of pro-MMP-9 and the plasma levels of other soluble markers of atherosclerosis and inflammation. A p-value <0.05 was considered to be statistically significant.

Results

The frequency of the alleles C and T for the $C^{-1562}T$ polymorphism were 90% and 10%, respectively. The frequency of the grouped alleles L and H for the microsatellite were 47% and 53%, respectively. The distribution of genotypes for the two polymorphisms studied here showed no deviation from Hardy-Weinberg equilibrium (all $p>0.05$). There were no significant differences in age, body mass index, systolic and diastolic arterial blood pressure, heart rate, cholesterol and triglycerides among genotype groups (Table 2, all $p>0.05$).

We found no significant differences in plasma pro-MMP-9 activity among the genotype groups for both *MMP-9* polymorphisms (Table 2, all $p>0.05$).

The estimated haplotype frequencies for the whole group of subjects are shown in Table 1. The gelatinolytic activity was completely inhibited by 5 mM EDTA or 1 mM 1,10-phenantroline (data not shown), thus confirming the bands in the gel as MMP activity. Curiously, we found no significant differences in plasma pro-MMP-9 activity among haplotype groups (all $p>0.05$, Figure 2)

Finally, we found no significant correlation between the circulating levels of pro-MMP-9 and the plasma

Table 2 Characteristics of subjects grouped by genotype.

Variable	Total (n=177)	$(CA)_n$ genotypes				$C^{-1562}T$ genotypes			
		LL (n=43)	LH (n=82)	HH (n=52)	p-Value[a]	CC (n=145)	CT (n=30)	TT (n=2)	p-Value[b]
Age, years	31.0±10.6	32.9±9.9	30.8±9.7	29.6±12.7	NS	30.8±10.5	31.8±10.6	32.0	NS
BMI, kg/m^2	25.7±4.1	25.5±3.5	26.2±4.4	25.2±4.1	NS	25.8±4.1	25.6±3.7	25.8	NS
SAP, mm Hg	119.3±22.4	122.2±10.9	121.6±13.1	123.1±14.2	NS	123.5±11.6	124.7±13.8	118.0	NS
DAP, mm Hg	70.7±15.4	70.5±11.9	71.7±10.7	76.1±10.3	NS	73.3±10.6	74.7±10.5	72.0	NS
HR, bmp	56.4±28.7	69.8±12.0	66.4±10.4	70.7±13.7	NS	67.9±10.5	66.6±14.4	60.0	NS
Total cholesterol, mmol/L	172.7±34.9	174.7±27.4	168.3±52.1	164.3±34.0	NS	173.4±34.2	168.7±36.5	189.2	NS
Triglycerides, mmol/L	115.4±66.6	118.6±62.3	112.7±76.5	116.0±59.6	NS	108.9±68.9	118.8±53.5	149.55	NS
Pro-MMP-2, arbitrary unit	2.72±0.73	2.58±0.51	2.74±0.82	2.80±0.73	NS	2.71±0.74	2.81±0.63	2.53	NS
Pro-MMP-9, arbitrary unit	2.07±0.93	2.00±1.01	2.24±0.95	1.85±0.79	NS	2.06±0.94	2.06±0.89	1.60	NS

Values are the mean±SD or the mean (for the TT genotype group). BMI, body mass index; SAP, systolic arterial blood pressure; DAP, diastolic arterial blood pressure; HR, heart rate; NS, non-significant. [a]Kruskal-Wallis test followed by the Dunn multiple comparison test comparing the three genotype groups. [b]Mann-Whitney test for CC subjects vs. CT/TT subjects.

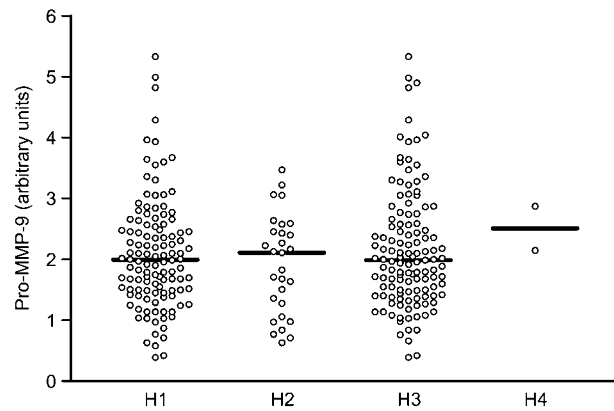

Figure 2 Plasma pro-MMP-9 activities in the four haplotype groups.
The bar shows the median value.

levels of sVCAM-1, sICAM-1, sP-selectin or MCP-1 (Figure 3, all $p>0.05$).

Discussion

The main novel findings reported here are that two functional *MMP-9* polymorphisms [the $(CA)_n$ and the $C^{-1562}T$] in the promoter region of the *MMP-9* gene probably do not have major influence on the circulating levels of MMP-9 in healthy subjects, either when considering their effects one by one or when considering their combined effects within MMP-9 haplotypes. These findings suggest that these functional polymorphisms may depend on the interaction with other factors (e.g., environmental) to modulate susceptibility to disease.

In the present study, we used gelatin zymography to examine whether functional *MMP-9* polymorphisms affect plasma MMP-9 activity because altered expression or activity of MMP-9 has been reported to play a role in a variety of pathological conditions, including neoplastic, cardiovascular and respiratory diseases (24–29), and the circulating level of MMP-9 has been suggested to be a clinically relevant blood-borne biochemical marker of diagnostic and prognostic value in many disease conditions (24, 25, 30–33). Curiously, MMP-9 plasma levels were shown to predict mortality in patients with coronary artery disease (24). These findings are consistent with the notion that altered plasma MMP-9 levels may reflect an increased susceptibility to disease, which could be based, at least in part, on a specific genetic background involving functional *MMP-9* polymorphisms. However, our results indicate no significant effects for the two functional *MMP-9* polymorphisms on the plasma levels of MMP-9 in healthy volunteers. Therefore, it is probable that these important polymorphisms affect MMP-9 expression and activity only when other disease risk factors are present.

Consistent with our findings, the basal expression of MMP-9 (which is low in most cell types) can increase by more than 100-fold under disease conditions (1). Therefore, one possible explanation for our

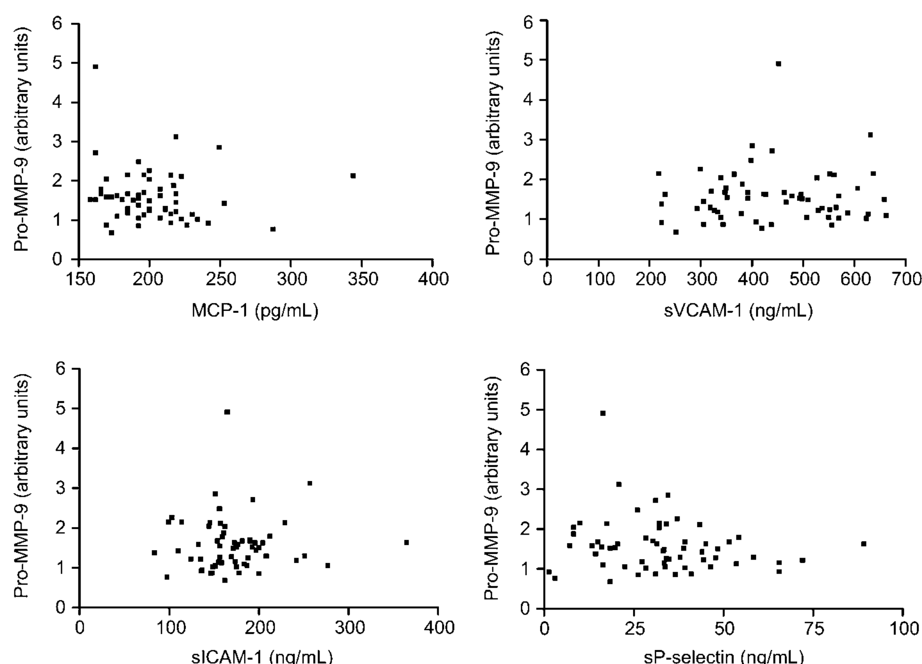

Figure 3 Relationship between circulating levels of pro-MMP-9 and soluble markers of atherosclerosis and inflammation. Lack of significant correlation between the circulating levels of pro-MMP-9 and the plasma levels of MCP-1, sVCAM-1, sICAM-1 or sP-selectin ($n=60$, all $p>0.05$).

findings is that the functionality of the two *MMP-9* polymorphisms studied here depend on the interaction with other factors causing increased MMP-9 expression (5, 12).

The analysis of haplotypes has been valued as a more powerful approach than the analysis of single polymorphisms (13, 14), because the study of haplotypes could eliminate inconsistencies commonly found in studies analyzing single polymorphisms one at a time (34, 35). For example, we have previously reported that three clinically relevant polymorphisms in the endothelial nitric oxide gene (eNOS) do not affect the circulating levels of nitric oxide products when analyzed one by one (36). However, *eNOS* gene haplotypes were significantly associated with different circulating levels of nitric oxide products in both healthy volunteers (36) and hypertensive patients (37). While MMP-9 haplotypes, including two functional *MMP-9* polymorphisms, did not affect the plasma levels of MMP-9 in the present study, it is possible that MMP-9 haplotypes may modify the circulating MMP-9 levels in patients, and thus affect disease susceptibility, as suggested by previous studies focusing on single *MMP-9* polymorphisms (2–8, 10, 11, 24, 38).

We found no significant correlation between the circulating levels of pro-MMP-9 and the plasma levels of cellular adhesion molecules involved in the early steps of leukocyte recruitment to the vessel wall (sVCAM-1, sICAM-1, sP-selectin) or with the plasma levels of MCP-1, which is a chemokine regulating leukocyte recruitment into sub-endothelial space (39). While the biological relevance of these soluble forms is not completely known, their levels correlate with cardiovascular disease activity (39). In this regard, the utility of these soluble markers of atherosclerosis and inflammation is apparently similar to that of MMP-9, which is now being validated as a clinically relevant marker of diagnostic and prognostic value in many disease conditions (24, 25, 30–33). However, our findings suggest that these markers are independent from each other, at least in healthy subjects.

Some limitations of our study should be taken into consideration. The relatively small number of volunteers ($n=177$) included in the present study may have limited the power to detect possible effects associated with rare alleles or haplotypes. However, our results provide evidence of lack of major effects associated with the genetic variations studied here. It is probably not worthy to carry out a larger study to test this hypothesis. Moreover, it is possible that other polymorphisms may affect pro-MMP-9 levels, although the two polymorphisms studied here are functional (2, 9). Finally, we have not examined whether there are gender-specific effects of the polymorphisms in the MMP-9 promoter region.

In conclusion, our study shows no effects of the $C^{-1562}T$ and $(CA)_n$ *MMP-9* polymorphisms or haplotypes on the plasma levels of MMP-9 in healthy subjects. These findings suggest that functional polymorphisms in the promoter of the *MMP-9* gene are not linked with significant plasma MMP-9 variations in healthy subjects. Further studies examining how these functional polymorphisms and *MMP-9* haplotypes interact with disease risk factors are warranted.

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