

MMP12 -82 A>G Promoter Polymorphism in Bronchial Asthma in a Population of Central Bulgaria

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ABSTRACT

A characteristic feature of inflamed lungs in bronchial asthma (BA) is airway remodeling. Due to limited information on this topic in the literature, we aimed to explore the possible role of polymorphisms in the promoter region of the macrophage elastase gene *MMP12* 82A>G (rs2276109) as a predisposing factor for BA in an ethnic Bulgarian population. Using restriction fragment length polymorphism analysis of polymerase chain reaction–amplified fragments (PCR-RFLP), we performed genotype analysis of 58 patients and 119 control individuals. We found statistically significant differences in the distribution of genotypes ($P = .008$) and

alleles ($P = .004$) between patients and nonaffected controls. In the dominant model, carriers of the G allele genotypes had 3.6-fold lower risk for BA, compared with those with the AA genotype, after adjustment for age and sex (odds ratio [OR], -0.277 ; 95% confidence interval [CI], $.12-.65$; $P = .003$). The results of our study suggest that the variant G allele of the *MMP12* -82 A>G promoter polymorphism might be considered protective for development of BA in ethnic Bulgarian adults residing in central Bulgaria.

Keywords: bronchial asthma, MMP-12, polymorphism, risk, genotyping, PCR-RFLP

Abbreviations

BA, bronchial asthma; COPD, chronic obstructive pulmonary disease; ECM, extracellular matrix; MMPs, matrix metalloproteinases; MMP-12, macrophage elastase; SNP, single nucleotide polymorphism; AP-1, activator protein-1; PCR-RFLP, restriction fragment length polymorphism analysis of polymerase chain reaction–amplified fragments; PCR, polymerase chain reaction; dNTP, deoxynucleotide; ANOVA, analysis of variance; LSD, least significant difference; HEW, Hardy-Weinberg equilibrium; TIMPs, tissue inhibitors of metalloproteinases; MALU, murine lung macrophage; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; IL, interleukin; NE, neutrophil elastase; ..., nonapplicable; FEV1, forced expiratory volume: first second of forced breath; FVC, forced vital capacity; OR, odds ratio; CI, confidence interval

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Bronchial asthma (BA) is a common chronic disorder of the airways that is characterized by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammatory process.¹ The main pathological feature of BA is inflammation, in which many cells and cellular elements play a role.² Histological assessments of airways in patients with asthma, particularly patients with more severe disease, reveal injury to the epithelium and, often, loss of those cells. Because the airway epithelium is a rich source of inflammatory mediators and growth factors, injury to the epithelium may contribute to inflammation of the airways.³ Airway smooth-muscle cells are also recognized as immunomodulators in asthma.⁴

The main immune cells infiltrating the lungs in BA are eosinophils; however, neutrophils and macrophages are also more numerous in the airways, especially in older patients with moderate to severe asthma and in asthma–chronic obstructive pulmonary disease (COPD) overlap syndrome.⁵⁻⁷

The result of pathological changes in inflamed lungs in asthma is airway remodeling, the main features of which include hypertrophy of smooth-muscle cells in airways, significant hyperplasia of smooth-muscle cells, submucosal glands and goblet cells, proliferation of endothelial cells, and structural changes in the extracellular matrix (ECM; degradation and subsequent repair cycles and deposition of ECM proteins).^{5,8-10}

Matrix metalloproteinases (MMPs) are a family of proteinases that participate in degradation of ECM proteins. They are not only responsible for ECM degradation but also for the shedding of cell membrane proteins. In addition, they are able to process and cleave diverse bioactive mediators, such as growth factors, cytokines, and chemokines.^{11,12} MMPs are associated with a variety of normal and pathological conditions that involve matrix degradation and remodeling in different tissues (the endometrium during menstruation and the involuting breast, uterus, and prostate).¹³

MMP-12 (macrophage elastase) is a 54 kDa secreted proenzyme, known mainly by the elastolytic activity of the active form.¹⁴ Some evidence shows the role of MMP-12 in acute allergen-responsive proinflammatory and chronic airway remodeling in the lungs.¹⁵ Also, it has been shown that many cell types that participate in the pathogenesis of BA secrete different types of MMPs, including MMP-12,¹⁵⁻¹⁷ which is produced mainly by macrophages.¹⁸

MMP-12 has been reported to be associated with cigarette smoke-induced emphysema and macrophage migration. An increased expression of MMP-12 was reported in a mouse model of allergic airway inflammation. Indeed, *MMP12* has been proposed as one of the asthma candidate genes.¹⁶

The gene of MMP-12 is polymorphic. A single nucleotide polymorphism (SNP) in the promoter region for MMP-12 (-82A>G, rs2276109) was reported to influence the binding of the transcription factor activator protein-1 (AP-1). The A allele has been associated with higher promoter activity in cell culture models.^{17,19} Only a limited number of studies concern the effect of the aforementioned SNP in *MMP12* in BA. In one such study, better lung function was reported in children with asthma who have minor G allele genotypes, as well as in adults who smoke and also possess these genotypes.²⁰ However, no study in the literature, to our knowledge, has been designed for evaluation of *MMP12* -82A>G SNP as a predisposing factor in asthma. For this reason, we explored the possible role of *MMP12* -82A>G polymorphism in the development of BA in adults in an ethnic Bulgarian population from central Bulgaria.

Materials and Methods

Patients and Control Individuals

We performed a prospective case-control study including 58 ethnic Bulgarian patients with BA residing in the region of Stara Zagora, Bulgaria, and 119 nonaffected controls from the same region. The patients were recruited at the Clinic of Internal Medicine, University Hospital, Trakia University, Stara Zagora, Bulgaria. The control group consisted of 119 healthy volunteers (individuals not affected by lung diseases or cancer) from the same region of Bulgaria and the same ethnicity, Bulgarian. In both groups, age of inclusion in the study and smoking status were noted; in the patient group, age at diagnosis and duration of diseases were reported. Demographic and clinical data are presented in [Table 1](#). The study protocol was approved by the ethics committee at Medical Faculty, Trakia University, Stara Zagora, Bulgaria; written informed consent was obtained from all study participants before the study began.

Genomic DNA was isolated from 0.2 mL of whole blood using a commercial kit for isolation of genomic DNA from blood (GenElute Mammalian Genomic DNA Miniprep Kit, Merck KGaA). The quality and quantity of the isolated total DNA were evaluated spectrophotometrically (NanoDrop Spectrophotometer ND-1000, Thermo Fisher Scientific, Inc), with the ratio $\lambda 260 \text{ nm} / \lambda 260 \text{ nm}$ optical density as measure of genomic DNA purity. The DNA concentration in ng/ μL was calculated as the absorbance at $\lambda 260 \text{ nm} \times 50$.

Genotyping

Genotyping for the *MMP12* -82 A>G polymorphism (rs2276109) was performed by restriction fragment length polymorphism analysis of polymerase chain reaction-amplified fragments (PCR-RFLP)-based methods, as described by Joos et al.²¹ The reactions were performed in a total volume of 20 μL of reaction mix. The polymerase chain reaction (PCR) mix contained 2 μL 10 $\times$ PCR buffer, 2 μL 25 mM MgCl_2 , 2 μL 2 mmol/L deoxynucleotide (dNTP) mix (Fermentas), 1 U Taq DNA Polymerase (Thermo Fisher Scientific, Inc cat. no. E2500-04), 7.6 pmol of each primer, and 100 ng DNA. The sequences of the primers were as follows: *MMP12*F: 5'-GAG ATA GTC AAG GGA TGA TAT CAG G-3'; *MMP12*R: 5'-AAG AGC TCC AGA AGC AGT GG-3.

Table 1. Demographic and Clinical Data of Patients With Bronchial Asthma and Control Individuals, Genotyped for *MMP12* -82 A>G Polymorphism

Variable	Patients With Bronchial Asthma	Controls
No. (%)	<i>n</i> = 58	<i>n</i> = 119
Male	17 (29.3%)	59 (49.6%)
Female	41 (70.7%)	60 (50.4%)
Age at Study Inclusion, y		
Mean (SD)	54 (16.2)	59.35 (10.66)
Median (range)	59.5 (30.0–74.0)	58 (30–80)
Age at Diagnosis, y		
Mean (SD)	46.0 (11.6)	...
Median (range)	46.5 (29.0–62.0)	...
Duration of Disease, y		
Mean (SD)	7.94 (6.29)	...
Median (range)	8.5 (0–16.0)	...
Smoking Status	<i>n</i> = 58	<i>n</i> = 91
Nonsmokers	49 (84.5%)	60 (65.9%)
Former smokers	6 (10.3%)	9 (9.9%)
Current smokers	3 (5.2%)	22 (24.2%)
Smoking Habits (Packs/y)		
Mean (SD)	16.6 (15.11)	15.35 (9.92)
Median (range)	12.5 (3.0–40.0)	15 (5–44)
FEV1, % predicted		
Mean (SD)	71.00 (8.09)	97.67 (13.87)
FEV1/FVC, %		
Mean (SD)	66.60 (14.09)	77.53 (1.88)

..., nonapplicable; FEV1, forced expiratory volume: first second of forced breath; FVC, forced vital capacity.

The thermal profile of the amplification reactions included primary denaturing of template DNA for 5 minutes at 94°C, followed by 35 cycles of denaturation for 45 seconds at 94°C, annealing for 45 seconds at 57°C, and polymerization for 45 seconds at 72°C. The PCR reaction was terminated by a final extension for 10 minutes at 72°C.

We performed the digestion of the PCR product with 5U *Pvu II* (Thermo Fisher Scientific Inc, cat. no. ER0631) and 8 µL of each PCR product, in a final volume of 15 µL, for 16 hours at 37°C. The obtained restriction products were analyzed using 4% agarose gel stained with ethidium bromide. The results were documented using a gel documentation system (Synoptics Ltd).

After PCR reaction, the amplification product with primers for *MMP12* -82 A>G SNP was 199 bp in length. After the restriction reaction with *PvuII*, the wild-type A allele remained unchanged (199 bp in length). In the variant G allele, the restriction reaction resulted in 2 fragments—175 bp and 24 bp. The heterozygous genotypes were visualized by 2

fragments—one in length of 199 bp and the second with 175 bp (the third restriction fragment, of 24 bp in length, was not visualized on the electrophoregrams) (**Figure 1**).

Statistical Analyses

Statistical analyses were performed using SPSS software, version 16.0 for Windows (SPSS Inc). Continuous variables were analyzed for normality of the distribution using Kolmogorov-Smirnov test (1-sample Kolmogorov-Smirnov *D* Test). When the level of significance in this test was $P < .05$, the hypothesis for normal distribution was rejected. Continuous variables with normal distribution were compared between 2 or more independent groups using Student *t* testing or 1-way analysis of variance (ANOVA) testing with least significant difference (LSD) posthoc analysis; we compared variables with nonnormal distribution by using Mann-Whitney *U* testing or Kruskal-Wallis *H* testing, respectively.

We checked SNPs for deviation from Hardy-Weinberg equilibrium (HWE) in controls and in patients. The differences in the frequencies of distribution between patients and controls were analyzed in contingency tables using the χ^2 test, or Fisher exact test, when needed. The odds ratios and 95% confidence interval were calculated by binary logistic regression, with age and sex as covariates. Factors with $P < .05$ were considered statistically significant.

Results

After PCR reaction, the amplification product was 199 bp in length. After restriction reaction, the wild-type A allele remained unchanged (in size of 199 bp). As for the variant G allele, the restriction with *PvuII* resulted in 2 fragments—175 bp and 24 bp. The heterozygotes were visualized by 2 fragments—one in length of 199 bp and second with 175 bp (the third restriction fragment of 24 bp in length is not visible on the electropherogram) (**Figure 1**).

The genotype distribution in controls and patients did not deviate from the HWE values ($P = .99$ for each). Our comparison of the genotype distribution according to *MMP12*

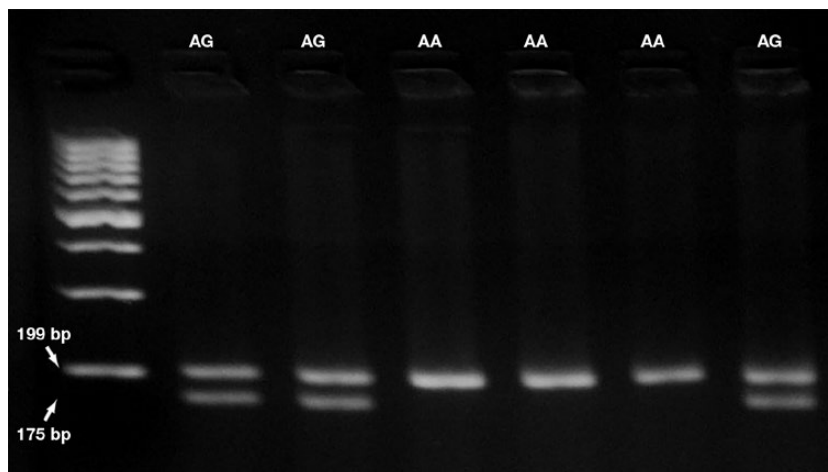


Figure 1

Allele distribution in control individuals and patients with asthma according to *MMP12* -82 A>G single nucleotide polymorphism (SNP).

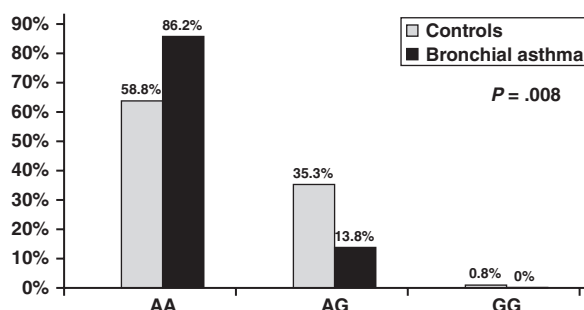


Figure 2

Visualization of restriction fragment length polymorphism analysis of polymerase chain reaction–amplified fragments (RFLP-PCR) products for genotyping of individuals for *MMP12* -82 A>G single nucleotide polymorphism (SNP) in 4% agarose gel electrophoresis.

-82 A>G SNP between patients and controls revealed a statistically significant difference ($P = .008$). In the 58 patients with BA, 50 patients (86.2%) had the homozygous AA genotype and 8 patients (13.8%) were heterozygous (AG). We did not identify any patient as being homozygous for the G allele (GG). Among the 119 controls, the distribution was as follows: 76 patients (63.9%) had the AA genotype, 42 (35.3%) were heterozygous (AG), and only 1 (0.8%) had the GG homozygous genotype (**Figure 2**).

Genotypes containing at least 1 variant G allele were less frequent (AG + GG, 8 [13.8%]) in patients with BA than in controls (AG + GG, 43 [36.1%]) (**Table 2**). Thus, in the dominant model, carriers of the G-allele genotypes appeared to have 4.2-fold lower risk for BA, compared with patients possessing the AA genotype (OR = 0.238; 95% CI, .12–.64; $P = .002$) (**Table 2**). The significance of the observed associations also remained after the adjustment for age and sex in the cohorts (OR = 0.277; 95% CI, .12–.65; $P = .003$) (**Table 2**), and the G-allele genotypes (AG + GG) conferred 3.6-fold lower risk than the AA genotype.

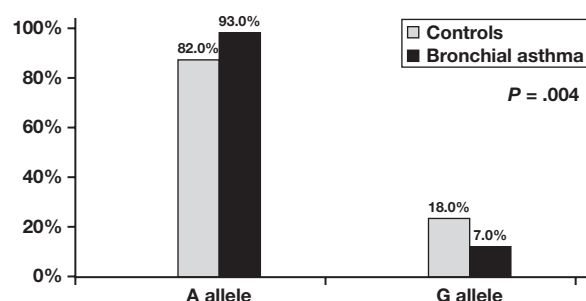
Carriers of the heterozygous AG genotype had 3.56-fold lower risk for BA (after the adjustment for age and sex), compared with those with the AA genotype (OR = 0.281; 95% CI, .12–.66; $P = .004$; **Table 2**).

We also found a statistically significant difference for allele distribution ($P = .004$), and the variant G allele determined 3.06-fold lower risk for asthma (OR = 0.327; 95% CI, .15–.71; **Figure 3**; **Table 2**). No associations were discovered between the genotypes and the sex, age at disease onset, disease duration, or spirometric parameters of lung functioning.

Table 2. Genotype and Allele Frequencies of the *MMP12* -82 A>G Gene Polymorphism in Patients With Bronchial Asthma and Control Individuals

<i>MMP12</i> -82 A>G	No. (%)		OR (95% CI), <i>P</i> Value	*OR (95% CI), <i>P</i> Value
	Patients n = 58	Controls n = 119		
Genotype Distribution				
AA	50 (86.2)	76 (63.9)	1.0 (reference)	1.0 (reference)
AG	8 (1.38)	42 (35.3)	0.297 (.13–.67), <i>P</i> = .004	0.280 (.12–.66), <i>P</i> = .004
GG	0	1 (.8)	...	...
AG + GG vs AA	8 (13.8)	43 (36.1)	0.238 (.12–.64), <i>P</i> = .002	0.277 (.12–0.65), <i>P</i> = .003
AG vs GG + AA	50 (86.2)	77 (64.7)	0.293 (.13–.68), <i>P</i> = .004	0.281 (.12–.66), <i>P</i> = .004
Allele Distribution				
-82A	108 (93.1)	194 (81.5)	1.0 (reference)	...
-82G	8 (6.9)	44 (18.5)	0.327 (0.15–0.71), <i>P</i> = .004	...

*, adjusted for sex and age; OR, odds ratio; CI, confidence interval; ..., nonapplicable.

**Figure 3**

Genotype distribution in control individuals and patients with asthma according to *MMP12* -82 A>G single nucleotide polymorphism (SNP).

Discussion

Many cell types play a role in the pathogenesis of bronchial asthma, including airway epithelium, smooth-muscle cells, eosinophils, and macrophages. The results reported by Woodruff et al²² showed an increased mass of airway smooth-muscle cells along the bronchial tree, with evidence of cell hypertrophy and hyperplasia in bronchial asthma. Human airway smooth-muscle cells can secrete MMPs and their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs), which have a role in the immunomodulatory mechanisms regulating ECM composition in patients with asthma.²³ Alveolar macrophages, which are known to play an important role in acute and chronic lung inflammation, are the major source of MMP-12. Immunohistochemical examinations revealed

MMP-12 staining in bronchial epithelia, alveolar macrophages, and bronchial smooth muscles and lung alveoli in rats—the MMP-12-positive macrophages were significantly increased after antigen challenge.¹⁶ It has been shown¹⁵ that macrophage MMP-12 plays an important role in the development of the inflammatory response to allergic lung injury.

In the present study, we have explored the role of *MMP12* -82 A>G polymorphism in the development of bronchial asthma and found that the variant G allele might have a protective effect. MMP gene expression is regulated by numerous stimulatory and suppressive factors that influence multiple signaling pathways. AP-1 complexes play a critical role in the regulation of several MMPs, including MMP-12. Basal and inducible levels of MMP gene expression can be influenced by genetic variations that may, in turn, influence the development or progression of several diseases.

One of the SNPs determined in the promoter region of MMP-12, an A>G transition at position -82 (*MMP12* -82A>G), has been reported to influence promoter activity because the A allele was associated with higher AP-1 binding affinity in *in vitro* studies with U937 and murine lung macrophage (MALU) cells. Thus, this allele has resulted in approximately 1.2-fold higher promoter activity, compared with the less-common G allele.^{19,24}

Our results showed that the G allele, as well as G genotypes (AG, GG), were less frequent in the patient group, conferring approximately 3.6-fold lower risk for development of bronchial asthma after adjustment for age and sex. So far, to our knowledge, no other study in the literature describes an effect of *MMP12* -82A>G SNP on the risk for BA.

The *MMP12* -82A>G SNP has been explored by Hunninghake et al.²⁰ as a factor influencing lung function in children with asthma; it was suggested that the variant G allele was associated with better lung function. The same study group also reported that the variant G allele was related to a reduction of risk among smokers for COPD and other chronic inflammatory diseases. Our data suggest a similar association: carriers of G-allele genotypes have approximately 3-fold lower risk for COPD in a population from central Bulgaria.²⁵ Two other studies confirm the protective effect of the variant G allele of the aforementioned promoter polymorphism for COPD.^{26,27}

Two previous studies^{21,28} have found that another functional polymorphism in the gene of MMP-12 had significant influence on the risk for BA. SNP (rs652438), a missense A>G transition in exon 8, causes a change of asparagine to serine at position 357 (p.Asn357Ser; N357S) in the polypeptide chain of the enzyme. This amino acid replacement is in the hemopexin domain, which is responsible for substrate binding; substrate binding is supposed to interfere with the catalytic activity of the enzyme. In vitro experiments with airway epithelial cells have shown that the variant-allele enzyme has lower enzyme activity.²⁹ Variant allele genotypes were associated with higher risk for childhood and combined asthma (adults plus children) in Japanese populations (ethnicity unknown)²⁹, as well as with asthma severity in Japanese adult patients (ethnicity unknown)²⁹ and in young patients (exact age range not specified) from the United Kingdom.¹⁷

Studies using mice deficient in metalloelastase (*MMP-12*^{-/-}) have demonstrated that macrophage recruitment in lungs and emphysema induced by long-term exposure to cigarette smoke were linked to MMP-12.^{15,18} *MMP-12*^{-/-} mice develop less severe acute lung injury, relative to their wild-type counterparts (*MMP-12*^{+/+}), expressing normal levels of metalloelastase. In the same group, the researchers report a reduction in proinflammatory cytokines and chemokines (eg MIP-1 α , MCP-1, TARC, and IL-5), which are important for the recruitment of inflammatory cells into the lungs.¹⁵

The expression of MMP-12 is modulated by variety of factors. Besides the higher binding affinity of the more common A allele of the *MMP12* -82 A>G promoter polymorphism, several cytokines and growth factors involved in the pathogenesis of asthma (interleukins, interferons, epidermal growth factor [EGF], vascular endothelial growth factor

(VEGF), tumor necrosis factor [TNF]- α , TGF- β) have been shown to induce the binding of AP-1 to MMP gene promoters and to stimulate the expression of MMP enzymes.²⁴

The differential expression of MMPs has been associated with asthma pathogenesis. Besides their roles in degrading ECM components, MMPs are also involved in inflammatory cell trafficking, host defenses, and tissue repair. MMP-12 is found to activate MMP-2 and MMP-3 and to cleave elastin, fibronectin, vitronectin, heparin sulfate, and basement membrane components such as type IV collagen and laminin, which process facilitates macrophages to penetrate the injured tissue.^{14,30}

The transcriptional factor AP-1 is a dimeric complex composed of Jun (c-Jun, JunB, or JunD) and Fos (FosB, c-Fos, Fra-1, or Fra-2) proteins. It has been shown that IL-1 β induces a time- and concentration-dependent increase in MMP-12 mRNA expression. Similar effects were noted for TNF- α . Interleukin (IL)-1 β and TNF- α enhanced c-Jun activation and nuclear binding; when combined together, they had an additive effect. The effects of those cytokines on c-Jun activation were directly correlated with their activities on MMP-12 release.³⁰

At the same time, MMP-12 has the ability to induce the release of proinflammatory cytokines and chemokines. A number of MMPs (including MMP-12) are able to shed the membrane bound TNF- α and thus to increase the bioavailability of that inflammatory mediator.³¹

Proteases do more than just play a role in the development of chronic lung diseases. It appears that products of their activity are also important in these pathological conditions. Structural proteins or fragments of structural proteins generated by proteolytic enzymes have the ability to induce chemotaxis of monocytes and neutrophils. Intact and degraded collagens, including collagenase-generated peptides, are chemotactic for monocytes.³²

Elastic fibers are some of the major components of ECM of the lung parenchyma and airways. It has been shown⁴ that in fatal asthma, elastic fibers are damaged in the large airways, and that elastic fiber content is decreased at the subepithelial and alveolar attachment levels. A positive correlation between elastic fibers and increased expression of MMP-12 in airway smooth-muscle cells in patients with BA, including those with fatal cases, was reported.⁴

ELN-441 is a proteolytical product from elastin and the measurement of its release from elastin-rich tissues, marked so that MMP-12 is able to degrade elastin from insoluble lung tissue. Further, it has been shown³³ that elastin degradation fragments, particularly a MMP-12 generated repeated sequence fragment, act as a chemoattractant for monocytes and fibroblasts in vitro, and that autoimmune response to elastin fragments has been identified.

Interactions between neutrophil elastase (NE) and the MMP-12 proteolytic systems were observed, with each augmenting the destructive capacity of the other: MMP-12 may degrade the serine protease inhibitor, and α 1-antitrypsin and NE may degrade the tissue inhibitor of MMP, namely, TIMP-1. NE may also be required for the proteolytic activation of pro-MMP-12.¹⁸

Conclusion

According to our results, the variant G allele of the *MMP12* -82 A>G promoter polymorphism could be considered protective for development of BA. This effect might be attributed to the possible lower gene expression and protein levels of MMP-12 in carriers of the G allele, leading to weaker destruction capacity within bronchial walls and in ECM remodeling in asthma. More studies are required to unravel the relationship between *MMP12* -82 A>G promoter polymorphism and BA. **LM**

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