# Gene polymorphisms and antigen levels of matrix metalloproteinase-1 in type 2 diabetes mellitus coexisting with coronary heart disease

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**Abstract**

**Background and aim:** Diabetes mellitus is a major risk factor for coronary heart disease (CHD). Matrix metalloproteinases (MMPs) can play a pivotal role in the remodelling of extracellular matrix associated with the development of atherosclerosis. Therefore, the aim of the study was to compare the distribution of genotypes and frequency of alleles of two polymorphisms of the MMP-1 gene promoter, an A/G substitution and a 1G/2G insertion, in correlation with antigen levels of matrix metalloproteinase-1 (MMP-1) in type 2 diabetic patients with or without CHD as well as individuals with normal glucose level without CHD.

**Methods:** Genotypes of 115 patients with type 2 diabetes mellitus (T2DM) and a subpopulation of 66 patients with coexisting CHD as well as 120 non-diabetic control subjects were determined by PCR-based restriction fragment length polymorphism (PCR-RFLP).

**Results:** We demonstrated that antigen levels of MMP-1 in the serum of diabetic patients were significantly higher than those of individuals with normal glucose metabolism ($p < 0.05$). Elevated levels of MMP-1 positively correlated with CHD occurrence in T2DM patients ($p < 0.01$). The distribution of genotypes revealed higher frequency of the 2G/2G polymorphism variant in diabetic patients with CHD [OR 5.76, 95% CI (1.24; 26.87)], thus suggesting its strong association with high level of MMP-1. In T2DM patients with coexisting CHD, a higher frequency of the 2G allele of 1G/2G [OR 1.74, CI 95% (1.01; 2.99)] and the G allele of A/G polymorphism [OR 2.15, 95% CI (1.22; 3.80)] was also found.

**Conclusion:** Our results suggest that type 2 diabetes mellitus is linked with elevated blood level of MMP-1, and polymorphisms of the promoter region of its gene might be associated with CHD.

**Key words:** type 2 diabetes mellitus, coronary heart disease, metalloproteinases, genetic polymorphism

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## Introduction

Proteolytic enzymes, including matrix metalloproteinases (MMP), may play an important role in the development of type 2 diabetes mellitus (T2DM) and cardiovascular disorders. Diabetic-related vascular complications including coronary heart disease (CHD) and hypertension (HT) are the leading cause of morbidity and mortality in diabetic patients [1, 2]. Because MMPs appear to be involved in monocyte invasion and vascular smooth muscle cell migration, their deregulation may be a critical factor in the development of vascular lesions [3]. On the other hand, MMP activity can be affected by reactive oxygen species generated by elevated levels of circulating glucose [4]. Recent findings indicate that expression and activities of MMP-1 and MMP-9 are enhanced by high glucose concentration in human primary cultured endothelial cell monocyte-derived macrophages [5]. The promoter region of many MMP genes contains nuclear factor κB, activator protein-1 (AP-1), stimulatory protein (SP-1), and phorbol ester responsive elements. Nuclear factors κB and AP-1 are sensitive to oxidative stress and

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contribute to the involvement of glucose in the regulation of MMP activity, which may depend on the sequence of the promoter of MMP genes [6].

*MMP-1* (interstitial collagenase) is a major member of the MMP family, primarily because of its specificity for type 1 collagen proteins, which are the main component of the interstitial matrix [7]. Two single nucleotide polymorphisms of the *MMP-1* gene promoter have been described: a guanine to adenine substitution at the – 519 position (the G/A polymorphism) and a guanine insertion at – 1607 (the 1G/2G polymorphism) [8, 9].

In the present work we determined the antigen levels of MMP-1 and the distribution of genotypes and frequency of alleles of two polymorphisms of the *MMP-1* gene promoter: an A/G substitution and a 1G/2G insertion. Genotypes of T2DM patients with and without CHD as well as individuals with normal glucose level without CHD were analysed.

## Methods

### Subjects

One hundred fifteen patients with type 2 diabetes mellitus (46 men and 69 women, mean age 65.8±11.99) were enrolled in this study. Among those patients 66 subjects (18 men and 48 women, mean age 58.2±16.91) presented with CHD, 120 individuals with normal glucose metabolism served as controls (44 men and 76 women, mean age 56.3±19.8). All patients were recruited from two internal medicine wards. All subjects included in the study were unrelated Caucasians. T2DM was diagnosed at least six months before enrolment in the study. We defined diabetes as: 1) a report of having been informed of having diabetes, and/or 2) use of oral hypoglycaemic drugs or insulin, or 3) having a fasting plasma glucose ≥126 mg/dl, or 4) having a 2-h glucose OGTT ≥200 mg/dl. CHD was diagnosed if the patients had a history of myocardial infarction or showed abnormal electrocardiographic findings. The study was approved by the local ethics committee and written informed consent was obtained from all participants before entering the study.

### MMP-1 antigen levels

Blood samples for the MMP-1 serum antigen levels measurement were taken from the cubital vein after overnight fasting. MMP-1 was quantified in the serum by sandwich enzyme linked immunosorbent assay (ELISA) using the Biotrak MMP-1 human ELISA system (Amersham Pharmacia Biotech, Little Chalfont, England). Absorbance was measured at 450 nm and the antigen levels in ng/ml were obtained from standard curves.

### Determination of the MMP-1 promoter genotype

DNA was isolated from peripheral blood leukocytes by proteinase K digestion and phenol/chloroform extraction. Genotypes of the 1G/2G polymorphism in the MMP-1 promoter were determined by PCR-based *Xmn*I restriction fragment length polymorphism [8]. The following primers were used: 5'-TCG TGA GAA TGT CTT CCC ATT-3" (forward primer) and 5'-TCT TGG ATT GAT TTG AGA TAA GTG AAA TC-3" (reverse primer). Two mismatches were introduced in the reverse primer annealed to the proximity of the polymorphic site, creating a recognition sequence for restriction endonuclease *Xmn*I (5'-GAANNNNTTC-3'), when the DNA template contained 1G but not 2Gs at the polymorphic site. Thus, *Xmn*I would cleave PCR products derived from the 1G allele but not those obtained from the 2G allele. Genotypes of the G/A polymorphism were determined by PCR-based *Kpn*I restriction fragment length polymorphism [10]. The following primers were used: 5'-CAT GGT GCT ATC GCA ATA GGG T-3' (forward primer) and 5'-TGC TAC AGG TTT CTC CAC ACA C-3' (reverse primer). KpnI digestion would cleave PCR products derived from the A allele but not those derived from the G allele. The PCR was carried out in an MJ Research, INC thermal cycler, model PTC-100 (Waltham, MA, USA), in a total volume of 25 µl, containing 50 ng genomic DNA, 10 pmol each primer (Eurogentec, Seraing, Belgium), 200 mmol each dATP, dCTP, dGTP and dTTP (Boeringer, Mannheim, Germany), 20 mmol Tris-HCl (pH 8.4), 50 mmol KCl, 2 mmol $MgCl_2$ for 1G/2G polymorphism and 1.5 mmol $MgCl_2$ for G/A polymorphism, 1 unit Taq polymerase. The thermal cycling conditions were 1 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 56°C for the 1G/2G polymorphism and 51°C for the G/A polymorphism and 30 s at 72°C. PCR-amplified DNA was digested with 3 U *XmnI* for 1G/2G polymorphism and 4 U KpnI for G/A polymorphism in a total volume of 18 µl. The mixtures were incubated at 37°C for 16 h. 15 µl aliquots of the digest were electrophoresed on a 10% horizontal polyacrylamide gel (PAGE) and visualised by ethidium bromide staining (Figure 1).

### Statistical analysis

The allelic frequencies were estimated by gene counting and genotypes were scored. The significance of the differences of observed alleles and genotypes between groups was tested using the $\chi^2$ test. The ORs and 95% CIs were calculated using a logistic regression model. The t-test (for normal distribution) or Mann-Whitney test (for non-normal distribution) was used to compare each parameter between two groups. An ANOVA test was used to identify parameters that would make significant differences between more than two groups; Scheffe's test was then used to assess the significance of difference in each identified parameter between any two groups. A p value <0.05 was considered significant. Analyses were performed using STATISTICA 6.0 software (Statsoft, Tulsa, OK, USA).

## Results

A significant increase of the MMP-1 antigen levels in type 2 diabetic patients was observed in comparison with non-diabetic subjects (controls). Diabetic patients with

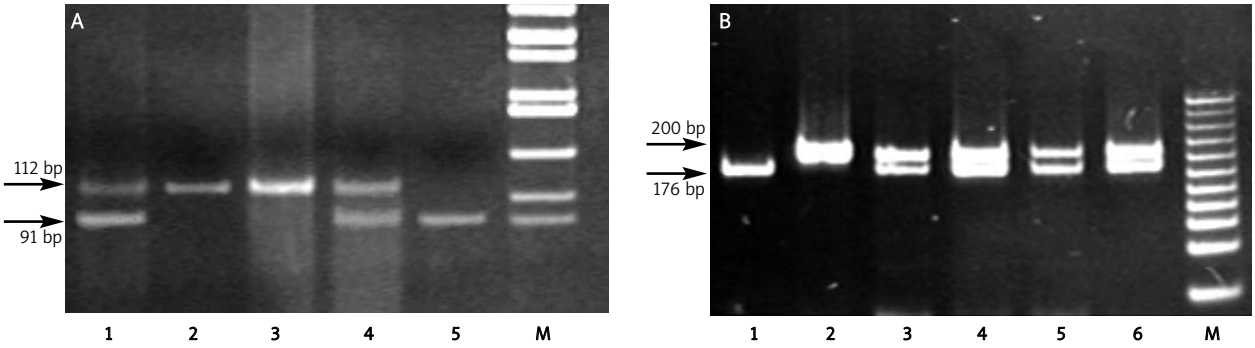


**Figure 1.** Typical results of the *Xmn*I (A) and *Kpn*I (B) restriction endonuclease digestion of PCR products of *MMP-1* gene promoter analysed by 10% polyacrylamide gel electrophoresis, stained with ethidium bromide and viewed under UV. **A** – lanes 1 and 4 display bands for a heterozygote 1G/2G; lanes 2 and 3 – a homozygote 2G/2G; lane 5 – a homozygote 1G/1G. Lane M – DNA molecular marker. **B** – lane 1 displays a band for a homozygote A/A; lane 2 – a homozygote G/G, lanes 3–6 – a heterozygote A/G. Lane M – DNA molecular marker

**Table I.** Average MMP-1 antigen level in serum of type 2 diabetes mellitus (DM) patients with or without CHD and subjects with normal glucose metabolism (controls)

| Genotype | Type 2 DM (n=94)* | Controls (n=99) |
|---|---|---|
| | Average MMP-1 [ng/ml] Median (quartiles) | Median (quartiles) |
| 1G/1G | 7.32 (6.40; 8.07) | 6.21 (5.49; 7.08) |
| 1G/2G | 8.61 (7.11; 9.95) | 7.73 (4.68; 9.76) |
| 2G/2G | 13.92 (8.11; 15.18)** | 12.79 (7.15; 15.39)** |
| A/A | 9.95 (7.87; 14.22) | 7.08 (5.49; 9.23) |
| A/G | 8.09 (6.17; 10.61) | 8.61 (5.14; 11.75) |
| G/G | 7.89 (7.30; 9.77) | 7.87 (6.40; 15.61) |
| Total | 9.95 (8.07; 12.87) | 7.89 (5.76; 10.69)* |

** p=0.05*
*** p <0.01*

| Genotype | Type 2 DM with CHD (n=62)* | Type 2 DM (n=32) |
|---|---|---|
| | Average MMP-1 [ng /ml] Median (quartiles) | Median (quartiles) |
| 1G/1G | 7.60 (6.60; 8.29) | 7.26 (6.27; 7.92) |
| 1G/2G | 8.99 (7.14; 10.69) | 8.27 (6.35; 9.87) |
| 2G/2G | 13.92 (8.11; 14.96)** | 12.85 (8.09; 14.96)** |
| A/A | 9.59 (7.79; 13.36) | 9.95 (9.97;12.93) |
| A/G | 8.11 (6.77; 11.49) | 7.69 (5.77; 9.03) |
| G/G | 8.27 (7.39; 11.26) | 7.57 (7.21; 8.57) |
| Total | 8.27 (6.52; 15.19) | 6.41 (3.98; 8.91) |

** p=0.01*
*** p <0.01*

CHD had higher level of *MMP-1* compared with diabetic patients without CHD (p <0.01) (Table I). Additionally, the level of MMP-1 in all investigated samples with 2G/2G genotype (p <0.01) was significantly higher than the level in samples with genotypes 1G/1G and 1G/2G.

Table II shows the distribution of genotypes and frequency of alleles of the 1G/2G polymorphism in patients with T2DM and controls. There were no significant differences in the distribution of genotypes between these two groups. Additionally, we did not observe any difference in the frequency of the 1G and 2G alleles between groups.

The distribution of the 1G/2G polymorphism in diabetic patients with or without CHD is presented in Table III. A significant difference was found in genotype distribution between analysed groups ($\chi^2$=10.727[2]). The obtained results revealed higher frequency of 1G/2G polymorphism in diabetic patients with CHD [OR 5.76, 95% CI (1.24; 26.87)] compared to diabetic patients without CHD. In T2DM patients coexisting with CHD higher frequency of 2G allele of the 1G/2G polymorphism [OR 1.74, CI 95 % (1.01; 2.99)] was also found.

Table IV shows the distribution of genotypes of the A/G polymorphism in patients with type 2 diabetes mellitus and non-diabetic subjects. There were no differences in the distribution of the genotypes or the A and G allele frequencies between groups. However, there was a significant difference in the distribution of the A/G polymorphism between diabetic patients with and without CHD ($\chi^2$=11.553) (Table V). It was found that the percentage of the G allele was higher in type 2 patients with CHD than in type 2 diabetic patients without it [OR 2.15, CI 95% (1.22; 3.80)].

## Discussion

Patients with type 2 diabetes mellitus have a greater than 3-fold risk of cardiovascular disease [11]. High glucose has been shown in many cell types to trigger signalling cascades leading to upregulation of MMP expression and its activity [12, 13]. Recently, Lobmann et al. demonstrated elevated levels of matrix metalloproteinases in cultivated fibroblasts of diabetic patients [14]. Kumar et al. observed

**Table II.** Distribution of genotypes and frequency of alleles of the 1G/2G polymorphism in patients with type 2 diabetes mellitus (DM) and subjects with normal glucose metabolism (controls). OR analysis

| Genotype | Patients with type 2 DM (n=115)* | | Controls (n=120) | | OR (95% CI) |
|---|---|---|---|---|---|
| | Number | Frequency | Number | Frequency | |
| 1G/1G | 32 | 0.29 | 32 | 0.27 | 1.06 (0.59; 1.88) |
| 1G/2G | 59 | 0.51 | 60 | 0.50 | 1.05 (0.63; 1.76) |
| 2G/2G | 24 | 0.20 | 28 | 0.23 | 0.86 (0.47; 1.61) |
| | | $\chi^2$=0.684 | | | |
| 1G | 123 | 0.53 | 124 | 0.52 | 1.07 (0.75; 1.54) |
| 2G | 107 | 0.47 | 116 | 0.48 | 0.93 (0.63; 1.34) |

**Table III.** Distribution of genotypes and frequency of alleles of the 1G/2G polymorphism in patients with type 2 diabetes mellitus (DM) with coexisting coronary heart disease (CHD) and without it (controls). OR analysis

| Genotype | Patients with type 2 DM and CHD (n=66) | | Patients with type 2 DM (n=49) | | OR (95% CI) |
|---|---|---|---|---|---|
| | Number | Frequency | Number | Frequency | |
| 1G/1G | 17 | 0.26 | 18 | 0.37 | 0.6 (0.27; 1.33) |
| 1G/2G | 36 | 0.54 | 29 | 0.59 | 0.83 (0.39; 1.75) |
| 2G/2G | 13 | 0.20 | 2 | 0.04 | 5.76 (1.24; 26.87) |
| | | $\chi^2$=10.727 | | | |
| 1G | 70 | | 65 | 0.66 | 0.57 (0.33; 0.98) |
| 2G | 62 | 0.530.47 | 33 | 0.34 | 1.74 (1.01; 2.99) |

**Table IV.** Distribution of genotypes and frequency of alleles of the A/G polymorphism in patients with type 2 diabetes mellitus (DM) and subjects with normal glucose metabolism (controls). OR analysis

| Genotype | Patients with type 2 DM (n=115)* | | Controls (n=120) | | OR (95% CI) |
|---|---|---|---|---|---|
| | Number | Frequency | Number | Frequency | |
| A/A | 56 | 0.49 | 63 | 0.53 | 0.86 (0.51; 1.43) |
| A/G | 35 | 0.30 | 30 | 0.25 | 1.31 (1.74; 2.33) |
| G/G | 24 | 0.21 | 27 | 0.22 | 0.91 (0.48; 1.69) |
| | | $\chi^2$=1.964 | | | |
| A | 147 | 0.64 | 156 | 0.65 | 0.95 (0.65; 1.39) |
| G | 83 | 0.36 | 84 | 0.35 | 1.04 (0.72; 1.53) |

**Table V.** Distribution of genotypes and frequency of alleles of the A/G polymorphism in patients with type 2 diabetes mellitus (DM) with coronary heart disease (CHD) and without it (controls). OR analysis

| Genotype | Patients with type 2 DM and CHD (n=66)* | | Patients with type 2 DM (n=49) | | OR (95% CI) |
|---|---|---|---|---|---|
| | Number | Frequency | Number | Frequency | |
| A/A | 26 | 0.45 | 30 | 0.6 | 0.41 (0.19; 0.88) |
| A/G | 24 | 0.33 | 15 | 0.3 | 1.29 (0.59; 2.85) |
| G/G | 16 | 0.22 | 5 | 0.1 | 2.82 (0.95; 8.31) |
| | | $\chi^2$=11.553 | | | |
| A | 76 | 0.58 | 75 | 0.75 | 0.42 (0.23; 0.74) |
| G | 56 | 0.42 | 25 | 0.25 | 2.15 (1.22; 3.80) |

an increased concentration of MMP-8 and -9 in the gingival tissue of diabetic patients and suggested that these changes may contribute to the failure of the healing process in the diabetic condition [15]. Previously, Noda et al. found significantly higher levels of MMP-2 in patients with proliferative diabetic retinopathy and suggested that the activity of MMP-2 was involved in the formation of the fibrovascular tissues [16]. These findings indicate that type 2 diabetes mellitus may be associated with the elevated blood level of different MMPs, which may favour the development of vascular complication. Matrix metalloproteinase-1 (interstitial collagenase) is a substantial enzyme for interstitial collagen degradation in the myocardium. Increased myocardial MMP-1 activity was reported to be accompanied by structural remodelling of the myocardium, which includes myocardial hypertrophy and fibrosis of intramyocardial arteries in advanced hypertensive heart disease [17, 18]. Additionally, upregulation of MMP-1 gene transcription may be associated with ischaemic cardiomyopathy in humans [19]. These findings suggest that the expression of MMP-1 can be considered as an important factor in the development of CHD. In our study we observed a significant increase of the *MMP-1* antigen levels in type 2 diabetic patients in comparison with non-diabetic subjects. Additionally, we demonstrated that an elevated level of *MMP-1* was positively correlated with CHD occurrence in diabetic patients.

Metalloproteinases expression occurs under a tightly regulated mechanism [20]. The signalling pathway mediating the high glucose level and MMP expression is not completely understood. Analysis of the MMP promoter has identified essential response elements including activator protein-1 (AP-1) and Ets, which regulates the transcription of the human gene in natural killer (NK) cells. High glucose has been shown to activate AP-1 and growth factors of NF-κB [21, 22], PDGF and TGF-β [21-24]. Numerous studies have indicated that polymorphisms in the promoter region of MMP genes might be associated with acute coronary syndrome, myocardial infarction and progression of vascular complications in diabetic patients [25-29]. Therefore, we searched for common functional variation in the promoter sequence of the MMP-1 gene that may be associated with coronary artery disease in diabetic patients. The insertion of an extra guanine at the – 1607 position in the MMP-1 gene promoter creates a binding site for the transcription factor Ets, 5"-GGAT-3", adjacent to an AP-1 site at – 1602. Promoters containing the 2G allele display significantly higher transcriptional activity than 1G promoters [8]. The A/G polymorphism is located close to the start of the transcription site, but its effect on the expression of the *MMP-1* gene is not known [9].

In subjects enrolled in the control group, the distribution of the 1G/2G polymorphic variant of the promoter sequence of the *MMP-1* gene was comparable to other populations studied, including Caucasian and Chinese populations [30-34]. However, in Japanese individuals the genotype frequencies were different, since Hirata et al. revealed that the 2G/2G polymorphic variant appeared with 2-fold higher, and 1G/1G polymorphic variant with nearly 4-fold lower frequency compared to our population [35].

In our study, the analysis of the distribution of genotypes in diabetic patients revealed higher frequency of the 2G/2G polymorphism variant in the T2DM group with CHD than in diabetic patients without CHD (20 vs. 4%). In addition, we found an association between 2G/2G genotype and high MMP-1 level. These findings suggested a strong association of the 2G/2G genotype with coronary heart disease progression in diabetic patients. We also noted significantly higher frequencies of the G allele of A/G polymorphism in the T2DM group with CHD; however, this polymorphism did not seem to have an impact on MMP-1 level.

This work indicates that type 2 diabetes mellitus is associated with elevated blood level of *MMP-1*, which may contribute to coronary heart disease. Moreover, our results suggest that polymorphism of the promoter region of the *MMP-1* gene may be linked with T2DM coexisting with CHD, and therefore it can be considered as a potential prognostic marker of cardiovascular disorders in diabetic patients. Further investigations are needed to explain the potential role of MMP-1 in diabetes and its complications.

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# Polimorfizm regionu promotorowego i poziom antygenu metaloproteazy macierzowej 1 u chorych na cukrzycę typu 2 ze współistniejącą chorobą niedokrwienną serca

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## Streszczenie

**Wstęp:** Cukrzyca typu 2 sprzyja szybszemu rozwojowi miażdżycy, a powikłania sercowo-naczyniowe są główną przyczyną zachorowalności i śmiertelności w tej grupie chorych. Metaloproteazy macierzy (MMP) odgrywają istotną rolę w remodelingu macierzy pozakomórkowej prowadzącym do rozwoju miażdżycy. Wydaje się, że polimorfizm genów promotora MMP-1 może mieć wpływ na rozwój powikłań sercowo-naczyniowych u chorych na cukrzycę typu 2 (T2DM).

**Cel:** Porównanie dystrybucji genotypów i częstości alleli dwóch polimorfizmów promotora genu MMP-1: substytucja A/G i insercja 1G/2G, w odniesieniu do poziomu metaloproteazy macierzy 1 (MMP-1) u chorych na T2DM z chorobą niedokrwienną serca i bez tej choroby oraz u osób bez zaburzeń gospodarki węglowodanowej i bez choroby niedokrwiennej serca.

**Metody:** Do badania zakwalifikowano 115 chorych na T2DM, przy czym u 66 z nich rozpoznano współistniejącą chorobę niedokrwienną serca. Grupę kontrolną stanowiło 120 zdrowych osób – bez cukrzycy i choroby niedokrwiennej serca. Genotyp osób zakwalifikowanych do badania określono metodą PCR-RFLP (ang. *PCR – based restriction fragment length polymorphism*, PCR-RFLP).

**Wyniki:** Poziomy MMP-1 w osoczu chorych na T2DM były istotnie wyższe niż u osób z prawidłową gospodarką węglowodanową ($p < 0,05$). Wykazano dodatnią korelację pomiędzy poziomem MMP-1 a występowaniem choroby niedokrwiennej serca u chorych na cukrzycę typu 2 ($p < 0,01$). Zaobserwowano również większą częstość występowania wariantu polimorficznego 2G/2G u chorych na cukrzycę ze współistniejącą chorobą niedokrwienną serca [OR 5,76, 95% CI (1,24; 26,87)]. Sugeruje to silny związek choroby niedokrwiennej serca z poziomem MMP-1. U chorych na cukrzycę ze współistniejącą chorobą niedokrwienną serca zaobserwowano ponadto zwiększoną częstość występowania allela 2G polimorfizmu 1G/2G [OR 1,74, 95% CI (1,01; 2,99)] i allela G polimorfizmu A/G [OR 2,15, 95% CI (1,22; 3,80)].

**Wnioski:** Uzyskane wyniki sugerują podwyższenie poziomu MMP-1 u chorych na T2DM oraz związek określonych wariantów polimorficznych regionu promotora genu dla MMP-1 z występowaniem choroby niedokrwiennej serca.

**Słowa kluczowe:** cukrzyca typu 2, choroba niedokrwienna serca, metaloproteazy, polimorfizm

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