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**Objectives:** The aim of this study was to analyze functionally significant polymorphisms of matrix metalloproteinases genes (*MMP-1, -2, -3, -8, -9*) for their association with duodenal ulcer (DU) in the Caucasian population from Central Russia.

**Methods:** The study sample included 364 DU patients (208 had *H. pylori* and 156 were uninfected) and 347 controls (*H. pylori*-negative). Ten polymorphisms of the *MMP-1, -2, -3, -8, -9* genes were examined for association with DU by the logistic regression analysis (used the three main genetic models). The polymorphisms of the *MMP-9* gene associated with DU and 59 proxy variants ($r^2 \geq 0.80$) were studied *in silico* for their functionality.

**Results:** Allele G of rs17576 and haplotype GG [rs17576-rs3787268] of the *MMP-9* gene may increase risk for DU ($_{adj}$OR = 1.46-2.09, $p_{perm} \leq 0.006$ and $_{adj}$OR =1.60, $p_{perm}$=0.016 respectively). Five SNPs of the *MMP-9* gene may increase risk for *H. pylori*-positive DU: alleles T of rs3918242 ($_{adj}$OR = 1.95, $p_{perm}$ = 0.007), G of rs17576 ($_{adj}$OR = 1.68-2.81, $p_{perm} \leq 0.002$), and A of rs17577 ($_{adj}$OR = 1.96, $p_{perm}$ = 0.008), haplotypes GG [rs17576-rs3787268] ($_{adj}$OR =1.95, $p_{perm}$=0.006) and GGC [rs17576-rs3787268-rs2250889] ($_{adj}$OR =1.96, $p_{perm}$=0.006). These loci and 59 proxy SNPs may have functionally significant epigenetic effects, amino acid replacements in the MMP9, and correlate with the expression and alternative splicing of 17 and 6 genes respectively.

**Conclusions:** Polymorphisms rs17576 and rs3787268 of the *MMP-9* were associated with DU and five *MMP-9* gene SNPs were associated with *H. pylori*-related DU in Caucasians of Central Russia.

**Key words:** Association; Duodenal ulcer; *H. pylori*; Matrix metalloproteinases; SNP

## 1. Introduction

Duodenal ulcer (DU) is a peptic ulcer disease affecting the duodenum. In the general population, the peptic ulcer prevalence is estimated at approximately 5–10% (Lanas and Chan, 2017). DU may be caused by various factors, e.g., *Helicobacter pylori* infection, some medications, e.g., aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs), alcohol and tobacco consumption, dietary factors, stress, etc. (Kuna et al., 2019; Lanas and Chan, 2017). Both NSAIDs and *H. pylori* infection induce secretion of gastric acid and damage the mucus barrier that allow gastric acid to cause inflammation and ulceration of the duodenal lining (Zhang et al.,

2013). On the other hand, far not all people using NSAIDs or infected by *H. pylori* develop DU. This assumes that individual susceptibility is important for the mucosal damage to begin (Kuna et al., 2019). DU is a multi-factorial disease and thus determined by a complex of environmental and genetic factors (Datta De and Roychoudhury, 2015).

Matrix metalloproteinases (MMPs) play a key role in extracellular matrix (ECM) degradation, one of the causes of gastrointestinal ulcer (Shahin et al., 2001; Tarnawski, 2005). MMPs comprise a family of neutral endopeptidases capable to degrade extracellular matrix proteins and remodel connective tissue (Cui et al., 2017). In addition to this, MMPs activate some chemokines, thus contributing to the recruitment of white blood cells into inflamed tissues (Kruidenier et al., 2006). MMPs are overexpressed in *H. pylori*-associated gastrointestinal ulcers (Hellmig et al., 2006; Pender and MacDonald, 2004). The MMP proteins can be expressed by many cell types in response to various signals from soluble factors or cell-matrix interactions (Parks et al., 2004). In *H. pylori*-infected mucosa, epithelial cells are apparently one of the major producers of MMPs (Mori et al., 2003). In addition to *H. pylori* infection (Bebb et al., 2003), other major stimuli for MMP production are cytokines produced by mucosal T cells and macrophages (Pender and MacDonald, 2004).

Despite the apparent significance of *MMP* for the development of DU, the association of these genes with the disease has been studied poorly: we were able to find only three articles on this topic (Shaymardanova et al., 2016; Shan et al., 2010; Yeh et al., 2010). Shaymardanova et al. (2016) analyzed seven loci from five *MMP* genes (rs1799750 and rs494379 *MMP-1*, rs2285052 *MMP-2*, rs3025058 *MMP-3*, rs3918242 and rs17576 *MMP-9*, rs2276109 *MMP-12*) in DU patients from the Republic of Bashkortostan (Russia) and showed the association of two polymorphisms, rs494379 *MMP*-1 and rs17576 *MMP*-9, with the disease. Yeh et al. (2010) studied loci of three *MMP* genes (*MMP-3* -1612 6A/5A, *MMP-7* -181 A/G, and *MMP-9* exon 6 A/G) for their association with DU in *H. pylori*-infected Taiwanese patients and found that the *MMP-3* promoter polymorphism (-1612 6A/5A) might contribute to DU susceptibility after *H. pylori* infection in Taiwanese females (a 2.4-fold increased risk of DU for the 6A6A genotype as compared to the 5A allele carriers). On the other hand, no association with the disease was detected for rs3918242 *MMP–9* in Chinese children (Shan et al., 2010).

The available literature data, despite being contradictory, suggests that *MMP* genes may contribute to DU. This study analyzed the association of ten loci of the *MMP-1, -2, -3, -8, -9* genes with DU in Caucasians from Central Russia.

## 2. Materials and methods

### *2.1. Study participants*

The study sample included 364 DU patients and 347 controls. To be recruited for the study, the participants should meet the following inclusion criteria: Russian ethnicity (self-reported) and birthplace in Central Russia (Reshetnikov et al., 2015; Litovkina et al., 2014).

DU was diagnosed by clinical/endoscopic examination. The group of control included otherwise healthy participants without any symptoms of gastrointestinal disease (Minyaylo, 2020). Endoscopy was not performed in the participants from the control group because, in addition to ethical reasons, the chance to find an active ulcer in asymptomatic individuals is very low (García-González et al., 2003). Those individuals who were on long-term treatment with NSAIDs, corticosteroids, and aspirin were excluded.

The infection of *H. pylori* in DU participants was diagnosed by the histologic examination of endoscopic biopsies. Among 364 patients with DU, 208 had *H. pylori* and 156 were uninfected. Serology testing in the controls was used to detect *H. pylori*-specific IgG (*H. pylori*-positive controls were excluded from the further analysis. All participants were evaluated for the possible presence of psycho-social stressful factors such as numerous home/work stressful situations, the unsatisfactory (or absence) of social support, stressful family situations (single/separated/widowed), poor social or/and economic participants status (Moskalenko et al., 2019a).

The study protocol was approved by the Regional Ethics Committee at the medical institute of Belgorod State National Research University. All participants were asked to sign an informed consent prior to entering the study. All medical examinations of the participants were conducted at the Gastroenterology Division of the St. Joasaph Belgorod Regional Hospital.

### *2.2. DNA isolation, SNPs selection, and genotyping*

About 4-5 ml of blood from each participant was drawn to a vacutainer tube (Vacutainer®) by a certified nurse. Total DNA was isolated from peripheral blood leucocytes using the phenol-chloroform protocol (Tikunova et al., 2017).

The selection of SNPs of the *MMP* genes was based on their previously reported associations with disorders of the digestive system (duodenal and gastric ulcer, cancer of gastric and other organs of digestive systems), predicted functional characteristics, and minor allele frequency >0.05 in Europeans (Ponomarenko et al., 2020a). The ten common SNPs of the five *MMP* genes were selected for the study: *MMP-1* (rs1799750), *MMP-2* (rs243865), *MMP-3* (rs679620), *MMP-8* (rs1940475), *MMP-9* (rs2250889, rs17577, rs3918249, rs17576, rs3787268, rs3918242) (Moskalenko et al., 2019b; Starikova et al., 2021). The SNPs functionality was assessed *in silico* by the available online at the HaploReg tool (Ward and Kellis, 2016). All 10 loci *MMP* genes included in this study had potential functional significance (Supplementary Table 1). According to previously published association studies, eight loci were associated with various diseases of the digestive system (duodenal and gastric ulcer, gastritis, esophageal and gastric cancer, other digestive cancers) (among them with peptic ulcer were associated 2 loci, rs1799750 *MMP3* and rs17576 *MMP9*) (Supplementary Table 2)

The selected loci were genotyped by the MassARRAY 4 system. About five percent of the samples were regenotyped at random (Golovchenko et al., 2020) and showed complete (100%) reproducibility.

*2.3. Data analysis*

The Hardy-Weinberg equilibrium of the observed genotype (allele) frequencies was assessed by the $\chi^2$-test. The associations of the candidate SNPs with DU were examined using the logistic regression analysis (according to the recessive, dominant, and additive models) (Ponomarenko et al., 2020b). The following covariates we used for the regression analysis: BMI as continuous variable, whereas stress, tobacco and alcohol consumption, positive peptic ulcer family history as categorical variables (data are presented in Table 1). The «Solid Spine» procedure of the linkage disequilibrium (at $D' > 0.80$) executed in the HaploView program (Barrett et al., 2005) was used to determine haplotype blocks. The association analysis (OR, 95% CI) and the permutation test (multiple comparisons adjustment) were carried out using PLINK v. 2.050 (Purcell et al., 2007). The value of $P_{perm} \leq 0.017$ was applied as the significance level assuming the Bonferroni correction

(n=3) based on the number of pairwise comparisons (control vs DU, control vs *H. pylori*-infected patients, and control vs *H. pylori*-uninfected patients).

*2.4. SNPs functionality effects*

The DU-associated genetic variants and their proxies were further studied *in silico* for their functional significance (Ponomarenko et al., 2021). The SIFT online tool (Kumar et al., 2009) was used to analyze missense SNPs. The regulatory potential was analyzed using HaploReg (Ward and Kellis, 2016) and RegulomeDB (Dong and Boyle, 2019). The RegulomeDB database values of the rank and the score were applied to estimate SNP regulatory potential: the higher RegulomeDB score (from 0 to 1) and lower RegulomeDB rank (from 1 to 7) indicate a higher regulatory potential (Dong and Boyle, 2019). The data from the GTExportal browser (The GTEx Consortium, 2017) was used to estimate the effect of the DU candidate loci on expression QTLs and splicing QTLs. Likewise, regulatory potential, eQTL, and sQTL parameters of the SNPs in LD with the DU-associated polymorphisms were assessed (Moskalenko et al., 2021). The polymorphisms linked ($r^2 \geq 0.80$) to the DU-associated ones were identified by HaploReg (Ward and Kellis, 2016).

**3. Results**

A summary of the phenotypic characteristics (demographic and clinical) of the enrolled subjects is provided in Table 1 and Table 2. The DU-affected participants had lower BMI, higher incidence of peptic ulcer in family history, stress, tobacco and alcohol consumption, as compared to the control subjects (p=0.004-0.0005) (data are presented in Table 1). Therefore, these variables were applied as covariates (continuous (BMI) and categorical (all other aforementioned demographic parameters) variables) in the association data analyses. The prevalence of frequent somatic pathologies such as essential hypertension (24.18% DU and 19.02% controls, p=0.11), heart ischemia (15.11% DU and 12.97% controls, p=0.47), heart atherosclerosis (10.98% DU and 8.93% controls, p=0.43), spine osteochondrosis (9.07% DU and 7.20% controls, p=0.44), osteoartrosis (6.87% DU and 5.19% controls, p=0.43), chronic bronchitis (4.67% DU and 4.03% controls, p=0.81), diabetes (2.20% DU and 3.17% controls, p=0.57), chronic glomerulonephritis/pyelonephritis (2.75% DU and 2.59% controls, p=0.99) among DU patients and controls were not significantly different (p>0.05).

*3.1. Association analysis*

The distribution of genotypes/alleles of the studied SNPs in the case and control groups is shown in Supplementary Table 3. All polymorphisms were in the HWE ($p>0.005$, $p_{bonf}>0.05$). The risk value for DU had allele G rs17576 (recessive model, the parameter of OR adjusted for covariates $_{adj}OR = 2.09$, $p_{perm} = 0.004$, power - 96.86%; additive model, $_{adj}OR = 1.46$, $p_{perm} = 0.006$, power - 93.66%) (Table 3).

Three SNPs of the *MMP-9* were associated with *H. pylori*- related DU: allele T of rs3918242 was associated according to the dominant model ($_{adj}OR = 1.95$, $p_{perm} = 0.007$, power - 95.98%), allele G of rs17576 - according to the additive ($_{adj}OR = 1.68$, $p_{perm} = 0.002$, power - 98.49%) and recessive ($_{adj}OR = 2.81$, , $p_{perm} = 0.001$, power - 99.73%) models, allele A of rs17577 - according to the dominant model ($_{adj}OR = 1.96$, $p_{perm} = 0.008$, power - 96.20%) (Table 4).

Haplotype GG [rs17576-rs3787268] of *MMP-9* was associated with DU ($_{adj}OR = 1.60$, $p=0.006$, $p_{perm}=0.016$). Also, *MMP-9* haplotypes GG [rs17576-rs3787268] and GGC [rs17576 - rs3787268 - rs2250889] were associated with *H. pylori*-related DU ($_{adj}OR = 1.95$, $p=0.0009$, $p_{perm}=0.006$ and $_{adj}OR = 1.96$, $p=0.001$, $p_{perm}=0.006$ respectively) (Fig. 1).

*3.2. Functional SNPs*

*Non-synonymous SNPs*. Among the five DU- and *H. pylori*-positive DU-associated polymorphisms of the *MMP-9* gene, three SNPs (rs17576, rs17577, rs2250889) cause amino acid replacements in the encoded protein (Gln279Arg, SIFT predictor value "tolerated"; Arg668Gln, SIFT predictor value "deleterious"; Arg574Pro, SIFT predictor value "tolerated" respectively).

*Regulatory effects*. According to the RegulomeDB, five *MMP9* loci associated with DU and *H. pylori*-positive DU are characterized by significant epigenetic potential. Variant rs17576 *MMP9*, individually associated with both DU and *H. pylori*-positive DU, had RegulomeDB rank 4 (transcription factor (TF) binding + DNAse-1 hypersensitivity peak) and RegulomeDB score 0.61. Another polymorphism, rs17577, had RegulomeDB rank = 2b (TF binding + any motif + DNase Footprint + DNase peak) and RegulomeDB rank score equal 0.79. The other *H. pylori*-positive DU-associated SNPs *MMP9* gene had RegulomeDB rank and RegulomeDB score equals 4-5 and 0.59-0.61 respectively. Data of the HaploReg showed that all five polymorphisms were located in the DNAse hypersensitivity region, four loci had a DNA position in the histone modification region corresponding to enhancer (H3K4me1 and H3K27ac) and promoter (H3K9ac and

H3K4me3) elements in several tissues/organs and the twelve motifs site to the factors of transcription (TFs); two SNPs - in the protein-bound region (Supplementary Table 1). Herewith, alleles of the five SNPs of the *MMP-9* gene, which are risk variants for *H. pylori*-positive DU (Table 4), decrease affinity to the ten TFs and increase affinity to two TFs (Supplementary Table 4).

In addition to the five *H. pylori*-positive DU-associated SNPs, 59 proxy SNPs were analyzed for their functionality (Supplementary Table 5). Seven SNPs (including four missense mutations) were mapped to *MMP9* exons, 24 and 28 loci were in introns and intergenic regions, respectively. All 59 proxy SNPs possessed a significant regulatory potential; several of them had pronounced epigenetic effects (Supplementary Table 5). For example, rs10432735 (linked to rs3918242, $r^2$=0.89) is located in the DNAase I hypersensitive region (21 tissues), in the several protein-bound regions (POL2, ZNF143, YY1, CTCF, ELF1, FOSL1, HMGN3) and a motif DNA regions (Pax-4, ZNF219, Zfp281). Importantly, both the *H. pylori*-positive DU-associated SNPs and their proxies manifested significant epigenetic effects in the target organs of DU in an adult (gastric, small intestine, duodenum mucosa and smooth muscle, mucosa and smooth muscle of stomach) and fetus (small intestine and stomach).

*Expression and splicing QTLs*. According to the GTExportal database, five DU- and *H. pylori*-positive DU-associated *MMP9* loci and 50 proxy SNPs affected the mRNA transcript level of seventeen genes (e.g., *ZNF335, CD40, SLC12A5, MMP9,* etc.) (Supplementary Table 6 and Supplementary Table 7) and might affect alternative splicing of six genes (e.g., *CD40, SLC12A5, PLTP,* etc.) (Supplementary Table 8 and Supplementary Table 9) in more than 20 various tissues/organs.

## 4. Discussion

The present study reports the association of several *MMP-9* polymorphic variants with DU in Caucasians from the central region of Russia: variant allele G rs17576 and haplotype GG[rs17576-rs3787268] of the *MMP-9* gene increased risk for DU ($_{adj}$OR = 1.46-2.09 and $_{adj}$OR =1.60 respectively). Also, five *MMP-9* SNPs increased risk for *H. pylori*-related DU: alleles T of rs3918242 ($_{adj}$OR = 1.95), G of rs17576 ($_{adj}$OR = 1.68-2.81), and A of rs17577 ($_{adj}$OR = 1.96), haplotypes GG[rs17576-rs3787268] ($_{adj}$OR =1.95) and GGC[rs17576-rs3787268-rs2250889] ($_{adj}$OR =1.96).

MMP-9 or gelatinase-B is a type IV collagenase located on chromosome 20q11.2-q13.1. MMP-9 is expressed by a variety of cells including epithelial cells, macrophages, T-cells, etc. (Cui et al., 2017). MMP-9 contributes to the degradation of large substrates such as elastin, various collagen types, fibronectin, laminin, proteoglycan, etc., and may cause ECM breakdown and loss of tissue integrity (Cui et al., 2017). Previous studies demonstrated an important role of ECM degradation in gastrointestinal ulceration (Shahin et al., 2001; Tarnawski, 2005). In addition, DU is associated with infiltration of the duodenal mucosa by monocytes, lymphocytes, plasma cells, neutrophils, which release proinflammatory cytokines (IL-6, IL-1, IL-8, TNF-α) (Lanas and Chan, 2017; Zhang et al., 2013). There is evidence that cytokines released by mucosal macrophages and T cells are also major inducers of MMP production (Pender and MacDonald, 2004).

The literature data about the association of *MMP-9* gene polymorphisms with DU is scarce and contradictory. Shaymardanova et al. (2016) reported the significant association of the AG rs17576 genotype (OR=1.57) in a multiethnic cohort from the Republic of Bashkortostan (Russia). Our results are in general agreement with these findings: allele G of rs17576 *MMP-9* was shown to elevate the risk of DU in the Caucasian population from Central Russia. On the contrary, Yeh et al. (2010) did not find a significant association of rs17576 with *H. pylori*-positive DU in Taiwanese. Similarly, no association with DU was reported for rs3918242 in Chinese (Shan et al., 2010) and in the multiethnic sample from the Republic of Bashkortostan (Shaymardanova et al., 2016), which contradicts the results of the present study. On the other hand, our results are in agreement with those of Shaymardanova et al. (2016) who did not find an association of rs1799750 *MMP-1* with DU. In summary, our study is the first to report the association of the *MMP-9* polymorphic variants with *H. pylori*-infected DU.

According to our results, *MMP-9* SNPs were associated with *H. pylori*-related DU but not with *H. pylori*-negative DU. There is evidence that in *H. pylori*-infected mucosa, epithelial cells are among the major producers of the MMP proteins (Mori et al., 2003). Furthermore, *H. pylori* by itself can stimulate gastric epithelial cells to release MMPs (Bebb et al., 2003). *H. pylori* infections apparently induce the release of MMP-9 by activating nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) (Mori et al., 2003). Antral gastric mucosa of *H. pylori*-infected patients with gastritis manifested a ten-fold increase of the *MMP-9* gene expression and 19-fold higher MMP-9 activity versus the uninfected patients (Bergin et al., 2004). The

MMP-9 serum levels were higher in individuals with *H. pylori*-positive gastritis than that in uninfected controls (Rautelin et al., 2009). A significant proportion of *H. pylori*-infected individuals (80%-85%) develop mild antrum and body gastritis characterized by hypergastrinemia with normal gastric acid levels (Gravina et al., 2018), while about 10%-15% of *H. pylori*-infected patients develop prevalent antrum gastritis characterized by hypergastrinemia and elevated gastric mucosa secretion resulting in duodenal ulceration (Censini et al., 2001).

The associations of the *MMP-9* polymorphisms determined in the present study may be backed by various functional effects of these loci and their proxies as suggested by the results of the *in silico* analysis. The predicted effects include amino acid replacements in the MMP-9 protein, alteration of expression and alternative splicing of several genes (e.g., *CD40, SLC12A5, MMP9, ACOT8, SNX21, PLTP*), etc. Importantly, these effects were predicted in both the target organs of DU (e.g., small intestine, duodenal mucosa and smooth muscle, and the others) and those involved in the pathophysiology of the disease (e.g., the frontal cortex of the brain, pituitary, blood, thyroid, adrenal gland, adipose (visceral and subcutaneous), etc.) (Lanas and Chan, 2017). The functional significance of the loci analyzed in the present study was also predicted for cardiovascular diseases (arterial hypertension) in the studied population of Central Russia (Moskalenko et al., 2019a; Moskalenko et al., 2019b; Moskalenko et al., 2021). Worth noting that some cardiovascular diseases (e.g, coronary artery disease) and body fat-related traits have a significant positive SNP-based genetic correlation with peptic ulcer disease (Wu et al., 2021), so that *MMP-9* gene polymorphisms and their proxies may be among syntropic genes contributing to the pathophysiology of DU and cardiovascular diseases.

**5. Conclusion**

Allele G rs17576 and haplotype GG [rs17576-rs3787268] of the *MMP-9* gene may increase risk for DU ($_{adj}$OR = 1.46-2.09 and $_{adj}$OR =1.60 respectively). Also, five SNPs of the *MMP-9* gene may increase risk for *H. pylori*-positive DU: alleles T of rs3918242 ($_{adj}$OR = 1.95), G of rs17576 ($_{adj}$OR = 1.68-2.81), and A of rs17577 ($_{adj}$OR = 1.96), haplotypes GG[rs17576-rs3787268] ($_{adj}$OR =1.95) and GGC [rs17576-rs3787268-rs2250889] ($_{adj}$OR =1.96). These loci and 59 SNPs linked to them appear to have functionally significant epigenetic effects, amino acid replacements in MMP9, may affect the expression and alternative splicing of 17 and 6 genes respectively.

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**Disclosure of any Conflict of interest**

The authors declare no competing interests.

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