INTRODUCTION Rheumatoid arthritis (RA) is an inflammatory autoimmune disease.\(^1,2\) It leads to chronic inflammation and tissue destruction, especially of the synovial membrane, cartilage, and bone. Environmental, genetic, and epigenetic factors participate simultaneously in the etiopathogenesis of RA. Environmental triggers include smoking, stress, and bacterial and viral infections.\(^3-7\)

In reference to genetic risk factors, mainly the single nucleotide polymorphisms (SNPs) were studied.\(^3\) Epigenetic modifications such as DNA methylation, microRNA expression, long non-coding RNAs, and histone alterations have been reported as elements associated with the regulation of the eukaryotic genome.\(^5-9\) Of these epigenetic mechanisms, the presence of CpG dinucleotide methylation, precisely, the presence of 5-methylcytosine (5-MC), is the widely studied process involved in the regulation of genes in human autoimmune disorders, including RA.\(^1,8\)

The first reports focused on CpG islands, which have been found in the majority of gene promoters. CpG islands contain a large percentage of CpG dinucleotides and have been associated with long and stable gene silencing.\(^10\) More recent publications have shown differences in the patterns of 5-MCs in gene enhancers or in DNA motifs responsible for binding transcription factors, as well as sequences outside of promoters, for example, gene-poor locations and extended transcriptionally inactive regions known as partially methylated domains.\(^10,11\)

Several interferon regulatory factors (IRFs) have been linked to autoimmunity. Among these, IRF5 was initially reported as a transcription factor associated with the defense response to viruses and positive regulation of interferon

**KEY WORDS**
DNA methylation, epigenetic, IRF5, rheumatoid arthritis, single nucleotide polymorphism

**ABSTRACT**

**INTRODUCTION** The interferon regulatory factor 5 (IRF5) gene is implicated in the toll-like receptor signaling pathway and has proinflammatory and chemotactic effects, but its role in the pathogenesis of rheumatoid arthritis (RA) remains unclear. Since the pathobiology of RA shares some similarities with other autoimmune diseases, we tested the hypothesis that RA may be associated with IRF5-related pathways, as has been reported for systemic lupus erythematosus and Sjögren syndrome.

**OBJECTIVES** The aim of the study was to investigate the association between the presence of methylation in the IRF5 promoter and the morbidity and severity of RA as well as with levels of inflammatory markers.

**PATIENTS AND METHODS** A total of 146 unrelated individuals, 122 patients with RA and 24 healthy controls, were enrolled in the study. All RA patients were genotyped with regard to the following polymorphisms in the IRF5 gene: rs10488631, T>C and rs4728142 G>A. The methylation analysis included 52 patients with RA and 24 healthy controls. A quantitative real-time methylation-specific polymerase chain reaction was used to evaluate methylation status.

**RESULTS** We found differences between patients with RA and healthy controls in the methylation pattern of the promoter region. The methylation level was 43.6% lower in RA patients than in controls (median [interquartile range], 0.79 [0.6–1.13] vs 1.4 [1.16–1.66]; \(P = 0.0001\)). Variant rs4728142 G>A was more common in seronegative patients with RA.

**CONCLUSIONS** The methylation profile of the IRF5 promoter may be used as a new potential marker of RA, which is independent of current criteria of disease activity.

**ORIgINAL ARTICLE**

**IRF5 promoter methylation as a new potential marker of rheumatoid arthritis**

Marek Cieśla\(^1\), Bogdan Kolarz\(^1\), Maria Majdan\(^2\), Dorota Darmochwał-Kolarz\(^1\)

\(^1\) Institute of Clinical and Experimental Medicine, Faculty of Medicine, University of Rzeszów, Rzeszów, Poland

\(^2\) Department of Rheumatology and Connective Tissue Diseases, Medical University of Lublin, Lublin, Poland

**Correspondence to:** Marek Cieśla, MSc, Institute of Clinical and Experimental Medicine, Faculty of Medicine, University of Rzeszów, ul. Kapieżu 2a, 35-959 Rzeszów, Poland, phone: +48 17 851 68 51, e-mail: ciesla_marek@wp.pl

**Received:** April 1, 2019.

**Revision accepted:** May 27, 2019.

**Published online:** June 6, 2019.

Pol Arch Intern Med. 2019; 129 (6): 370-376
doi:10.20452/pamw.14863

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production. Recent studies have demonstrated that IRF5 is associated with chronic and acute inflammation and responsible for the development of various autoimmune diseases, including RA, inflammatory bowel disease, systemic lupus erythematosus (SLE), and Sjögren syndrome (SS). According to the Ensembl database, the IRF5 gene is located on chromosome 7 in location 128,937,457–128,950,038 on the forward strand with reference to assembly GRCh38. The gene encodes 15 splice variants and is associated with interferon signaling (α, β, and γ) and immune response pathways, including cytokine signaling. According to the KEGG PATHWAY database, the IRF5 gene is implicated in the toll-like receptor signaling pathway, which is directly involved in RA pathogenesis. IRF5 is responsible for proinflammatory and chemotactic effects. The first is manifested by activation of interleukin (IL) 6, IL-12, IL-1β and tumor necrosis factor α (TNF-α). The chemotactic effect is associated with migration of cells, for example, neutrophils, and the following molecules: IL-8, regulated upon activation normal T-cell expressed and secreted, and macrophage inflammatory protein 1 α and β.

An SNP with rs10488631, T>C, located in the 3' region of the IRF5 gene has been reported to be associated with susceptibility to RA, but there has been no consensus as to whether the polymorphism is associated with seronegative or seropositive patients. IRF5 rs4728142, G>A, located in the promoter region has been reported to be common in SLE, SS, and systemic sclerosis, but there are insufficient data to establish a relationship with RA.

Since the pathobiology of RA shares many similarities with other systemic autoimmune diseases, we hypothesized that RA may be associated with IRF5-related pathways, as previously investigated for SS and SLE. The aim of the current study was to investigate whether the methylation pattern of the IRF5 promoter is associated with the morbidity and severity of RA, as well as to assess the possible relationship between methylation and levels of inflammatory markers. We also aimed to verify the hypothesis that SNPs in IRF5 may be considered genetic risk factors for RA.

PATIENTS AND METHODS

Patients A total of 146 unrelated individuals, 122 patients with RA and 24 healthy controls, were enrolled in the study. From the RA group, 52 patients with high disease activity and remission were selected for an epigenetic study. Disease severity was estimated using the disease activity in 28 joints (DAS28) score based on erythrocyte sedimentation rate (ESR). The Ethics Committee of the Medical University in Lublin approved the study (protocol number KE-0254/7/2016), and all participants provided written informed consent. All individuals were from the local population. Whole blood and serum samples were collected and stored at a temperature of −80°C until analysis.

Methylation analysis DNA was extracted from 200 µl of frozen whole blood according to the manufacturer’s protocol using the GeneMATRiX Quick Blood DNA Purification Kit (Buxr, Gdansk, Poland). A 1-µg DNA sample was converted by sodium bisulfite using the EZ DNA Methylation Gold Kit (Zymo Research, Irvine, California, United States) according to the manufacturer’s recommendations, but the elution volume was increased to 50 µl. DNA was stored at a temperature of −80°C until downstream analysis. The IRF5 promoter region was found in the Eukaryotic Promoter Database (EPD; https://epd.epfl.ch/cgi-bin/get_doc?db=hgEpdNew&format=genome&entry=IRF5_1). Using the EPD motif tool (based on JASPAR core 2018 vertebrates, as part of EPD; for details follow the link above), we found predictive binding sites for transcriptional repressor CTCF (the cutoff P value was 0.0001) at positions −224 and −17 relative to the transcription start site. The primers flanked the region from −308 to −192 bp downstream of TSS and partially covered the predicted binding sites of CTCF. Graphical representation of the studied locus is presented in Supplementary material, Figure S1.

Primers were designed using the MethPrimer Software, version 1.0. Their sequences are presented in Table 1. Specificity of primers was tested in silico using BiSearch e-polymerase chain reaction (PCR) online tool (http://bisearch.ensembl.ims.hu). The ability to amplify specific sequences was evaluated using fully methylated and unmethylated DNA controls (EpiTect PCR Control DNA Set, Qiagen, Hilden, Germany). PCR products were visualized in a 2% agarose gel.

To normalize the input of DNA after bisulfide conversion, the promoter region free of CpG sites in the β-actin gene (ACTB) was amplified. The primers flanked a similar region to that presented by Menigatti et al., but their sequences were manually redesigned. Quantitative real-time methylation-specific PCR (Q-MSP) was used to analyze methylation status. The methylation status of the IRF5 promoter was evaluated using primers complementary to the methylated target sequence (primers named: IRF5_methyl_sense and IRF5_methyl_antisense; detailed characteristics of primers are presented in Table 1). The Q-MSP reaction contained 300 nM of each primer for both IRF5 and ACTB, as well as 2 µl of bisulfide-treated DNA. The reaction was performed using ExiLEN'T SYBR Green master mix (EXIQON, Vedbaek, Denmark) in the COBAS z480 Real Time PCR System under the thermal cycling conditions given in the mix manual, except that the annealing/elongation step was performed at 62°C for 1 minute. Then, the PCR melt curve analysis was performed. All samples were evaluated in triplicate. The efficiency of Q-MSP, for both ACTB and IRF5, was evaluated using the Livak and Schmittgen method. Data were analyzed using the Advanced Relative Quantification module (LightCycler 480 SV, version 1.5.1.62 SP2 – UDF v.2.0.0.0, Roche, Mannheim, Germany)
with the maximum second derivative selected as the calculation model. The results were expressed as a normalized ratio.

**Genotyping of IRF5 polymorphisms** Genotypes of IRF5 rs4728142 (G>A, assay ID, C___2691222_10, Thermo Fisher Scientific, Massachusetts, United States) and rs10488631 (T>C, assay ID, C___2691242_10, Thermo Fisher Scientific) were ascertained by an allelic discrimination test using the TaqMan Genotyping assay and the Endpoint Genotyping module of the COBAS z480 Real-Time PCR System (LightCycler 480 SW, version 1.5.1.62 SP2 – UDF v.2.0.0, Roche).

**Statistical analysis** Depending on the distribution, as assessed by the Shapiro–Wilk test, quantitative values were presented as mean (SD) or median (interquartile range [IQR]). Differences between 2 independent groups were compared by the t test or the Mann–Whitney test. The analysis of variance Kruskal–Wallis test and a post hoc multiple comparison analysis were used to assess the differences between controls and patients divided into groups based on DAS28 scores. The relationship between 2 continuous variables was analyzed by the Spearman rank correlation coefficient. Qualitative parameters were given as numbers with percentage and were evaluated using contingency tables with the χ² test with Yates correction or 1-tailed Fisher exact test to determine the frequency of SNP (seropositive vs seronegative). A P value of less than 0.05 was considered significant. Logistic regression was used to evaluate the odds ratio between tested SNPs and seropositivity. The analysis was performed with the STATISTICA version 13 (StatSoft Inc., Tulsa, Oklahoma, United States). The SNP frequency was also evaluated using Bayesian contingency tables calculated in JASP version 0.8.6 (JASP, Amsterdam, the Netherlands). We tested the hypothesis that seronegative patients (RF-negative) have a higher minor allele frequency. A Bayes factor (BF₁₀) over 3 was considered sufficient to support our hypothesis. Linkage disequilibrium between rs4728142 and rs10488631 was evaluated by LDlink application, version 3.6.29 (Supplementary material, Figure S2).

**RESULTS IRF5 methylation** The methylation analysis included 52 RA patients and 24 healthy controls. Patients with RA were divided into 2 groups based on DAS28 score: patients with high disease activity (DAS28 >5.1; n = 30) and those in remission (DAS28 ≤2.6; n = 22). Detailed characteristics of patients with RA and healthy controls included in the methylation analysis are presented in Table 2.

Overall, patients with RA included in the methylation analysis had a 43.6% lower methylation level than the control group (median [IQR], 0.79 [0.6–1.13] vs 1.4 [1.16–1.66]; P = 0.0001). No difference was observed between patients with RA with high disease activity and those in remission (median [IQR], 0.82 [0.66–1.21] vs 0.76 [0.56–0.97]), but differences were found between the high-activity group and controls, and between the remission group and controls (P = 0.01 and P = 0.001, respectively). The comparison of methylation status between patients with RA and controls is presented in Supplementary material, Figure S3. Methylation in patients with high disease activity and in remission was 41.4% and 45.7% lower, respectively, than in healthy controls and was similar to the results obtained for all patients with RA.

Generally, the groups did not differ according to treatment, except in the high-activity group, where significantly more patients were treated with steroids. The main treatment agent, methotrexate, had a similar frequency in both groups and was used to treat most patients at similar doses (median [IQR], 20 [15–25] vs 20 [12.5–25] mg wk⁻¹; P = 0.41). Methylation status did not correlate with ESR (r = 0.16), C-reactive protein level (CRP; r = 0.06) or DAS28 (r = 0.08). Correlations between the methylation level and

### Table 1: Characteristics of primers and amplicons

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Sequence 5’3’</th>
<th>Amplicon size, bp</th>
<th>Amplicon location with reference to assembly GRCh38 [chromosome:start:end:strand]</th>
<th>Primer complementary to methylated/unmethylated sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>ACTB sense</td>
<td>GGTGGTGATGGAGGAGGTTAG</td>
<td>115</td>
<td>7:5532117:5 532232-1</td>
<td>Independent of methylation status</td>
</tr>
<tr>
<td></td>
<td>ACTB antisense</td>
<td>CTCCTAAAAATTACAAAAACCACAACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRF5</td>
<td>IRF5_methyl_sense</td>
<td>AATTGAGTATTGACGGGAGGTTAC</td>
<td>116</td>
<td>7:128937673:128937789:1</td>
<td>Methylated sequences</td>
</tr>
<tr>
<td></td>
<td>IRF5_methyl_antisense</td>
<td>CTCCCCCCAAATACAAAAACGGACG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IRF5_unmethyl_sense</td>
<td>TGAGATTGATGGGAGGTTACGG</td>
<td>113</td>
<td>7:128937676:128937789:1</td>
<td>Unmethylated sequences</td>
</tr>
<tr>
<td></td>
<td>IRF5_unmethyl_antisense</td>
<td>CTCCCTAAAAATACAAAAACACAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a CpG sites in primer sequence are in bold.

Abbreviations: ACTB, β-actin; IRF5, interferon regulatory factor 5.

---

**Figure S2**

**Figure S3**

**Table S2**
DISCUSSION

The current study is the first to demonstrate that the methylation pattern in IRF5 affects morbidity rates in RA and is not associated with the type of treatment or disease severity based on DAS28 scoring. A genome-wide association study combined with epigenome-wide association study by Liu et al. 30 has shown a potential role of differences in density of DNA methylation as a mediator of genetic risk of RA. These epigenetic changes were presented as differentially methylated positions (DMPs) and were compiled by SNPs DAS28 are presented in Supplementary material, Figure S4.

IRF5 genotyping

Of 122 patients with RA, 82 were rheumatoid factor (RF)-positive and 40 were RF-negative. The groups did not differ with regard to sex and major clinical parameters. As expected, in the RF-positive group, there were more patients who were positive for anticitrullinated protein antibodies. Detailed patient characteristics are presented in Table 3. Genotyping data are presented in Supplementary material, Tables S1–S3.

The variant frequency (both genotypes GA and AA; see also a dominant model in Supplementary material, Table S3) of rs4728142 was substantially lower (BF₁₀ = 3.68; Fisher exact test, P = 0.04) in the RF-positive group (n = 56; 68.3%) than in seronegative patients (n = 34; 85%). The variant frequency (both TC and CC; see also a dominant model in Supplementary material, Table S3) of rs10488631 was different (BF₁₀ = 1.949; Fisher exact test, P = 0.08) in the seropositive group (n = 23; 28%) as compared with seronegative patients (n = 17, 43.5%). The distribution of genotypes was in accordance with the Hardy–Weinberg equilibrium (P = 0.47 and P = 0.62, respectively). Tested SNPs were in linkage equilibrium (D' = 0.7, r² = 0.09; details in Supplementary material, Figure S2).

Individuals included in the methylation analysis (n = 76) had a different methylation level with regards to rs10488631 genotypes. Carriers of TC genotype (n = 22) had about 24% lower methylation than TT homozygous patients (n = 54) (median [IQR], 0.82 [0.55–1.20] vs 1.08 [0.74–1.60]; P = 0.045).

DAS28 are presented in Supplementary material, Figure S4.

IRF5 methylation in rheumatoid arthritis

TABLE 2

Characteristics of the individuals included in the methylation analysis

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RA in high disease activity (n = 30)</th>
<th>RA in remission (n = 22)</th>
<th>Controls (n = 24)</th>
<th>P value (high disease activity vs remission)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y, mean (SD)</td>
<td>53.6 (12.3)</td>
<td>48.7 (12.7)</td>
<td>52.9 (8.6)</td>
<td>0.21</td>
</tr>
<tr>
<td>Female sex, n (%)</td>
<td>22 (73.3)</td>
<td>21 (95.5)</td>
<td>18 (75)</td>
<td>0.09</td>
</tr>
<tr>
<td>Disease duration, y, median (IQR)</td>
<td>10 (2–16)</td>
<td>9.5 (3.5–16)</td>
<td>NA</td>
<td>0.96</td>
</tr>
<tr>
<td>RF-positive, n (%)</td>
<td>23 (76.7)</td>
<td>12 (54.5)</td>
<td>None</td>
<td>0.17</td>
</tr>
<tr>
<td>ACPA-positive, n (%)</td>
<td>29 (96.7)</td>
<td>20 (90.9)</td>
<td>None</td>
<td>0.78</td>
</tr>
<tr>
<td>ESR, mm/h, median (IQR)</td>
<td>54 (28–67)</td>
<td>7 (2–11.5)</td>
<td>15 (7–19)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CRP, mg/l, median (IQR)</td>
<td>15.72 (8.54–27.2)</td>
<td>0.45 (0.13–4.09)</td>
<td>0.58 (0.19–1.97)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VAS PGA, median (IQR)</td>
<td>67.5 (52–73)</td>
<td>8 (3–9.5)</td>
<td>NA</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VAS PhGA, median (IQR)</td>
<td>59 (49–70)</td>
<td>5 (1.5–7)</td>
<td>NA</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At least on methotrexate</td>
<td>23 (76.7)</td>
<td>15 (68.2)</td>
<td>NA</td>
<td>0.72</td>
</tr>
<tr>
<td>At least on biologics</td>
<td>7 (23.3)</td>
<td>11 (50)</td>
<td>NA</td>
<td>0.09</td>
</tr>
<tr>
<td>At least on steroids</td>
<td>23 (76.7)</td>
<td>7 (31.8)</td>
<td>NA</td>
<td>0.003</td>
</tr>
<tr>
<td>Single-line therapy</td>
<td>4 (13.3)</td>
<td>7 (31.8)</td>
<td>NA</td>
<td>0.2</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>2 (6.7)</td>
<td>3 (13.6)</td>
<td>NA</td>
<td>0.71</td>
</tr>
<tr>
<td>Biologics</td>
<td>0</td>
<td>1 (4.5)</td>
<td>NA</td>
<td>0.88</td>
</tr>
<tr>
<td>Steroids</td>
<td>1 (3.3)</td>
<td>0</td>
<td>NA</td>
<td>0.88</td>
</tr>
<tr>
<td>Other cDMARD</td>
<td>1 (3.3)</td>
<td>3 (13.6)</td>
<td>NA</td>
<td>0.39</td>
</tr>
<tr>
<td>Double-line therapy</td>
<td>17 (56.7)</td>
<td>10 (45.5)</td>
<td>NA</td>
<td>0.6</td>
</tr>
<tr>
<td>Methotrexate + steroids</td>
<td>13 (43.3)</td>
<td>1 (4.5)</td>
<td>NA</td>
<td>0.01</td>
</tr>
<tr>
<td>Methotrexate + biologics</td>
<td>2 (6.7)</td>
<td>4 (18.2)</td>
<td>NA</td>
<td>0.4</td>
</tr>
<tr>
<td>Steroids + biologics</td>
<td>0</td>
<td>1 (4.5)</td>
<td>NA</td>
<td>0.88</td>
</tr>
<tr>
<td>Other</td>
<td>2 (6.7)</td>
<td>4 (18.2)</td>
<td>NA</td>
<td>0.4</td>
</tr>
<tr>
<td>Triple-line therapy</td>
<td>9 (30)</td>
<td>5 (22.7)</td>
<td>NA</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Abbreviations: ACPA, anticitrullinated protein antibodies; CRP, C-reactive protein; cDMARD, classic disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; IQR, interquartile range; NA, not applicable; RA, rheumatoid arthritis; RF, rheumatoid factor; VAS PhGA, visual analog scale physician global assessment; VAS PGA, visual analog scale patient global assessment.
TABLE 2

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RA-positive (n = 82)</th>
<th>RA-negative (n = 40)</th>
<th>P value (RF-positive vs RF-negative)</th>
<th>RA overall (n = 122)</th>
<th>Controls (n = 24)</th>
<th>P value (RA overall vs controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y, mean (SD)</td>
<td>53.9 (12.4)</td>
<td>48.7 (11.5)</td>
<td>0.02</td>
<td>52.2 (2.3)</td>
<td>52.9 (8.6)</td>
<td>0.99</td>
</tr>
<tr>
<td>Female sex, n (%)</td>
<td>69 (84.1)</td>
<td>34 (85)</td>
<td>0.89</td>
<td>103 (84.4)</td>
<td>18 (75)</td>
<td>0.41</td>
</tr>
<tr>
<td>Disease duration, y, median (IQR)</td>
<td>11 (4–19)</td>
<td>10 (4–16)</td>
<td>0.66</td>
<td>10 (3–16)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>RF-positive, n (%)</td>
<td>82 (100)</td>
<td>0</td>
<td>NA</td>
<td>82 (67.2)</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>ACPA-positive, n (%)</td>
<td>78 (95.1)</td>
<td>26 (65)</td>
<td>&lt;0.0001</td>
<td>104 (85.2)</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>ESR, mm/h, median (IQR)</td>
<td>27 (13–51)</td>
<td>21 (10.5–35)</td>
<td>0.08</td>
<td>26 (8–57.5)</td>
<td>15 (7–19)</td>
<td>0.001</td>
</tr>
<tr>
<td>DAS28, n (%)</td>
<td>4.1 (1.5)</td>
<td>3.7 (1.7)</td>
<td>0.22</td>
<td>3.9 (1.6)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CRP, mg/l, median (IQR)</td>
<td>8.2 (1.39–17.47)</td>
<td>3.41 (0.47–15.72)</td>
<td>0.22</td>
<td>8.54 (0.53–19.12)</td>
<td>0.58 (0.19–1.97)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VAS PGA, median (IQR)</td>
<td>28 (9–62)</td>
<td>27 (10.5–54)</td>
<td>0.94</td>
<td>27 (9–61)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>VAS PhGA, median (IQR)</td>
<td>20 (5–50)</td>
<td>20 (10.5–44.5)</td>
<td>0.62</td>
<td>20 (7–49)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: DAS28, disease activity score; others, see Table 2

Associated with the RA genotype. These DMP–SNP pairs mainly involved the major histocompatibility complex region. There were 3 potential DMPs associated with the RA phenotype detected in the IRF5 gene; however, the mentioned DMPs did not have associated SNPs, thus no DMP–SNP pairs were created. Therefore, the differences in DNA methylation in IRF5 were not considered as a mediator of genetic risk of RA.30 The positions of 2 DMPs were located near the region considered in our study, but about 71 bp and 180 bp upstream of our target sequence. The study by Liu et al confirmed that the choice of this region, which is a possible binding site for the transcription factor CTCF, was appropriate. In turn, based on the hypothesis that different density in DNA methylation in well-defined regulatory regions, for example, transcriptional factor binding sites or gene enhancers, is important to gene expression,10,11 we believe that our study region may be involved in IRF5 regulation.

Decreased methylation in patients with RA may lead to overexpression of the IRF5 gene, which is responsible for the inflammatory response.12,13 In our study, patients with high disease activity and those in remission showed no differences in methylation. Moreover, no correlations were found between methylation and DAS28, CRP, or ESR. We expected that lower methylation, and thus potentially higher gene expression, would be found in the group with disease exacerbation. When formulating this hypothesis, we focused on the DAS28 score of calculating disease activity. One of its components is CRP or ESR. CRP is an acute phase protein, while ESR is a laboratory parameter dependent on acute phase proteins. Most of these proteins are stimulated by IL-6.31 IRF5 is involved in the production of cytokines such as IL-6, TNF-α, IL-12, and chemokines.32 We assumed that a low DAS28 score in remission might be associated with a lower production of proinflammatory molecules, and thus higher methylation of IRF5, but this did not prove to be true.

We have demonstrated for the first time that epigenetic activity of the IRF5 promoter is independent of disease activity. Widely used drugs such as methotrexate or biologics may inhibit cytokines and thus acute phase protein secretion, and may also affect DAS28 score.33–35 Furthermore, nonspecific agents (eg, nonsteroidal anti-inflammatory drugs or sulfasalazine) and specific monoclonal antibodies (eg, anti-TNF-α) may inhibit chemokine production.34–36 There have been reports focusing on the associations between treatment and changes in the methylation profile.22,27 Wan et al showed that methylation of CpGs decreased when systemic steroid therapy was prescribed in a group of patients with chronic obstructive pulmonary disease. Interestingly, one of the reported genes with reduced methylation was another IRF family member, IRF7, which is also involved in the toll-like receptor signaling pathway27 and may suggest that the methylation for this IRF family is a key regulatory mechanism. In contrast, Imgenberg-Kreuz et al22 reported no association between CpG methylation and treatment with glucocorticoids in SLE. In our study, patients with RA with high disease activity were more often treated with steroids, in contrast to the remission group (Table 2). The methylation levels were similar in both groups, which indicates that steroids had no influence on the methylation pattern. In addition, we suggest that the drugs used for RA treatment do not interfere with epigenetic deregulation of IRF5. In our study, both high-activity and remission groups were in general treated similarly, but neither group achieved a methylation level similar to that in the control group. The lack of differences in the methylation pattern in patients with RA indicates that the IRF5 gene is continually active in these patients, in contrast with healthy controls, which might contribute to disease exacerbation, especially from remission to an active stage. In this context, IRF5-targeting drugs may potentially find a place in RA treatment. IRF5 could be inhibited...
by genetic knockouts or posttranslational modifications, such as tyrosine kinases. Although most of these strategies were tested in animal or cell models, further laboratory and clinical research is needed,12,38-40 as a focus on IRF5-related pathways may be important for a better understanding of inflammatory processes. We believe that changes in IRF5 promoter methylation may be responsible not only for morbidity of RA but also of other chronic autoimmune diseases, as similar findings have been found in patients with SLE and SS.5,21,41

The test material was a limitation in our methylation analysis. We evaluated frozen whole blood, including all nucleus cells, while the analysis of the individual populations such as T and B cells, monocytes, and neutrophils or systemically circulating cells may help understand the role of IRF5 in RA. There are models that estimate the proportions of the leukocyte subpopulations that may be useful to better understand the methylation status. Mentioned models include direct or indirect approaches for estimation of cell proportions. A direct approach is based on a total blood count; however, in our study these data were unavailable. The indirect methods mainly include prediction of cell-subtypes percentage based on gene expression or methylation with the use of purified cells. This method was also unavailable in our study because we used frozen material, no other samples were available, for example the sorted subpopulations or at least the peripheral blood mononuclear cells.42 On the other hand, blood is the easiest material to obtain and store. This means that IRF5 methylation in whole blood can be used as a new molecular marker of RA. Despite the fact that most methylation studies used cell-type correction, there were some studies that did not use it and provided some interesting data.8

Our findings may be considered as preliminary, and further studies in a larger cohort should be performed to replicate and expand the results.

It is important to mention IRF5 polymorphisms and their association with RA. In previous reports, SNPs in IRF5 have mainly been associated with seronegative patients in various European populations.18,43,44 As regards the SNPs studied here, risk allele C in rs10488631 was reported to enhance the IRF5 expression and thereby promote inflammation, and was associated with seronegative patients.18 Contrary to these findings, Vernerova et al18 showed that rs10488631 is associated with seropositive patients. In our study, we found no arguments to support the hypothesis that this variant may be associated with RF-positive or RF-negative patients. The other variant with rs4728142 was reported to be associated with RA autoantibody positivity.23 Stahl et al23 also analyzed both SNPs (rs4728142 and rs10488631) and found that their effects were independent. In contrast, Wang et al19 reported that the variants located on 5' and 3' are associated with, and are equally responsible for, the development of RA.

To the best of our knowledge, this study is the first to show that rs4728142 may be associated with RF-negative patients with RA, which is in line with the previously reported trend between seronegative patients and location of SNPs at the 5' site of IRF5.18 Furthermore, our observations support the hypothesis that risk factors for RA are mostly located at the 5' site of the IRF5 gene, and further studies of the interactions between those SNPs and epigenetic changes in the promoter region may provide new information on susceptibility to, and morbidity of, RA. The analysis of our genotyping study was underpowered due to the small group size.

As was described above, there were other studies focused on the influence of SNP on methylation levels.39,41 Imingenberg-Kreuz et al41 demonstrated an association between methylation density and 3 SNPs (rs4728142, rs17339836, rs10954213) in IRF5-TNPO locus in patients with SS. However, we did not find a similar association for rs4728142, but carriers of TC genotype in rs10488631 had lower methylation levels. The limitation of these findings is the lack of CC homozygotes and a small group size, but our data and the previous reports18,38 may suggest that methylation is associated with genotype and may be considered a mediator of genetic risk, as was previously suggested by Liu et al.39

To conclude, the epigenetic profile of the IRF5 promoter may be used as a new potential marker of RA, which is independent of disease activity, treatment, and inflammatory markers such as ESR or CRP, but may be related to SNPs. The inclusion of epigenetic as well as genetic (SNPs) markers among classification criteria may contribute to an earlier recognition of autoimmune disorders (eg, in the predisease stage) and may play a potential role in targeted therapy.

SUPPLEMENTARY MATERIAL
Supplementary material is available at www.mp.pl/pamw.

ARTICLE INFORMATION

CONTRIBUTION STATEMENT MC conceived the concept of the study, designed and performed the study, analyzed the data, and drafted the manuscript. BK recruited the patients, collected the samples, and analyzed the data. BK, MM, and SD-K critically reviewed the final version of the manuscript.

CONFLICT OF INTEREST None declared.

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