ORIGINAL ARTICLE

Associations between single-nucleotide polymorphisms of *RFC-1*, *GGH*, *MTHFR*, *TYMS*, and *TCII* genes and the efficacy and toxicity of methotrexate treatment in patients with rheumatoid arthritis

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KEY WORDS

gene polymorphism, methotrexate, rheumatoid arthritis

ABSTRACT

INTRODUCTION The differences in drug efficacy and adverse reactions may be caused by genetic variations in drug metabolism between individuals.

OBJECTIVES The aim of the study was to evaluate the effect of gene polymorphisms on the efficacy of therapy and side effects in patients with rheumatoid arthritis (RA) treated with methotrexate (MTX).

PATIENTS AND METHODS A total of 273 Caucasian patients with RA were treated with MTX for at least 6 months or stopped MTX because of adverse effects. Seven polymorphisms (*RFC-1* c.80G>A, *GGH* c.-401C>T, *MTHFR* c.1298A>C and c.677C>T, *TYMS 2R/3R*, *TYMS* 6-bp deletion, and *TC*II c.593T>C) were examined for their effects on MTX efficacy and toxicity. Genomic DNA was obtained from peripheral blood leukocytes.

RESULTS Of all patients, 53% reported some adverse effects during at least 1 visit, which led to MTX withdrawal in 17% of the patients. Adverse effects were more frequent in patients with the *MTHFR* 677T allele than in those with the 677CC genotype (odds ratio [OR], 1.97; $P = 0.01$) and in those with the *GGH* 401CC genotype than in those with the *GGH* 401CT and TT genotypes (OR, 3.8; $P = 0.05$). Furthermore, the *MTHFR* 677T allele was associated with increased activity of aminotransferases (OR, 3.4; $P = 0.02$). MTX-related hepatotoxicity and alopecia were more common in patients with the *RFC-1* 80AA genotype (OR, 3.5, $P = 0.01$; OR, 2.4, $P = 0.04$; respectively). A more rapid positive response to MTX therapy was demonstrated in *MTHFR* 677CC homozygotes (OR, 3.4; $P = 0.001$). There were no other associations between single-nucleotide polymorphisms and the efficacy of MTX treatment.

CONCLUSIONS The *MTHFR* 677CC and *GGH* 401TT and CT genotypes were associated with a reduction in the number of MTX-related adverse events. Future allele and genotype analyses may help identify the subsets of RA patients with an increased risk of adverse effects.

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INTRODUCTION Despite the fact that numerous new drugs have been introduced to the market, methotrexate (MTX) remains the gold standard in the therapy of rheumatoid arthritis (RA). Good response to therapy is achieved in 35% to 65% of the patients, and in 10% to 30% of the patients, MTX therapy is discontinued because of adverse effects.[1,2] Genetic predisposition may play a role in therapy individualization. There are numerous potential enzymes and transport proteins participating in MTX transport to the cell and in its metabolism and cell excretion. The main enzymes

and the MTX metabolic pathway are presented in FIGURE 1. MTX is actively transported to the cell by a protein called reduced folate carrier (RFC-1). RFC-1 polymorphisms may lead to alteration of the enzyme's activity.[3]

Inside the cell, MTX undergoes polyglutamination with folylpolyglutamyl synthetase (FPGS). Polyglutamyl MTX forms (MTXPG) are less rapidly expelled from the cell; therefore, MTX's half-time inside the cell is extended. Reversion of polyglutamination reaction, requiring the participation of the folylpolygammaglutamyl hydrolase enzyme (GGH), facilitates MTX removal from the cell. High GGH concentrations were associated with resistance to MTX.[4] In recent years, several single-nucleotide polymorphisms within the *GGH* gene that may affect GGH activity have been identified. The polymorphism c.-401C>T (rs3758149) in the *GGH* promoter region increases GGH expression and may affect intercellular MTXPG concentrations.[5]

Enzymes affected by MTX are, among others, thymidylate synthase (TYMS) and methylenetetrahydrofolate reductase (MTHFR). TYMS is one of the basic enzymes necessary for DNA synthesis. MTXPG, by inhibiting TYMS activity, contributes to a reduction of the folate pool and inhibition of cell proliferation.[6] Three polymorphisms in the *TYMS* gene have been studied so far: a varied number of repetitions of a 28-nucleotide zone, a G>C exchange in the 12th nucleotide in the second repetition of the 3R allele, and a 6-bp deletion in the 3´UTR (3´UTR 6-bp ins/del, rs16430). TYMS enzymatic activity rises with an increasing number of repeated 28-bp sequences.[7]

Another mechanism of MTX's action is the indirect inhibition of folate metabolism by methylenetetrahydrofolate reductase blocking. MTX affects the general pool of intercellular folates and thus affects the enzyme. Martin et al.[8] analyzed the DNA of 240 individuals of various races and demonstrated the presence of 62 single-nucleotide polymorphisms. The best-known polymorphism, c.677C>T (rs1801133) of the *MTHFR* gene, leads to a replacement of alanine by valine in codon 222 (pAla222Val). This results in the formation of a thermo-labile MTHFR variant with reduced activity of the enzyme. The c.677C>T polymorphism may lead to a reduction of 5-methyltetrahydrofolate concentrations and accumulation of 5,10-methylenetetrahydrofolate resulting in hyperhomocysteinemia.[9,10] The second polymorphism, c.1298A>C (rs1801131) of *MTHFR*, leads to the substitution of alanine for glutamine at codon 429 (p.Glu420Ala).[11] The change causes a reduction in the enzyme's activity by approximately 40%. Data on the effect of polymorphisms in the *MTHFR* gene on the efficacy of MTX therapy and the development of adverse effects during RA therapy are ambiguous.[12,13]

Hyperhomocysteinemia may be the result of folic acid and vitamin B_{12} deficiency, among others. Approximately 25% of vitamin B_{12} is bound to transcobalamin II (TCII). Improper cobalamin binding to TCII negatively affects the process of cobalamin transport to cells and becomes one of the causes of vitamin B_{12} deficiency. Several polymorphisms have been described within the gene encoding TC. These changes may affect vitamin B_{12} binding to TC or alter the recognition of holo-TC by TC receptors on target cells (FIGURE 1).[14]

A number of studies on the polymorphisms of genes involved in the MTX metabolism have focused on the simultaneous evaluation of multiple polymorphisms in an attempt to determine a genetic profile that would be characterized by the highest efficacy and lowest incidence of adverse effects.[15-17]

The aim of this study was to determine the effect of polymorphisms in the *MTHFR*, *RFC-1*, *TYMS*, *GGH,* and *TC* genes on the efficacy of MTX therapy and related adverse effects in patients with RA.

PATIENTS AND METHODS A total of 273 patients with RA were included in the study. All patients were treated with MTX for at least 6 months or the drug was discontinued earlier because of adverse effects. The characteristics of the patients are presented in TABLE 1. Thirty-three patients were excluded from the analysis because they missed some of the follow-up visits and the data regarding the activity of the disease and adverse effects were incomplete. The final analysis included 240 patients: 196 women (82%) and 44 men (18%). During the follow-up period, 82 patients (34%) discontinued the therapy with MTX: 40 (17%) because of side effects and 42 (17.5%) because of no improvement.

The following inclusion criteria were applied: 1) consent to participate in the study; 2) confirmed RA based on the criteria of the American Rheumatology Association; 3) active form of the disease: erythrocyte sedimentation rate (ESR), >30 mm/h, or C-reactive protein (CRP), >1.5 mg/dl, or both; minimum 4 painful and 3 swollen joints; Disease Activity Score 28 (DAS28), >3.2; 4) age over 18 years; 5) women and men with reproductive potential had to use reliable contraception; 6) use of nonsteroidal anti-inflammatory drugs and corticosteroids in stable doses allowed.

The exclusion criteria were as follows: 1) pregnancy or breastfeeding; 2) coexistence of other systemic diseases of connective tissue besides RA; 3) clinically significant impairment of hepatic and renal function; 4) alcohol abuse; 5) infection with hepatotropic viruses; 6) infections resistant to therapy; 7) ongoing history of cancer if no cure was achieved; 8) uncontrolled diabetes; and 9) patient unwilling or unable to cooperate.

The study was approved by the Ethics Committee at the Wroclaw Medical University.

MTX was administered orally at an initial dose of 10 to 15 mg once a week. If at least a moderate improvement was not achieved and there were no significant adverse effects, the dose was titrated to a maximum level of 25 mg/wk.

Clinical and biochemical activity of the disease was evaluated in the study. Clinical evaluation

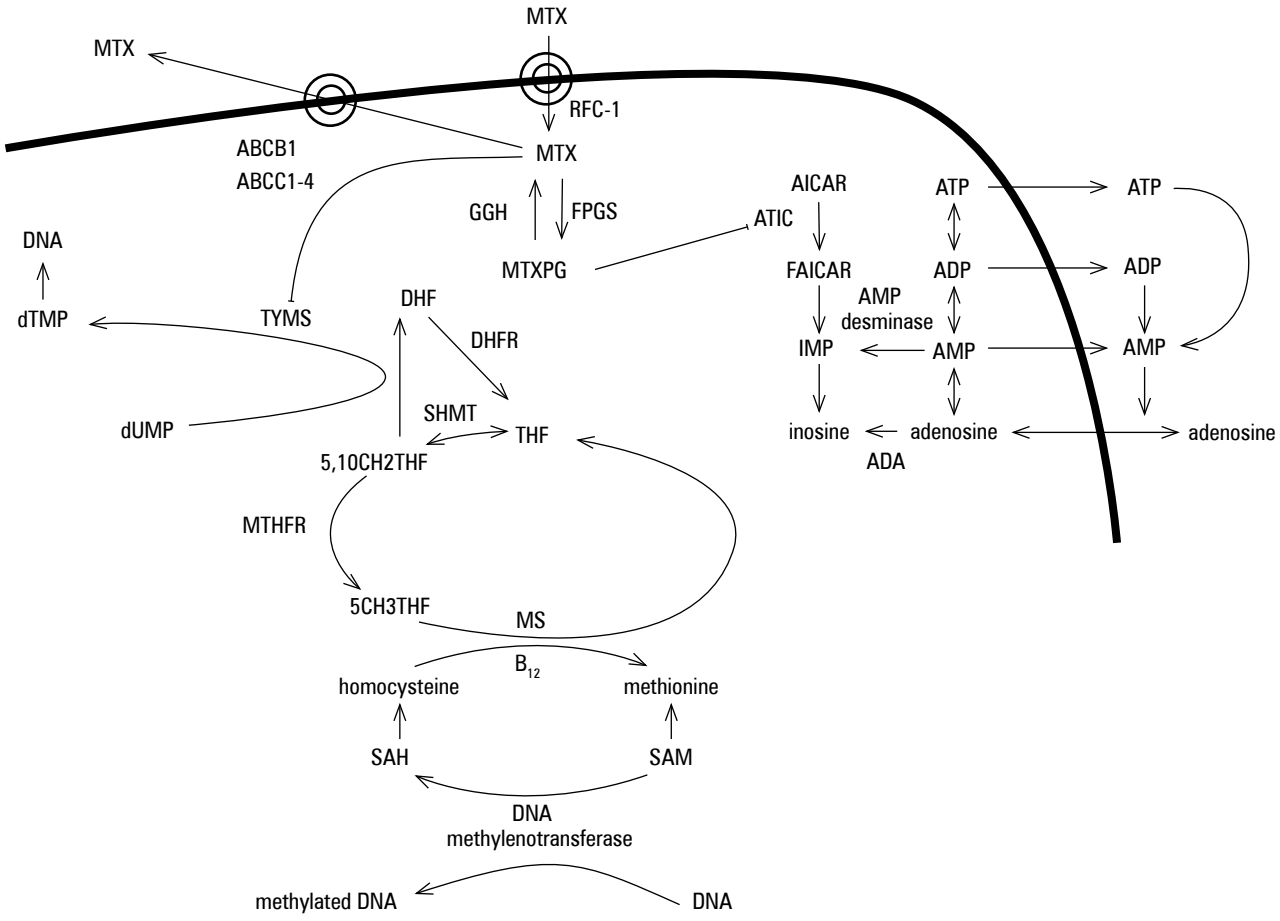

FIGURE 1 Cellular metabolism of methotrexate

Abbreviations: 5,10-CH2-THF, 5,10-methylene tetrahydrofolate; 5-CH3-THF, 5-methyl tetrahydrofolate; ABCB1, ABCC1-4, ABC transporters; ADA, adenosine deaminase; ADP, adenosine diphosphate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMP, adenosine monophosphate; ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; ATP, adenosine triphosphate; DHF, dihydrofolate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; DHFR: dihydrofolate reductase; FAICAR, 10-formyl 5-aminoimidazole-4-carboxamide ribonucleotide; FH2, dihydrofolate; FPGS, folylpolyglutamate synthase; GGH, γ-glutamyl hydrolase; IMP, inosine monophosphate; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; MTX, methotrexate; MTX-PG, methotrexate polyglutamate; RFC-1, reduced follate carrier 1; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TYMS, thymidylate synthetase

was based on a medical history, number of painful and swollen joints, pain intensity assessed by the patient on a 100-mm visual analogue scale, disease activity assessed by the patient and physician as well as the results of laboratory tests (ESR, CRP). The parameters allowed us to determine the improvement according to the criteria of the European League Against Rheumatism (EULAR). Based on the efficacy of the therapy, patients were divided into 3 groups: group A, no response (DAS28 ≤0.6); group B, moderate efficacy of the therapy (0.6 <DAS28 ≤1.2); group C, good response to the therapy (DAS28 >1.2).

Moreover, in some analyses, a comparison was made between patients with no response (nonresponders) and those who had moderate or good response (responders), which was due to the limited number of the patients in each group (TABLE 1).

Safety of MTX was analyzed on the basis of a medical history, physical examination, and selected laboratory tests. The clinical and laboratory tests were completed before the start of the therapy and at 2, 4, and 6 months of the follow-up.

Considering adverse effects, the patients were divided into 3 groups: patients with no adverse effects; with mild and moderate adverse effects (1st and 2nd degree); and with severe adverse effects and effects resulting in the discontinuation of the MTX therapy (3rd and 4th degree).

Adverse effects were divided into those associated with the gastrointestinal tract, respiratory and nervous system, increased activity of aminotransferases, hematological complications, hair loss, infections, and others. All patients received from 5 to 15 mg of folic acid 24 to 48 h after MTX administration.

A total of 7 polymorphisms were evaluated. Six of them were associated with MTX metabolism and mechanism of action (*MTHFR* c.677C>T [rs1801133] and c.1298A>C [rs1801131] *RFC-1* c.80A>G [rs1051266], *TYMS* 2R/3R [rs45445694] and ins/del (rs16430), and *GGH* c.-401C>T [rs3758149]) and 1 was associated with TC (*TC* c.593T>C).

TABLE 1 Baseline characteristics of responders and nonresponders to methotrexate therapy

Characteristics	Responders (n = 183)	Nonresponders (n = 57)	*P* value
age, y	54 ±10	50 ±11	0.01
female sex	80	82	0.66
DAS28	4.2 ±0.8	4.7 ±0.6	0.001
seropositive patients (RF)	60	67	0.37
MTX dose	15 (12.5–25)	15 (12.5–25)	1
MTX as the first DMARD	81	68	0.04
supplementation with folic acid	100	100	1
concomitant glucocorticosteroids	66	63	0.68
concomitant NSAIDs	76	74	0.76
RA period acc. to Steinbrocker I+II/III+IV	46/54	50/50	0.60
smoking	39	35	0.59

Data are presented as mean ± standard deviation, percentage, or mean (minimum–maximum)

Abbreviations: DAS28, 28-joint count Disease Activity Score; DMARD, disease-modifying antirheumatic drug; MTX, methotrexate; NSAIDs, nonsteroidal anti-inflammatory drugs; RA, rheumatoid arthritis; RF, rheumatoid factor

Genomic DNA was extracted from peripheral blood leukocytes with the Blood Mini Kit (A&A Biotechnology, Gdynia, Poland). The *MTHFR* c.677C>T, *MTHFR* c.1298A>C, *TYMS* 3`UTR1494insdel6bp, *RFC-1*, and *GGH* polymorphisms were analyzed by polymerase chain reaction (PCR)–restriction fragment length polymorphism. Amplification of DNA was performed in a PCR thermal cycler (MJ Research, Quebec, Canada). Digestion of the PCR products was conducted by appropriate restriction enzymes, namely, HinfI, MboII, DraiI, CfoI, and BslI (Fermentas, Vilnius, Lithuania), respectively. The polymorphisms in the *TCII* were analyzed by the PCR-amplification refractory mutation system. PCR products were analyzed by gel electrophoresis on agarose gel (2.5%) and visualized with SybrGREEN (Lonza, Rockland, Maine, United States).

To characterize and compare responders and nonresponders, descriptive statistics were calculated for age, duration of the disease, and evaluation of the inflammatory process activity using DAS28. The statistical significance of differences between the mean values or fraction of the parameters was determined with the *t* test for independent samples or the χ^2 test.

Relationships between: 1) polymorphisms (polymorphism combinations) and efficacy of the therapy; 2) polymorphisms (polymorphism combinations) and adverse effects; and 3) polymorphisms and duration of RA in years before the introduction of MTX, use of MTX as the first/next disease-modifying antirheumatic drugs (DMARDs), RA activity, rapid reaction to MTX, RA grade, MTX discontinuation, use of corticosteroids, and smoking were tested with the χ^2 test. The odds ratio (OR) and *P* value were calculated for 4-field tables.

RESULTS

Therapy efficacy A good response to therapy according to the EULAR criteria was observed at 2 months in 67 patients (28%) and at 6 months in 79 (33%). A moderate improvement was demonstrated at 2 and 6 months in 101 patients (42%) and 104 patients (43%), respectively, and no improvement or deterioration was demonstrated in 72 patients (30%) and 57 patients (24%), respectively. No effect of sex, the period of RA progression, use of corticosteroids, or smoking on the efficacy of the therapy was demonstrated. However, higher efficacy of the therapy was demonstrated in patients with a less active course of the disease at the beginning of the therapy ($P < 0.01$) and if MTX was used as the first DMARD ($P < 0.01$) (TABLE 1).

Adverse effects in patients treated with methotrexate Adverse effects occurred in 128 patients (53%), and in 40 of them (17%), they resulted in the discontinuation of the therapy (TABLE 2). We observed that adverse effects were significantly more common in patients with a more active course of the disease, in whom MTX was used as a second DMARD, and hair loss was more common among older patients. Serious adverse effects were significantly more common when MTX was introduced in patients with RA lasting for a longer period of time ($P < 0.01$). Patients were divided into 2 subgroups with regard to disease activity: those with DAS28 >4 and with DAS28 <4. Adverse effects were observed significantly more often in patients with more active RA at the start of MTX therapy (OR, 6.96; $P = 0.002$).

Gene polymorphisms and efficacy of therapy and adverse effects The genotype distribution of the MTHFRc.677C>T (rs1801133) and c.1298A>C (rs1801131), RFC-1 c.80A>G, TYMS 2R/3R (rs45455694) and 6bp ins/del (rs16430), GGH c.-401C>T (rs3758149), and TC c.593T>C polymorphisms was determined in 240 patients.

Compliance with the Hardy–Weinberg equilibrium (HWE) was shown for *MTHFR*c.677C>T ($P = 0.93$), *TYMS* 2R/3R ($P = 0.06$), *TYMS* 6bp ins/del ($P = 0.97$), *RFC-1* c.80A>G ($P = 0.93$) and the *GGH* c.-401C>T ($P = 0.45$). A lack of

TABLE 2 Adverse effects during methotrexate therapy

Type of an adverse effect	Total	Withdrawal of therapy
gastrointestinal	80 (33.0)	23 (9.5)
nausea and vomiting	50	15
abdominal pain	27	6
diarrhea	3	2
increased aminotransferase activity	18 (7.5)	5 (2.0)
hematological	8 (3.3)	4 (1.6)
anemia	3	0
leucopenia	2	2
trombocytopenia	2	1
pancytopenia	1	1
pulmonary	7 (2.9)	6 (2.5)
alopecia	28 (12.0)	0 (0)
fatigue, headache, myalgia	34 (14.0)	0 (0)
infections	9 (3.8)	1 (0.4)
skin reactions	2 (0.8)	1 (0.4)
MTX-associated nodulosis	2 (0.8)	0 (0)
total number of patients with adverse effects (number of patients)	128 (53)[a]	40 (17)

Data are presented as number (percentage) of patients.

a the number of patients is not equal to the number of adverse effects because some patients experienced more than 1 adverse effect (number of patients: 128; number of adverse effects: 188)

Abbreviations: see TABLE 1

compliance with HWE was observed for *MTHFR*c. 1298A>C (P <0.001) and *TC* c.593T>C (P <0.001).

The study showed no effect of the polymorphisms on the initial activity of the disease and age at the onset of the disease. A more rapid positive response to MTX therapy was demonstrated in *MTHFR* 677CC homozygotes (OR, 3.4; P = 0.001), and a higher number of patients in whom the therapy was discontinued because of adverse effects was observed among patients with the T allele (OR, 2.5; P = 0.02). There was no other association between the 7 single-nucleotide polymorphisms and the efficacy of MTX treatment.

The incidence of adverse effects in the study group in relation to individual polymorphisms was also analyzed (TABLE 3). The effect of combinations of the *RFC-1, TYMS, GGH*, and *MTHFR* genotypes on the incidence of adverse effects is presented in TABLE 4. Adverse effects occurred significantly more often in patients with genotypes *MTHFR* 677TT and 677CT than in those with 677CC (61% and 45%, respectively; OR, 1.97; P <0.01) (TABLE 4). A higher incidence of adverse effects was also found in the group of patients with genotype *GGH* 401CC compared with those with genotypes *GGH* 401CT and *GGH* 401TT (79% and 52%, respectively; OR, 3.8; P = 0.053) and in patients with at least 1 *TYMS* 3R allele compared with other patients (56% and 40%, respectively; OR, 1.97; P = 0.056) (TABLE 4).

TABLE 3 Effect of genotypes on the incidence of adverse effects

Polymorphism	Genotype	Adverse effects			
		no, n (%)	yes, n (%)	P value χ^2	OR (95% CI); P value
MTHFR 677	CC	62 (55)	50 (45)	0.052	1.97 (1.18–3.31); 0.01 CT+TT vs CC
	CT	39 (38)	63 (62)		
	TT	10 (40)	15 (60)		
MTHFR 1298	AA	63 (46)	74 (54)	0.95	0.94 (0.56–1.57); 0.81 AC+CC vs AA
	AC	47 (46)	53 (53)		
	CC	1	0		
TYMS 2R/3R	3R/3R	30 (48)	33 (52)	0.36	1.97 (0.97–4.02);0.056 3R+2R/3R vs 2R
	2R/3R	56 (42)	78 (58)		
	2R/ 2R	23 (60)	15 (40)		
TYMS ins/del	ins/ins	64 (49)	66 (51)	0.81	1.26 (0.76–2.12); 0.37 ins/del+del vs ins
	ins/del	39 (43)	51 (57)		
	del/del	7 (44)	9 (56)		
RFC-1 80	GG	29 (40)	43 (60)	0.64	0.70 (0.40–1.22); 0.21 GA+AA vs GG
	GA	62 (51)	59 (49)		
	AA	19 (43)	25 (57)		
GGH 401	TT	56 (46)	66 (54)	0.11	3.84 (0.92–12.46); 0.053 CC vs TT+CT
	CT	52 (50)	51 (50)		
	CC	3 (21)	11 (79)		
TC 593	MM	49 (41)	71 (59)	0.08	1.58 (0.94–2.63); 0.08 MM vs MT
	MT	60 (52)	55 (48)		
	TT	0	0		

Abbreviations: CI, confidence interval; OR, odds ratio

TABLE 4 Effect of combinations of *RFC-1, TYMS, GGH*, and *MTHFR* genotypes on the incidence of adverse effects

Adverse effects	Genotypes		OR (95% CI)	*P* value
RFC-1, n (%)				
	AA	**GA+GG**		
↑ aminotransferase activity	7/44 (16)	10/194 (5)	3.48 (1.24–9.73)	0.01
alopecia	9/44 (20)	19/193 (10)	2.4 (0.98–5.63)	0.049
	AA+GA	**GG**		
↑ aminotransferase activity	12/183 (7)	5/76 (7)	1.0 (0.34–2.93)	0.99
alopecia	21/165 (13)	7/72 (10)	1.35 (0.55–3.34)	0.51
MTHFR 677, n (%)				
	TT	**CT+CC**		
all adverse effects	15/25 (60)	113/214 (53)	1.21 (0.53–2.78)	0.65
↑ aminotransferase activity	2/25(8)	16/215 (7)	1.08 (0.23–5.00)	0.92
	TT+CT	**CC**		
all adverse effects	78/127 (61)	50/112 (45)	1.97 (1.18–3.31)	0.01
↑ aminotransferase activity	14/126 (11)	4/114 (3)	3.43 (1.1–10.8)	0.025
TYMS 2R/3R, n (%)				
	3R	**2R+2R/3R**		
all adverse effects	33/63 (52)	93/172 (54)	0.93 (0.52–1.67)	0.82
	2R/3R+3R	**2R**		
all adverse effects	111/197 (56)	15/38 (40)	1.97 (0.97–4.02)	0.056
GGH, n (%)				
	CT+CC	**TT**		
all adverse effects	62/117 (53)	66/121 (54)	0.94 (0.56–1.56)	0.81
	CC	**CT+TT**		
all adverse effects	11/14 (79)	117/225 (52)	3.84 (0.92–12.46)	0.053

Abbreviations: see TABLE 3

The effect of individual polymorphisms on specific adverse effects was also analyzed. Increased aminotransferase activity was more common in patients with genotypes *MTHFR* 677CT and 677TT compared with those with genotype *MTHFR* 677CC (11% and 3%, respectively; OR, 3.4; $P = 0.025$) and in *RFC-1* 80AA homozygotes compared with *RFC-1* 80GA + *RFC-1* 80GG ones (16% and 5%, respectively; OR, 3.4; $P = 0.01$). Genotype *RFC-1* 80AA was also characterized by a higher risk of hair loss compared with *RFC-1* 80GA + *RFC-1* 80GG (20% and 10%, respectively; OR, 2.4; $P = 0.049$) (TABLE 4). The results of a logistic regression model studying the effects of polymorphisms on adverse effects are presented in TABLE 5.

Homocysteine and folic acid concentrations were not increased after 6 months of MTX therapy.

DISCUSSION MTX is used in more than 0.5 million patients with RA worldwide. Routinely determined clinical and laboratory parameters are weak prognosticators of the response to MTX therapy. In our study, we also found no effect of sex, the presence of rheumatoid factor, use of corticosteroids, smoking, or disease stage on the efficacy of the MTX therapy.

Based on published studies, it is difficult to state clearly which polymorphisms would be good predictive factors for evaluating the efficacy of the MTX therapy and the risk of adverse effects. Polymorphisms of genes encoding enzymes involved in the MTX transport to and from the cells, MTX polyglutamination, folate metabolism, and gene products that affect MTX by adenosine have been considered.

Previous studies have provided contradictory results concerning the role of the *RFC-1* polymorphism in the intracellular MTX concentration and the effectiveness of therapy. Dervieux et al.[18] demonstrated that RA patients with the *RFC-1* 80AA genotype had higher MTXP concentrations, lower numbers of swollen joints, and lower scores on the visual analogue scale than patients with the *RFC-1* 80G/G and G/A polymorphisms. However, the authors could not confirm the results in a prospective study on 48 patients.[16] Drozdzik et al.[19] studied the effect of the *RFC-1* c.80G>A polymorphism on the effectiveness of MTX therapy in 174 RA patients treated with MTX. The chance for achieving remission was 3.3-fold higher in patients with genotype 80AA than in those with 80GG. An increase in aminotransferase activity was also more common among patients with genotype 80AA, but because of the low number of patients, these differences were not significant.[19] However, the presence of the *RFC-1* 80A allele was also associated with a more common

TABLE 5 Effects of polymorphisms on adverse effects according to logistic regression model

Polymorphism	Genotype	Estimate	SE	t-ratio	*P* value	OR	–95% CI	+95% CI
constant		–2.799	1.192	–2.347	0.020	0.061	0.006	0.638
MTHFR 677	CT+TT vs CC	0.845	0.293	2.881	0.004	2.328	1.306	4.151
MTHFR 1298	AC+CC vs AA	0.064	0.291	0.221	0.825	1.067	0.601	1.893
TYMS 2R/3R	3R+2R/3R vs 2R	1.019	0.415	2.456	0.015	2.770	1.223	6.271
TYMS ins/del	ins/del+del vs ins	–0.119	0.287	–0.413	0.680	0.888	0.504	1.565
RFC-1 80	GA+AA vs GG	–0.424	0.320	–1.325	0.186	0.655	0.349	1.229
GGH 401	CC vs TT+CT	1.800	0.837	2.150	0.033	6.047	1.162	31.468
TC 593	MM vs MT	0.322	0.284	1.136	0.257	1.380	0.789	2.416
age		0.007	0.012	0.544	0.587	1.007	0.982	1.032

Abbreviations: SE, standard error; others, see **FIGURE 1** and **TABLE 3**

occurrence of adverse effects, especially in combination with the *TYMS* 3-UTR 6-bp del allele.[20] Different results related to the *RFC-1* polymorphism and adverse effects were obtained by other investigators. The *RFC-1* 80GG genotype significantly increased the risk of all adverse effects and was associated with a 15-fold increase in the risk of infection compared with the *RFC-1* AA/AG genotype.[17] However, Fukino et al.[21] did not show any effect of the *RFC-1* polymorphism on serum MTX concentrations in 100 patients with RA,[21] and several other studies did not confirm any effect of the polymorphism on the efficacy of therapy and adverse effects during the MTX therapy.[22,23] In this study, we demonstrated no association between the *RFC-1* c.80G>A polymorphism and the efficacy of therapy, but similarly to some other studies mentioned above, an increase in aminotransferase activity and hair loss were observed more often in patients with the *RFC-1* 80AA genotype. There are also other mechanisms allowing folate transport into the cell, which may explain the lack of the effect of the *RFC-1* polymorphisms in some studies.[24]

The clinical significance of polymorphisms in the *FPGS* and *GGH* genes has not yet been established. Several studies evaluated the effect of *GGH/FPGS* polymorphisms on MTX therapy in RA patients. Among 226 patients with RA treated with MTX, lower MTXPG concentrations were found in patients with the *GGH* 401TT genotype, and this could be associated with resistance to MTX. However, the authors failed to confirm those results in a prospective study.[18] The c.452C>T polymorphism in the *GGH* gene reduces the catalytic activity of GGH and increases the accumulation of long-chain MTXPG, which may affect the efficacy of MTX therapy.[25] However, van der Straaten et al.[26] did not find any effect of that polymorphism on the efficacy and incidence of adverse effects during MTX therapy. It seems that the ratio of GGH to FPGS activity is more important for the MTXPG concentration than the separate activities of these enzymes.[27] van der Straaten et al.[26] studied the effect of polymorphisms of the *FPGS* and *GGH* genes on therapy efficacy and adverse effects in 352 patients with RA treated with MTX. They demonstrated that individuals with the *GGH* 16C allele have a 2.9-fold higher chance for DAS reduction by over 1.2 compared with patients with the TT genotype. A lack of the effect of *GGH* c.16T>C and *GGH* c.452 C>T polymorphisms on the efficacy of therapy was confirmed in several other studies on patients with RA and inflammatory bowel disease.[22,23] In the present study, we evaluated only the *GGH* c.-401C>T polymorphism. We found no effect of this polymorphism on the efficacy of the therapy. However, we observed a higher incidence of adverse effects in the group of patients with the *GGH* 401CC genotype, which could be associated with higher cellular MTXPG levels.

The analysis of the effect of *MTHFR* polymorphisms in RA patients treated with MTX also provided contradictory results. van Ede et al.[13] evaluated the role of the c.677C>T polymorphism in 236 patients with RA. MTX therapy had to be discontinued more often because of adverse effects in patients with the T allele, mostly because of increased aminotransferase activity. The study did not demonstrate an effect of the genotype on the efficacy of therapy.[13] Studies performed in Hindu (150 patients) and Chinese populations (193 patients) did not show an effect of the c.677C>T polymorphism on the efficacy of therapy and the incidence of adverse effects. The authors explained this by the low number of patients, low doses of MTX (mean dose, 9.4 mg/wk), lack of folic acid use, and effect of other polymorphisms.[28,29] One of the very few studies which precisely defined the type and severity of adverse effects was a study by Kim et al.[30] The presence of 677CT or 677TT homozygotes was associated with a 4-fold higher incidence of adverse effects compared with CC homozygotes.[30] Different results were obtained in a study of 63 patients with RA, psoriatic arthritis, polymyositis, and ankylosing spondylitis.[31] The study showed that adverse effects were more common in a group of patients with the 677CC genotype. It should be noted that the study group was heterogeneous in relation to the type of disease, the number of patients was low, and adverse effects were analyzed as a whole.[31] In 3 other studies on

the effect of the *MTHFR* polymorphisms, a significant increase in the rate of adverse effects was demonstrated in the group of patients with the 677TT variant.[32-34] In our study, we observed only a more rapid positive response to MTX therapy and a trend for a better response to therapy in CC homozygotes. A higher incidence of adverse effects was found among patients possessing at least 1 T allele. An increase in aminotransferase activity was observed over 3 times more often in those patients.

Several studies also evaluated the effect of a second polymorphism within the *MTHFR*. In their meta-analysis involving 5 studies, Fisher et al.,[32] as in this study, did not show any effect of the c.1298A>C polymorphism on the incidence of adverse effects. Other studies also did not demonstrate any effect on therapy efficacy.[23,35]

Several studies also evaluated the role of the *TYMS* polymorphism among RA patients. Theoretically, patients with the 3R allele (mainly homozygotes) and with the 6-bp ins allele should experience fewer adverse effects, mostly related to tissues made of fast-dividing cells. However, this was not confirmed in all studies, which indicates the influence of numerous other factors on the development of adverse effects.[16,36] A role for those polymorphisms may be certified by the necessity for higher doses of MTX to achieve similar therapeutic effects in patients who were 3R/3R homozygotes and by the higher reduction of CRP levels found in patients who were homozygotes in relation to the 6-bp deletion in *TYMS*.[37] However, the results obtained in that study can hardly be compared with those of the present study, because in the case of both *TYMS* polymorphisms, the frequency of the allele is different in Asian and European populations, lower MTX doses were used, and there were different criteria of improvement. In their study on 214 patients with RA, Weisman et al.[38] demonstrated a more frequent incidence of hair loss in patients with the *TYMS* 2R/2R. Different results were published recently by Bohanec et al.[17] The *TYMS* 3R/3R polymorphism was associated with a more common incidence of hematological adverse effects, but it had no effect on the efficacy of therapy. Similarly to Bohanec et al.,[17] we could not demonstrate the effect of the *TYMS* 2R/3R polymorphism on the efficacy of therapy, but fewer adverse effects were observed in *TYMS* 2R homozygotes.

The results for *MTHFR*c. 1298A>C and *TC* c.593T>C polymorphisms should be interpreted with caution (despite the lack of correlation shown) because of the observed deviation from the HWE.

Furthermore, the use of a genome-wide association strategy is likely to reveal novel predictors of MTX response.[39] There is a chance that future individualization of therapy will allow to adapt the applied treatment to a particular molecular subtype of a disease and the patient's genotype. The further development of pharmacogenetics will contribute to improved efficacy of therapies and a reduction of adverse effects to a minimum and allow individualization of treatment. However, it should be noted that genetic predisposition is only one of the numerous factors affecting the effects of pharmacotherapies in individual patients.

Conclusions Studies on gene polymorphisms may allow to determine a group of patients with a predisposition to higher efficacy of MTX therapy or to the development of adverse effects during the therapy. Patients with the *MTHFR* 677TT and 677CT and *GGH* 401CC genotype have a higher risk of developing adverse effects. Because of the increased risk of hepatotoxicity, aminotransferase activity should be determined more often in patients with the *MTHFR* 677CT and 677TT genotype and in *RFC-1* 80AA homozygotes. Studies on the frequency of the 677T allele in the *MTHFR*, 401C allele in *GGH*, and 80A allele in *RFC-1* genes require continuation and evaluation in a large group of RA patients to determine their roles as factors predisposing to some adverse effects during MTX therapy.

Contribution statement JS and RS conceived the idea for the study and were responsible for study design, data interpretation, and final revision of the manuscript. PK and JP were responsible for data collection; LN, for statistical analysis; and JSz and PW, for the final revision of the manuscript. All authors edited and approved the final version of the manuscript.

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Zależność między polimorfizmami pojedynczych nukleotydów genów *RFC-1*, *GGH*, *MTHFR*, *TYMS* i *TCII* a skutecznością leczenia i działaniami niepożądanymi u chorych na reumatoidalne zapalenie stawów leczonych metotreksatem

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metotreksat, polimorfizm genów, reumatoidalne zapalenie stawów

STRESZCZENIE

WPROWADZENIE Różnice w skuteczności leku oraz w występowaniu działań niepożądanych mogą być spowodowane przez zmienność genetyczną w metabolizowaniu leków przez poszczególnych chorych.

CELE Celem pracy było określenie wpływu polimorfizmów genów na skuteczność terapii i działania niepożądane podczas leczenia chorych na reumatoidalne zapalenie stawów (RZS) za pomocą metotreksatu (MTX).

PACJENCI I METODY W badaniu uczestniczyło 273 chorych na RZS rasy kaukaskiej, leczonych MTX przez co najmniej 6 miesięcy lub u których leczenie MTX zostało przerwane z powodu działań niepożądanych. Badano wpływ siedmiu polimorfizmów genów (*RFC-1* c.80G>A, *GGH* c.-401C>T, *MTHFR* c.1298A>C and c.677C>T, *TYMS 2R/3R*, *TYMS* 6-bp deletion oraz *TC*II c.593T>C) na skuteczność i toksyczność MTX. Genomowy DNA izolowano z leukocytów krwi obwodowej.

WYNIKI U 53% chorych stwierdzono działania niepożądane w trakcie co najmniej jednej wizyty, a w przypadku 17% chorych spowodowały one odstawienie leczenia MTX. Działania niepożądane były częstsze u chorych z allelem *MTHFR* 677T niż u osób z genotypem 677CC (OR = 1,97; p = 0,01), a także częstsze u chorych z genotypem *GGH* 401CC niż u osób z genotypem *GGH* 401CT i TT (OR = 3,8; p = 0,05). Ponadto allel *MTHFR* 677T wiązał się ze zwiększeniem aktywności aminotransferaz (OR = 3,4; p = 0,02). Łysienie oraz hepatotoksyczność podczas terapii MTX były częstsze u chorych z genotypem *RFC*-1 80AA (odpowiednio OR = 3,5; p = 0,01 i OR = 2,4; p = 0,04). Szybszą dobrą odpowiedź na leczenie MTX obserwowano u homozygot *MTHFR* 677CC (OR = 3,4, p = 0,001). Nie było innych zależności między badanymi polimorfizmami pojedynczych nukleotydów a efektywnością terapii MTX.

WNIOSKI Genotypy *MTHFR* 677CC oraz *GGH*401 TT i CT wiązały się ze zmniejszeniem liczby działań niepożądanych u chorych na RZS leczonych MTX. Dalsza analiza alleli i genotypów może pomóc w identyfikacji podgrup chorych na RZS z większym ryzykiem wystąpienia działań niepożądanych.

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