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Polyglutamation of Methotrexate With Common Polymorphisms in Reduced Folate Carrier, Aminoimidazole Carboxamide Ribonucleotide Transformylase, and Thymidylate Synthase Are Associated With Methotrexate Effects in Rheumatoid Arthritis

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Objective. **Methotrexate (MTX) enters cells through the reduced folate carrier (RFC-1) and exerts part of its effects through polyglutamation to MTX polyglutamates (MTXPGs) and inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC) and thymidylate synthase (TS). We investigated the contribution of common genetic polymorphisms in RFC-1 (G80A), ATIC (C347G), and TS (28-bp tandem repeats located in the TS enhancer region [TSER*2/*3]) and of MTXPGs to the effect of MTX in patients with rheumatoid arthritis.**

Methods. **The study was cross-sectional. All patients received MTX for at least 3 months. The numbers of tender and swollen joints, the Visual Analog Scale (VAS) scores for the physician's global assessment of disease activity, and the modified Health Assessment Questionnaire scores were collected. Using the VAS score for the physician's assessment of patient's response to MTX, the population of patients was dichotomized into responders to MTX (VAS score ≤2 cm) and nonresponders to MTX (VAS score >2 cm). A pharmacogenetic index was calculated as the sum of homozygous variant genotypes (RFC-1 AA + ATIC 347GG + TSER *2/*2) carried by the patients. MTXPG concentrations were measured in red blood cells (RBCs) by high-performance liquid chromatography.**

Results. **The dose of MTX was not associated with the effects of MTX ($P > 0.05$). In contrast, increased RBC long-chain MTXPG concentrations (median 40 nmoles/liter; range <5–131 nmoles/liter) and an increased pharmacogenetic index were associated with a lower number of tender and swollen joints ($P < 0.05$) and a lower score for the physician's global assessment of disease activity ($P \leq 0.001$). Patients with RBC MTXPG levels of >60 nmoles/liter and carriers of a homozygous variant genotype were 14.0-fold (95% confidence interval [95% CI] 3.6–53.8) and 3.7-fold (95% CI 1.7–9.1), respectively, more likely to have a good response to MTX ($P \leq 0.01$).**

Conclusion. **These data suggest that measuring RBC MTXPG levels and/or the common polymorphisms in the folate–purine–pyrimidine pathway may help in monitoring MTX therapy.**

The folate antagonist methotrexate (MTX) is currently one of the most widely prescribed drugs for the treatment of rheumatoid arthritis (RA) (1,2). Although MTX is among the best-tolerated disease-modifying antirheumatic drugs, a major drawback of MTX therapy is great interpatient variability in the clinical response and the unpredictable appearance of a large spectrum of side effects that include gastrointestinal disturbances, alopecia, elevation of liver enzyme levels, and bone marrow suppression (3,4). Several well-controlled clinical trials have demonstrated that MTX decreases functional disability, with a maximum effect observable after 6 months of therapy (2,3). However, recent findings

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from a large cohort of patients with RA have surprisingly demonstrated that the time to maximal MTX effects is longer than initially thought, thereby raising the concern that the currently recommended dosage of MTX may be suboptimal (5). A possible explanation proposed by the authors was the lack of efficient monitoring of the therapeutic effect of MTX and the difficulty for the physician to rapidly individualize the dose-maximizing response to MTX.

As already proposed by various investigators (6–9), the field of pharmacogenetics may fulfill part of the need for innovative markers that help predict MTX response, and data support the hypothesis that a C677T polymorphism in methylene tetrahydrofolate reductase may help identify patients with an increased likelihood of MTX-related adverse events (10–13). MTX enters cells through the reduced folate carrier (RFC-1) and is activated by folylpolyglutamate synthetase to MTX polyglutamates (MTXPGs) (14). This γ-linked sequential addition of glutamic acid residues enhances the intracellular retention of MTX and promotes the sustained inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC), the last enzyme in the de novo purine synthesis pathway (15,16). This inhibition by MTXPGs promotes the accumulation of AICAR ribotide, a potent inhibitor of adenosine deaminase (17). The consequence is the buildup of adenosine, a potent antiinflammatory agent (18–20). Furthermore, MTXPGs are inhibitors of thymidylate synthase (TS) (21), which methylates deoxyuridine monophosphate to produce deoxythymidylate, the unique de novo source of thymidylate in the cell. Inhibition of TS by MTX causes cytotoxicity by deoxythymidine triphosphate pool depletion, leading to thymineless death (22).

It can be hypothesized that genetic polymorphisms in RFC-1, ATIC, and TS may account for part of the large interpatient variability in the therapeutic response to MTX. In fact, a G-to-A transition at position 80 of RFC-1 (G80A) was associated with clinical outcome in patients with acute lymphoblastic leukemia (23). In addition, an increasing number of variable-number 28-bp tandem repeats (TSER*2/*3 [2 or 3 28-bp tandem repeats]) in the 5′-untranslated promoter region of TS was associated with enhanced TS expression and a decreased response to MTX and 5-fluorouracil (24–26). However, to date, no polymorphisms in the de novo purine synthesis pathway (e.g., ATIC) have been associated with the therapeutic response to MTX.

We recently developed an analytical method for quantifying the concentration of MTXPG in red blood cells (RBCs) (27). In the present study, we applied this method to the routine monitoring of MTX therapy in patients with RA and investigated the contribution of 3 common polymorphisms (RFC-1 G80A, ATIC C347G, and TSER*2/*3) to the efficacy of MTX.

PATIENTS AND METHODS

Study design. The cross-sectional study was conducted at a single investigational site, a community-based rheumatology clinic in Knoxville. To be eligible, patients (age ≥18 years) had to meet the revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) for RA (28) and to have received low-dose MTX therapy for at least 3 months. Other treatments for RA that were allowed included low-dose corticosteroids (<10 mg/day) and folic acid supplementation (1 mg/day) for the prevention of side effects induced by MTX. The institutional review board approved the study, and patient consent was obtained.

Clinical and demographic characteristics of the patients were collected at the time of enrollment in the study. A 22-joint count (including the metacarpophalangeal joints, the proximal interphalangeal joints, wrists, and elbows) was used (29,30). Also, a physician's global assessment of disease activity (10-cm visual analog scale [VAS]), a patient's global assessment of disease activity (10-cm VAS), and a patient's assessment of physical function using the modified Health Assessment Questionnaire (M-HAQ) (31) were collected. The M-HAQ score was calculated using the average score for the 8 questions on the questionnaire that are addressed to the patient (dressing yourself, getting in and out of bed, lifting a full glass to your mouth, walking, washing and drying your entire body, bending down to pick up clothing, turning faucets on and off, getting in and out of a car). Scores for each item ranged from 0 (without any difficulty) to 3 (unable to do). In addition, a physician's assessment of patient's response to MTX using a 10-cm VAS was used. The physician's assessment of patient's response to MTX was scored from 0 (high response) to 10 (poor response). Clinical data were collected on case report forms at the time of the single study visit. The attending physician and each patient were blinded to the patient's MTXPG concentrations and their genotypes throughout the entire study.

High-performance liquid chromatography (HPLC) of MTXPG concentrations in RBCs. Red blood cell long-chain MTXPG concentrations were measured as described previously using an HPLC–fluorometry procedure with a postcolumn photooxidation technique (27). The technician performing the quantification of RBC MTXPGs (up to the penta order of glutamation) was blinded to patient information. Our preliminary analyses have shown that MTX triglutamate ($MTXPG_3$) is the predominant polyglutamate species in RBCs obtained from patients with RA and is strongly predictive of the total long-chain MTXPG concentrations, expressed as the sum of $MTXPG_3$ + $MTXPG_4$ + $MTXPG_5$ (R^2 = 0.94; n = 108 [data not shown]). Therefore, the RBC $MTXPG_3$ concentration was used as the marker of long-chain MTXPG concentrations ($MTXPG_{3–5}$). The quantification limit of the analytical method is 5 nmoles/liter packed RBCs, and the detection

limit is 2 nmoles/liter packed RBCs (for all MTXPG species) (27).

Genotyping procedures. Whole blood was drawn on the day of each patient's clinical visit, and genomic DNA was extracted using a Generation purification capture column (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. Total genomic DNA was quantified using a Hitachi U-2000 spectrophotometer (San Francisco, CA) at 260 nm.

The RFC-1 G80A polymorphism (resulting in a histidine-to-arginine substitution at codon 27 of RFC-1) was detected using a polymerase chain reaction (PCR)–restriction fragment length polymorphism method, as previously described (23). PCR amplification was performed with 5 ng of genomic DNA in a final volume of 50 μl containing 900 n*M* forward primer (5′-AGTGTCACCTTCGTCCCCTC-3′), 900 n*M* reverse primer (5′-CTCCCGCGTGAAGTTCTT-3′) (23), and 1× AmpliTaqGold master mix (Applied Biosystems, Foster City, CA). The PCR conditions consisted of a 5-minute initial denaturation at 95°C followed by 35 cycles with denaturation for 15 seconds at 95°C, annealing/extension at 60°C for 1 minute, with a final extension at 72°C for 7 minutes. A 20-μl PCR product (amplicon of 230 bp) was subjected to enzymatic digestion at 37°C using *Cfo* I (Promega, Madison, WI) for 3 hours. Individuals with the 80GG genotype presented 3 fragments (125 bp, 68 bp, and 37 bp), whereas individuals with the 80AA genotype presented 2 fragments (162 bp and 68 bp).

The ATIC C347G polymorphism (resulting in a threonine-to-serine substitution at position 116 of ATIC) was determined with a real-time TaqMan allelic discrimination method using fluorogenic 3′-minor groove binding probes. The forward primer sequence was 5′-CCTGCAATCTCTATCCCTTTGTAAA-3′, and the reverse primer sequence was 5′-TTCTGACTTACCAATGTCAATTTGCT-3′. Allelic discrimination was performed using the 347C fluorescent 5′-FAM–TCCAGGTGTAACTGTT–MGB-3′ and the variant 347G fluorescent 5′-VIC–CCAGGTGTAAGTGTTG–MGB-3′ probes. The final conditions for each primer and each probe were 900 n*M* and 200 n*M*, respectively, with 5 ng of genomic DNA and a 1× TaqMan master mix (Applied Biosystems). PCR conditions consisted of one 2-minute cycle at 50°C followed by a 10-minute cycle at 95°C followed by 40 cycles of 95°C for 15 seconds, 58°C for 15 seconds, and finally 60°C for 45 seconds.

The 28-bp variable-number tandem repeats (TSER*2/*3) in the 5′-untranslated promoter region of TS were measured using modifications of the method developed by Horie et al (32). A 10-ng genomic DNA was amplified in final conditions consisting of 900 n*M* forward primer (5′-GTGGCTCCTGCGTTTCCCCC-3′), 900 n*M* reverse primer (5′-CCAAGCTTCGCTCCGAGCCGGCCACAGGCATGGCGCGG-3′), 1.5 m*M* magnesium sulfate (Invitrogen, Carlsbad, CA), 1× PCR amplification buffer (Invitrogen), 0.5× PCR enhancer (Invitrogen), and 2.5 units Platinum Taq (Invitrogen). The PCR conditions (performed on an Eppendorf Mastercycler Gradient; Brinkmann, Westbury, NY) consisted of a 2-minute initial denaturation at 95°C followed by 35 cycles of a 30-second denaturation at 95°C followed by 60°C annealing for 30 seconds, 68°C extension for 1 minute, and finally a 72°C final extension for 7 minutes. PCR products were run on 3% agarose. Two 28-bp tandem repeats (TSER*2) consisted of a 220-bp amplicon, whereas three 28-bp tandem repeats (TSER*3) consisted of a 248-bp amplicon.

Statistical analysis. Because the integrity of the folate–purine–pyrimidine pathway is critical for cell survival, mutations that produce only subtle alterations in a key enzymatic step may be transmitted across generations (common polymorphism) but are likely to exhibit minimal effects, even in the context of a homozygous variant genotype. Therefore, we calculated a pharmacogenetic index as the sum of homozygous variant genotypes carried by an individual.

The homozygous variant genotypes were RFC-1 80AA, ATIC 347GG, and TSER*2/*2. Wilcoxon's exact test was used to assess the association of the dichotomous genotypes (homozygous variant versus homozygous wild-type and heterozygous) or dichotomous pharmacogenetic index (presence or absence of a homozygous variant genotype) with clinical variables. Multivariate linear regression analysis was performed, and results were adjusted for concomitant use of corticosteroids or folic acid. Using the physician's assessment of patient's response to MTX, the population of patients was dichotomized into responders to MTX (VAS score $\leq$2 cm) and nonresponders to MTX (VAS score $>$2 cm). Responders were compared with nonresponders using a multivariate logistic regression analysis, adjusting for use of corticosteroids or folic acid. The probability of the event (being a responder) was derived from a logistic regression model that included MTXPG and/or the pharmacogenetic index. The chi-square test was used as appropriate.

RESULTS

A total of 108 patients (76 women and 32 men) ages 65 years (range 36–90 years) who were undergoing MTX therapy for $>$3 months (median 65 months, range 3–266 months) were enrolled from December 2002 to May 2003 at a rheumatology clinic in Knoxville. Ninety-one patients (84%) received folic acid supplementation (1 mg/day), and 53 patients (49%) were receiving concomitant low-dose corticosteroids. Demographic data are presented in Table 1.

Poor association of MTX dosage with effects of MTX. The median weekly dose of MTX administered was 14 mg (range 5–25 mg). In a multivariate linear

Table 1. Characteristics of the 108 patients enrolled in the study*

Characteristic	Mean ± SEM
No. of tender joints (maximum 22)	5.0 ± 0.6
No. of swollen joints (maximum 22)	4.0 ± 0.5
Physician's global assessment of disease activity VAS score	3.5 ± 0.2
Patient's global assessment of disease activity VAS score	4.1 ± 0.2
Physician's assessment of patient's response to methotrexate VAS score	2.7 ± 0.2
Modified Health Assessment Questionnaire score	0.55 ± 0.05

* VAS = visual analog scale.

regression model including administration of folic acid and corticosteroids, MTX dosage was not associated with the number of tender joints ($P = 0.15$), the number of swollen joints ($P = 0.82$), the score for the physician's assessment of patient's response to MTX ($P = 0.71$), the M-HAQ score ($P = 0.09$), or the erythrocyte sedimentation rate (ESR) ($P = 0.16$). However, patients who had higher scores for physician's and patient's global assessment of disease activity had received higher dosages of MTX. These results are presented in Table 2.

Contribution of the RFC-1 80AA, ATIC 347GG, and TSER*2/*2 homozygous variant genotypes to the effects of MTX. In the 108 patients, the allelic frequency for the RFC-1 80A variant was 44%. Patients with the RFC-1 80AA genotype (21 patients) had a lower mean ± SD number of swollen joints (1.4 ± 0.4 versus 4.6 ± 0.6 [$P = 0.02$]), lower VAS scores for patient's and physician's global assessment of disease activity (2.2 ± 0.3 versus 3.8 ± 0.2 [$P < 0.01$] and 3.2 ± 0.3 versus 4.3 ± 0.2 [$P = 0.09$], respectively), and lower M-HAQ scores (0.35 ± 0.10 versus 0.60 ± 0.05 [$P = 0.02$]) compared with patients with the RFC-1 80GG (n = 34) or RFC-1 80GA (n = 53) genotype.

The allelic frequency for the ATIC 347G variant was 37%. The 18 patients who were carriers of the ATIC 347GG genotype had fewer swollen joints (1.9 ± 0.6 versus 4.5 ± 0.6 [$P = 0.06$]) and a lower score for physician's assessment of patient's response to MTX (1.8 ± 0.3 versus 2.8 ± 0.2 [$P = 0.02$]) compared with the 90 patients who were carriers of the ATIC 347CC (n = 47) or ATIC 347CG (n = 43) genotype. The distribution of the TSER*2/*3 tandem repeat polymorphism consisted of 23 carriers of the TSER*2/*2 genotype. These patients had a lower score for physician's global assessment of disease activity (2.9 ± 0.5 versus 3.6 ± 0.2 [$P = 0.049$]), a lower score for physician's assessment of patient's response to MTX (2.1 ± 0.4 versus 2.8 ± 0.2 [$P = 0.06$]), and a lower M-HAQ score (0.38 ± 0.08 versus 0.60 ± 0.05 [$P = 0.05$]) compared with carriers of a third 28-bp repeat (for TSER*3/*3, n = 21; for TSER*2/*3, n = 64). There was no other significant difference between genotype and MTX dosage or outcome variables ($P > 0.10$; data not shown).

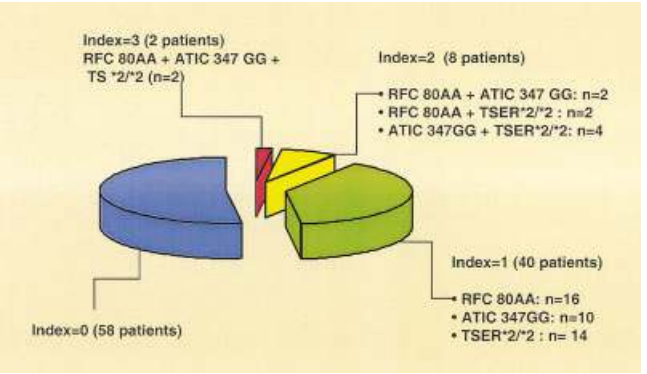

Figure 1. Pharmacogenetic index. The pharmacogenetic index corresponds to the sum of homozygous variant genotypes (RFC-1 80AA; ATIC 347GG; TSER*2/*2) carried by the patients. RFC = reduced folate carrier; ATIC = 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; TSER = thymidylate synthase enhancer region.

Contribution of RBC MTXPGs and the pharmacogenetic index to MTX efficacy. Among the 108 patients, 50 carried a homozygous variant genotype (40 patients with 1 homozygous variant genotype, 8 patients with 2 homozygous variant genotypes, and 2 patients with all 3 homozygous variant genotypes) (Figure 1). As shown in Table 3, individuals with at least 1 homozygous variant genotype responded better to MTX than did those with none of the homozygous variant genotypes.

The median RBC long-chain MTXPG concentration ($MTXPG_3$) was 40 nmoles/liter (range <5–131 nmoles/liter). In a multivariate regression analysis, the data revealed that both the MTXPG levels and the pharmacogenetic index contributed significantly to the effects of MTX (Table 4).

Table 2. Multivariate analysis of outcome variables with MTX dosage, adjusting for concomitant administration of corticosteroids and folic acid*

Variable	Global R^2	Regression estimate ± SEM for MTX dosage	*P*
No. of tender joints	0.021	0.17 ± 0.12	0.15
No. of swollen joints	0.003	0.02 ± 0.10	0.82
Physician's global assessment of disease activity VAS score	0.038	0.082 ± 0.044	0.06
Patient's global assessment of disease activity VAS score	0.088	0.136 ± 0.043	0.002
Physician's assessment of patient's response to MTX VAS score	0.006	0.014 ± 0.004	0.71
Modified Health Assessment Questionnaire score	0.035	0.017 ± 0.009	0.09

* MTX = methotrexate; VAS = visual analog scale.

Table 3. Contribution of the pharmacogenetic index to the effect of MTX*

Variable	Homozygous variant genotype 0 (n = 58)	Homozygous variant genotype ≥1 (n = 50)	*P*
MTX dosage, mg/week	14.3 ± 0.7	13.9 ± 0.7	0.60
MTXPG concentration, nmoles/liter of RBCs	42.6 ± 3.3	41.2 ± 3.3	0.62
No. of tender joints (maximum 22)	6.3 ± 0.9	3.6 ± 0.6	0.048
No. of swollen joints (maximum 22)	5.4 ± 0.8	2.6 ± 0.5	0.019
Physician's global assessment of disease activity VAS score	4.1 ± 0.3	2.8 ± 0.3	<0.001
M-HAQ score	0.67 ± 0.07	0.42 ± 0.06	0.010

* The pharmacogenetic index was calculated as the total number of reduced folate carrier (RFC-1) 80AA, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC) 347GG, and thymidylate synthase enhancer region (TSER) *2/*2 genotypes. MTX = methotrexate; MTXPG = MTX polyglutamate; RBCs = red blood cells; VAS = visual analog scale; M-HAQ = modified Health Assessment Questionnaire.

Higher concentrations of RBC long-chain MTXPGs were associated with a lower number of tender joints ($P = 0.040$), a lower number of swollen joints ($P = 0.021$), a lower score for physician's global assessment of disease activity ($P = 0.001$), and a lower score for the physician's assessment of patient's response to MTX ($P = 0.0003$). However, there was no association between RBC long-chain MTXPGs and the score for the patient's global assessment of disease activity on the VAS, the M-HAQ score ($P \geq 0.2$), and the ESR.

An increased pharmacogenetic index was associated with a decreased number of tender joints ($P = 0.012$), a decreased number of swollen joints ($P = 0.004$), a decreased score for the physician's global assessment of disease activity ($P = 0.0004$), a decreased score for the physician's assessment of patient's response to MTX ($P = 0.014$), and a decreased M-HAQ score ($P = 0.001$) (Figure 2 and Table 4). The frequency of coadministration of corticosteroids was similar between index groups ($P = 0.31$).

In a logistic regression model, higher MTX dosages were associated with higher MTXPG concentrations ($R^2 = 0.078$, $P = 0.003$), and MTXPG concentrations of >60 nmoles/liter were associated with a 14.0-fold (95% confidence interval [95% CI] 3.6–53.8 [$P < 0.001$]) higher likelihood of a VAS score of ≤2 cm for physician's assessment of patient's response to MTX (Figure 3). Interestingly, patients with the RFC-1 80AA genotype had RBC MTXPG levels of >60 nmoles/liter at a higher frequency than did those with the RFC-1 80GG or RFC-1 80GA genotype (38% versus 18% [$P = 0.051$]). There was, however, no difference in the mean (±SD) MTX dose between these 2 groups of patients (14.4 ± 1.0 mg versus 14.1 ± 0.5 mg [$P = 0.95$]).

Furthermore, an increased number of homozygous variant genotypes, as reflected by the pharmacogenetic index, was associated with an increased likelihood of having a VAS score of ≤2 cm for physician's assessment of patient's response to MTX ($P < 0.001$) (Figure 4). Patients with at least 1 homozygous variant genotype

Table 4. Linear regression of outcome variables with RBC MTXPGs and the pharmacogenetic index, adjusting for concomitant administration of corticosteroids and folic acid*

Variable	R^2	Regression estimate for RBC MTXPG concentrations ± SEM	*P*	Regression estimate for the pharmacogenetic index ± SEM	*P*
No. of tender joints (maximum 22)	0.086	−0.048 ± 0.023	0.040	−2.02 ± 0.79	0.012
No. of swollen joints (maximum 22)	0.106	−0.045 ± 0.019	0.021	−1.98 ± 0.66	0.004
Physician's assessment of disease activity VAS score	0.179	−0.026 ± 0.008	0.001	−1.00 ± 0.28	0.0004
Patient's assessment of disease activity VAS score	0.054	−0.005 ± 0.009	0.604	−0.72 ± 0.30	0.020
Physician's assessment of patient's response to methotrexate VAS score	0.166	−0.026 ± 0.007	0.0003	−0.60 ± 0.24	0.014
M-HAQ score	0.108	−0.002 ± 0.002	0.20	−0.22 ± 0.062	0.001

* RBC = red blood cell; MTXPG = methotrexate polyglutamate; VAS = visual analog scale; M-HAQ = modified Health Assessment Questionnaire.

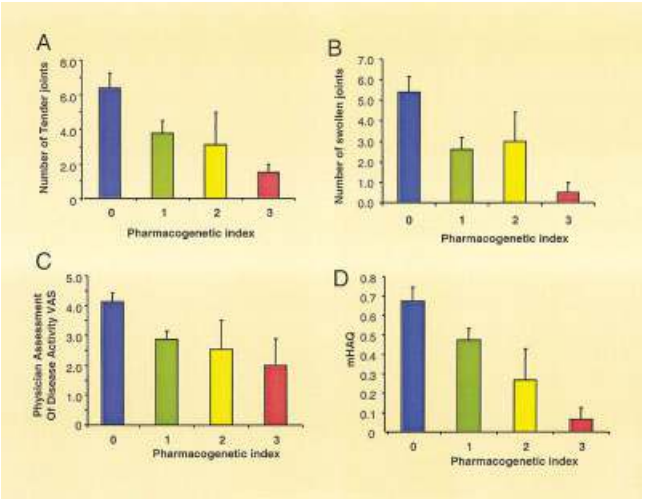

Figure 2. Effect of the pharmacogenetic index on clinical measures. An increased number of homozygous variant genotypes was associated with **A**, a lower number of tender joints ($P = 0.012$), **B,** a lower number of swollen joints ($P = 0.004$), **C,** a lower score for physician's assessment of disease activity on a visual analog scale (VAS) ($P = 0.0004$), and **D,** a lower score on the modified Health Assessment Questionnaire (M-HAQ) ($P = 0.001$). Bars show the mean and SEM.

were 3.7-fold (95% CI 1.7–9.1) more likely to exhibit a good response to MTX ($P = 0.01$). Finally, a multivariate logistic regression analysis including MTXPG levels

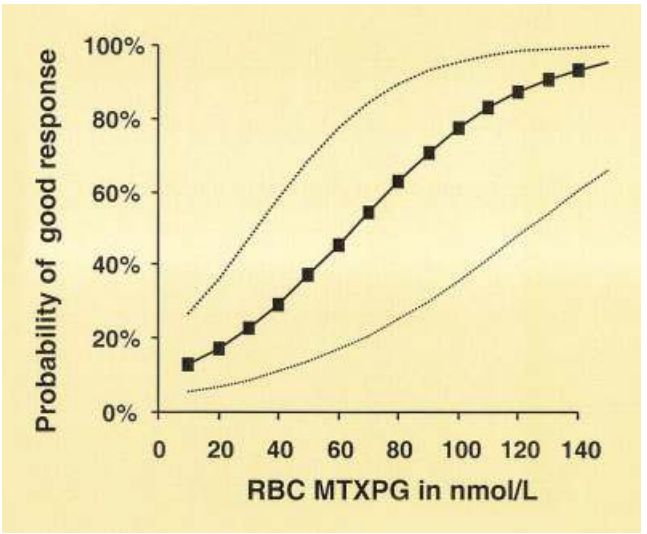

Figure 3. Effects of methotrexate (MTX) dosage and red blood cell (RBC) MTX polyglutamate (MTXPG) levels on therapeutic response. Patients with a visual analog scale (VAS) score of ≤2 cm for physician's assessment of patient's response to MTX (n = 57) were considered responders and were compared with patients with a score of >2 cm (nonresponders; n = 51). Solid line with squares shows the probability (*P*); dotted lines show the 95% confidence intervals (derived from the logistic regression) for a VAS score of ≤2 cm for physician's assessment of patient's response to MTX. $MTXPG_3$ estimate = 0.035 ± 0.01 ($P < 0.001$).

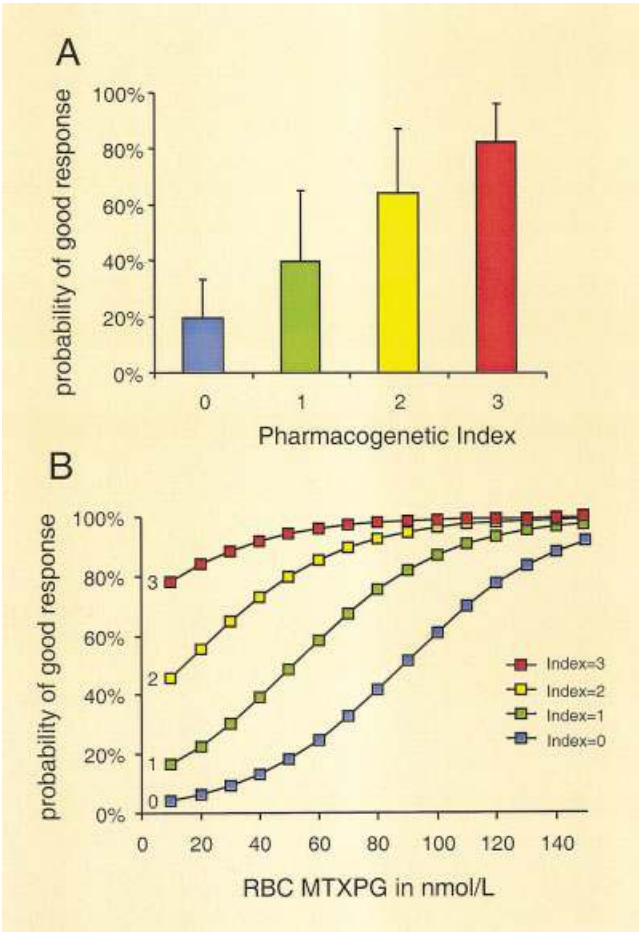

Figure 4. Effect of the pharmacogenetic index and methotrexate polyglutamate (MTXPG) levels on response to treatment with MTX. **A,** Probability of a good response to MTX (visual analog scale [VAS] score ≤2 cm) as the number of homozygous variant genotypes increases (pharmacogenetic index). Bars represent the probability and standard error. **B**, Probability of a good response to MTX (VAS score ≤2 cm) as the number of homozygous variant genotypes increases (pharmacogenetic index) with the red blood cell (RBC) MTXPG level.

and total pharmacogenetic index revealed that increased MTXPG levels tended to overcome the contribution of the genetic component to the therapeutic response (Figure 4).

DISCUSSION

This report is the first to describe the contribution of MTXPGs and common polymorphisms in RFC-1, ATIC, and TS to the effects of MTX in patients with RA treated with low-dose MTX. Results of recent studies have suggested that the MTX dosage is suboptimal in RA, and that innovative approaches may be

required to more rapidly maximize the effects of MTX (5).

The current study was cross-sectional rather than longitudinal but was useful in an initial examination combining the measurement of intracellular MTXPGs and certain MTX-related genetic polymorphisms. Because several investigators have advocated monitoring the therapeutic effect of MTX with measurement of MTXPGs in various diseases including RA (33–37), we cross-sectionally measured RBC MTXPG levels in a population of patients with RA who had been receiving MTX for at least 3 months. Because circulating erythrocytes lack folylpolyglutamate synthetase, MTXPGs in RBCs are representative of polyglutamation in bone marrow progenitors (38,39) and, therefore, are representative of MTXPG levels in less accessible tissues such as lymphocytes. Our data revealed that increased RBC MTXPG concentrations were associated with an increased response to MTX, and we identified a therapeutic threshold of 60 nmoles/liter RBC MTXPGs to be associated with a 14-fold higher likelihood of a VAS score of $\leq$2 cm for the physician's assessment of patient's response to MTX (i.e., a good response to MTX). This is consistent with previous findings in the treatment of RA (33,34), psoriasis (35), and cancer (36,37), and is consistent with the notion that the quantification of RBC MTXPG can be useful for practicing physicians to achieve rapid, effective dosing of MTX.

There is growing evidence that a part of the large interpatient variability in response to xenobiotics is related to genetic polymorphisms (40). In the present study, we evaluated the contribution of 3 common polymorphisms in the folate (RFC-1 G80A), de novo purine (ATIC C347G), and pyrimidine (TSER*2/*3) synthesis pathways to the effects of MTX therapy. Recent evidence suggests that the G80A polymorphism in RFC-1 is associated with altered folate/antifolate levels and is modestly associated with the risk for neural tube defect (23,41,42). Data suggest that individuals carrying the homozygous mutant 80AA genotype tend to have higher plasma folate and MTX levels (23,43) and higher RBC folate polyglutamate levels compared with those with the wild-type or heterozygous genotype (41). This latter finding is consistent with the observation that individuals with the RFC-1 homozygous mutant genotype (RFC-1 80AA) had a 2-fold higher frequency of MTXPG (>60 nmoles/liter) compared with those with the RFC-1 80GG and RFC-1 80GA genotypes. It is tempting to suggest that these higher levels may have contributed to the lower disease activity and improved scores for patient's assessment of disability (lower M-HAQ score) in individuals with the 80AA genotype, although the polymorphism could also directly impact disease activity through more subtle alteration in folate homeostasis (23,44).

Investigators have previously demonstrated that inhibition of the de novo purine synthesis pathway is an important component of the mechanism of MTX (15,17). MTXPGs are inhibitors of ATIC, a bifunctional enzyme that catalyzes the final steps in the de novo purine nucleotide biosynthetic pathway (45). The result is accumulation of AICAR and release of the antiinflammatory agent, adenosine (18–20). In the present study, we investigated the contribution of a threonine-to-serine substitution at position 116 of ATIC (C347G) to the effects of MTX, and our data suggest that patients carrying a homozygous variant genotype (347GG) may have an increased likelihood of response to MTX compared with those carrying a 347CC or 347CG genotype. These data are consistent with the hypothesis that MTX may produce part of its antiinflammatory effects through inhibition of ATIC. Whether the single-nucleotide polymorphisms may alter the enzymatic activity, thereby increasing the intracellular pools for the purine precursor AICAR, is not known.

Previous studies have demonstrated that TS levels increase by 10-fold 48 hours after activation of T lymphocytes (46), and evidence suggests that an increased number of tandem repeats in the TS promoter is associated with increased TS expression and a decreased response to 5-fluorouracil and MTX (24–26). In the present study, individuals homozygous for 2 tandem repeats had lower disease activity and improved response to MTX compared with patients with a third repeat. Therefore, our data suggest that inhibition of the pyrimidine synthesis is part of the mechanism of action of MTX in RA.

Because each of these common polymorphisms exhibits only a marginal phenotype, we calculated a pharmacogenetic index to demonstrate the additive association of these polymorphisms to MTX efficacy. Our data revealed that an increase in the number of variant homozygous genotypes was associated with an increased likelihood of response to MTX. Of course, it can be hypothesized that additional genetic markers in loci associated with MTX polyglutamation (e.g., folylpolyglutamate synthetase, γ-glutamyl hydrolase, multidrug resistance–associated protein, aldehyde oxidase) or MTX efficacy (e.g., dihydrofolate reductase, adenosine receptors) may also contribute to the efficacy of this drug.

Finally, the contribution of the total number of

homozygous mutant genotypes to the effect of MTX was evident at low concentrations of MTXPGs, while increased MTXPG concentrations tended to overcome the interpatient variability of the effects of MTX. This latter observation is of interest and can have direct applications in clinical practice, because individuals with no homozygous mutant genotypes and low MTXPG levels may require more aggressive MTX treatment to maximize polyglutamation and achieve efficacy.

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