Pharmacogenomic and Metabolic Biomarkers in the Folate Pathway and Their Association With Methotrexate Effects During Dosage Escalation in Rheumatoid Arthritis

Thierry Dervieux,[1] Neal Greenstein,[2] and Joel Kremer[2]

Objective. To evaluate the contribution of metabolites (methotrexate [MTX] and folate polyglutamate [PG] levels) and pharmacogenetic biomarkers in the folate pathway to the effects of MTX in patients with rheumatoid arthritis not previously treated with this antifolate.

Methods. Forty-eight MTX-naive adult patients were enrolled in a prospective longitudinal study. MTX therapy was initiated at 7.5 mg/week and was increased every 4–6 weeks until a therapeutic response was achieved. Response was assessed using the Disease Activity Score in 28 joints (DAS28). Red blood cell (RBC) MTX and folate PG levels were measured with 9 common polymorphisms in the folate pathway. Statistical analyses consisted of generalized linear models and multivariate regressions.

Results. After 6 months of therapy, the median weekly MTX dosage was 17.5 mg and the median decrease in the DAS28 was 2.0. There was a large interpatient variability in RBC MTXPG levels (median 35 nmoles/liter [interquartile range 28–51] at month 6). Patients with a lesser decrease in the DAS28 (fewer improvements) had lower RBC MTXPG levels ($P <$ 0.05) despite the higher MTX dose administered ($P <$ 0.05). RBC folate PG levels decreased significantly during treatment, and a lesser decrease in RBC folate PGs was associated with a lesser decrease in the DAS28 ($P <$ 0.05). Primary side effects were gastrointestinal and neurologic in nature. Risk genotypes associated with toxicity were in γ-glutamyl hydrolase (−401CC), 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (347GG), methylenetetrahydrofolate reductase (1298AC/CC), methionine synthase (2756AA), and methionine synthase reductase (66GG).

Conclusion. RBC MTXPG levels are a useful means by which to monitor therapy. The genetic associations presented generate hypotheses, and confirmation in independent cohorts is warranted.

Methotrexate (MTX) is the cornerstone of treatment of rheumatoid arthritis (RA) and acute lymphoblastic leukemia. MTX enters the cells through reduced folate carrier 1 (RFC-1) and is intracellularly converted to MTX polyglutamates (MTXPGs) by a γ-linked sequential addition of glutamic acid residues to MTX (1). This process is in competition with deconjugation by γ-glutamyl hydrolase (GGH). Polyglutamation of MTX enhances the intracellular retention of MTX and promotes the sustained inhibition of de novo purine synthesis along with the buildup of adenosine, a potent anti-inflammatory agent (2,3). MTX directly inhibits several enzymes of the folate pathway, including dihydrofolate reductase (DHFR), thymidylate synthase (TS), and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC) (for review, see ref. 4) (Figure 1). Other folate enzymes, such as methylenetetrahydrofolate reductase (MTHFR), are not directly inhibited by MTX, but their expression level may contribute to the antifolate effects of MTX through subtle alterations in the folate pools (4).

Since its widespread adoption as a disease-modifying antirheumatic drug (DMARD) in the 1980s (5), MTX has become the most widely used drug in the treatment of RA. Although MTX is among the best-

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[1]Thierry Dervieux, PharmD, PhD (current address: Proprius Pharmaceuticals, San Diego, California): Prometheus, San Diego, California; [2]Neal Greenstein, MD, Joel Kremer, MD: The Center for Rheumatology, Albany, New York.

Address correspondence and reprint requests to Joel Kremer, MD, The Center for Rheumatology, 1367 Washington Avenue, Suite 101, Albany, NY 12206. E-mail: jkremer@joint-docs.com.
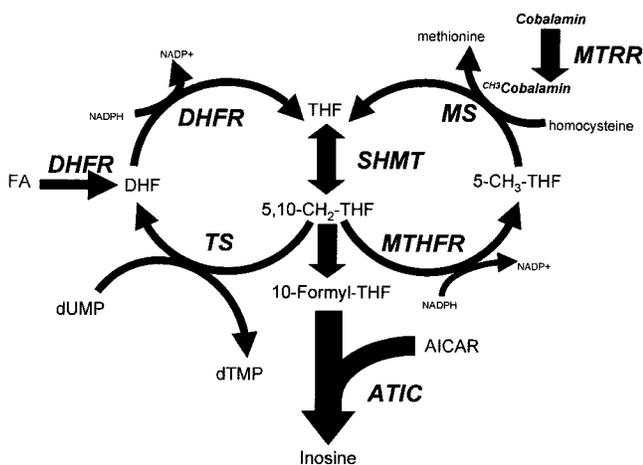
Figure 1. Methotrexate in the folate pathway. Dihydrofolate reductase (DHFR), hydrofolate reductase, thymidylate synthase (TS), and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC) are directly inhibited by methotrexate. FA = folic acid; DHF = dihydrofolate; THF = tetrahydrofolate; SHMT = serine hydroxymethyltransferase; MS = methionine synthase; CH_3 = methyl; MTRR = methionine synthase reductase; MTHFR = methylenetetrahydrofolate reductase; AICAR = 5-aminoimidazole-4-carboxamide ribonucleotide.

tolerated DMARDs, major drawbacks of MTX therapy are the large interpatient variability in clinical response and the unpredictable appearance of a large spectrum of side effects (6).

Recent evidence suggests that MTX dosing may be suboptimal in many patients with RA and that a therapeutic drug monitoring approach may help rapidly adjust the MTX dosage (7). Also, pharmacogenetic testing (8) may help to optimize therapy, and a large body of evidence suggests that single-nucleotide polymorphisms in the folate pathway (Figure 1) may contribute to the efficacy and toxicity of this antifolate (9).

Because ~95% of a given MTX dose is metabolized within 24 hours after administration, there is little value in monitoring low-dose MTX therapy using plasma concentrations. However, erythrocyte MTXPG levels are associated with MTX effects (10–12); therefore, monitoring of MTX therapy using those measurements may help in choosing a MTX dose that maximizes clinical response.

It is now well established that patients with poor folate status have an increased risk of MTX-related gastrointestinal and hematologic side effects and that folic acid supplementation can decrease the risk of MTX-related toxicity (13–15). However, because MTX may exert part of its pharmacologic effects through folate depletion, it might be anticipated that folic acid

supplementation could also decrease the efficacy of MTX. Some studies have investigated the contribution of folate supplements to the effects of MTX, but the published data are controversial, and the effects of folic acid supplementation on MTX efficacy are not clearly established (16). In the present study, we investigated the contribution of metabolic and pharmacogenetic biomarkers to the effects of MTX in patients with RA.

PATIENTS AND METHODS

Patients and study protocol. The study was a prospective longitudinal study in adult (≥18 years) patients with RA who were naive to MTX treatment. All patients had to meet the revised diagnostic criteria for RA of the American College of Rheumatology (formerly, the American Rheumatism Association) (17). All patients were studied at a single center (The Center for Rheumatology, Albany, NY). Patients provided consent prior to enrollment, and an Institutional Review Board approved the study. Oral MTX therapy was initiated at a dosage of 7.5 mg/week and was increased by 2.5 mg/week every 4–6 weeks (monthly visit) until a therapeutic response was achieved.

The clinical assessment at each study visit consisted of a tender and swollen joint count, patient's assessment of disease activity (pain rating on a 100-mm visual analog scale [VAS]), physician's assessment of disease activity, and determination of C-reactive protein (CRP) levels. The decision to modify the MTX dosage was made at the discretion of the physician and was based upon both efficacy and toxicity considerations. Treating physicians were blinded to the results of the red blood cell (RBC) MTXPG, RBC folate polyglutamate (PG), and pharmacogenetic biomarker measurements throughout the entire study. Concurrent medications allowed during the study included corticosteroids, sulfasalazine, and hydroxychloroquine. Patients were started on 1 mg of folic acid daily at the time of MTX initiation.

The occurrence of side effects was recorded at the time of each study visit. MTX side effects were defined as those affecting the gastrointestinal tract (nausea, diarrhea, stomatitis, dyspepsia, elevation of aspartate aminotransferase [AST] levels above the upper limit of normal [40 units/liter]) (18), central nervous system (headache, lethargy), hematopoietic system (white blood cell count <3,500/mm³, hemoglobin level <80 gm/liter, mean corpuscular volume [MCV] >120 fl), and the lung (cough, dyspnea, pulmonary infiltrate). Pulmonary side effects were related to MTX in the absence of upper respiratory symptoms. The leukocyte count, hemoglobin value, and liver AST level were measured on the day of the study visit using standard laboratory methods.

Toxicity was assessed at each study visit by asking each patient if he or she had experienced any side effects, and each category of MTX-related side effects was explored. A standardized questionnaire was used, and all data were recorded on case report forms. These data consisted of the presence of nausea, dyspepsia, diarrhea, headache, lethargy, stomatitis, alopecia, and pulmonary side effects (cough, dyspnea, pulmonary infiltrates). Patients were evaluated by the same physicians at the time of each study visit (JK and NG). Side effects

were graded as mild, moderate, or severe. Whole blood from each patient was drawn into tubes containing EDTA and shipped overnight from Albany, NY, to our remote location in San Diego, CA.

Laboratory measurements. RBC long-chain MTXPG concentrations (nmoles/liter of RBCs) were measured using a postcolumn photo-oxidation high-performance liquid chromatography–fluorometry technique (19). RBC folate PGs (expressed as nmoles/liter of RBCs) were measured using a radioassay (Bio-Rad, Richmond, CA). Genomic DNA was extracted from EDTA-treated whole blood using standard methods. Polymorphisms in MTHFR C677T (alanine to valine substitution at codon 222), MTHFR A1298C (glutamine to alanine substitution at codon 429), methionine synthase (MS) A2756G (aspartic acid to glycine substitution at codon 919), methionine synthase reductase (MTRR) A66G (methionine to isoleucine substitution at codon 22), serine hydroxymethyltransferase (SHMT) C1420T (leucine to phenylalanine at codon 474), and ATIC C347G (threonine to serine substitution at codon 116) were determined using a real-time TaqMan allelic discrimination method with fluorogenic 3′–minor groove binding probes (Assay-by-Design; Applied Biosystems, Foster City, CA) (10,20). Primers, probes, and polymerase chain reaction conditions are available upon request. Polymorphisms in GGH (−401C/T), TS (variable number of 28-bp tandem repeats; thymidylate synthase enhancer region [TSER] *2/*3), and RFC-1 (G80A, histidine to arginine substitution at codon 27) were measured as previously described (10,17).

The Disease Activity Score in 28 joints (DAS28) for each patient (21) was calculated as follows using the tender joint count (maximum 28), swollen joint count (maximum 28), CRP level (in mg/liter), and the patient's assessment of disease activity: $DAS28 = 0.56 \times \text{(tender joint count)}^{1/2} + 0.28 \times \text{(swollen joint count)}^{1/2} + 0.36 \times \ln(CRP + 1) + 0.14 \times \text{(patient's assessment of disease activity)} + 0.96$ (22). In 2 patients, the DAS28 was calculated using the erythrocyte sedimentation rate and the appropriate formula (http://www.das-score.nl/DAS_CRP.html).

Response to therapy was assessed using the European League Against Rheumatism (EULAR) response criteria (change in DAS28 from baseline and level attained) (23). A therapeutic response was defined as good, moderate, or poor. Patients presenting a moderate to good response were categorized as "responders" and were compared with nonresponders (i.e., those with a poor response). The percentage change in the physician's assessment of the patient's disease activity or, alternatively, the percentage change in the DAS28 score from baseline was also calculated.

Statistical analysis. Group comparisons were performed using Wilcoxon's rank sum test. Longitudinal data were analyzed using a generalized linear model. Multivariate linear or logistic regression was also used. Side effects were analyzed using the percentage of each 4–6-week period in which side effects occurred (per patient). Adjustments for concurrent medications (DMARDs, nonsteroidal antiinflammatory drugs [NSAIDs], and prednisone) were made as appropriate. Statistical analyses were performed using SAS software (release 8.2; SAS Institute, Cary, NC). In the pharmacogenetic post hoc analysis, no adjustments for multiple testing using the conservative Bonferroni method (which inflates the type II error while minimizing the type I error) were

Table 1. Clinical characteristics of patients in the study*

Age, median (IQR) years	55 (45–64)
Disease duration, median (IQR) years	1.0 (0.3–5.0)
Low-dose corticosteroid treatment, no. (%)	28 (58.3)
Folic acid supplementation, no. (%)	46 (95.8)
Concurrent DMARD treatment, no. (%)	14 (29.2)
Hydroxychloroquine	8 (16.7)
Sulfasalazine	6 (12.5)
Concurrent NSAID treatment, no. (%)	30 (62.5)
DAS28, median (IQR)	
Baseline	5.1 (4.5–6.0)
Month-4 visit	3.5 (2.7–4.2)
Month-6 visit	3.1 (2.3–3.7)
Physician's assessment of disease activity on 10-cm VAS, median (IQR)	
Baseline	5.6 (4.8–6.6)
Month-4 visit	2.6 (1.4–3.7)
Month-6 visit	2.1 (0.7–4.6)
Percentage of 4–6-week period with side effects, mean (95% confidence interval)	
Any side effects	45 (0–100)
Gastrointestinal tract	32 (0–94)
Nausea	17 (0–61)
Diarrhea	11 (0–67)
Dyspepsia	14 (0–67)
Stomatitis	4 (0–28)
AST >40 units/liter	2 (0–17)
Central nervous system	28 (0–67)
Headache	13 (0–67)
Lethargy	20 (0–67)
Alopecia	3 (0–17)
Cough	9 (0–61)
Dyspnea	0.3 (0–0)

* IQR = interquartile range; DMARD = disease-modifying antirheumatic drug; NSAID = nonsteroidal antiinflammatory drug; DAS28 = Disease Activity Score in 28 joints; VAS = visual analog scale; AST = aspartate aminotransferase.

made, and the associations reported are only hypothesis generating. *P* values less than 0.05 were considered significant.

RESULTS

Patients. A total of 48 patients enrolled from November 2002 to March 2004 received MTX for a mean ± SEM of 6.9 ± 1.4 months. All patients were followed up for 4 consecutive visits while taking MTX (a baseline visit followed by 4 subsequent visits every 4–6 weeks), and 35 patients (73%) were followed up for 6 consecutive visits (a baseline visit with 6 subsequent visits every 4–6 weeks). Patient demographics and characteristics are presented in Table 1. Allele frequencies for common polymorphisms are shown in Table 2. Genotype frequencies did not deviate from the Hardy-Weinberg equilibrium (data not shown). None of the genotypes was significantly associated with RBC MTXPG levels (data not shown).

Efficacy. At the month-4 visit, a total of 11 patients (23%) exhibited a poor response to therapy,

and 36 patients (75%) responded to therapy. The median MTX dosage was 15 mg/week (interquartile range [IQR] 12.5–15). A generalized linear model indicated that the MTX dose was not associated with response ($P = 0.63$), while lower RBC MTXPG levels resulted in a lower likelihood of response (mean ± SEM generalized linear model estimate 0.034 ± 0.020; $P = 0.095$). A lesser decrease (less improvement) in the physician's assessment of disease activity (percentage change from baseline to month 4) was associated with higher weekly MTX doses administered (generalized linear model estimate 0.043 ± 0.017; $P = 0.0097$) and lower RBC MTXPG levels (generalized linear model estimate -0.013 ± 0.004; $P = 0.0002$). These observations remained unchanged after adjustment for concurrent medications (DMARDs, NSAIDs, and prednisone).

At the month-6 visit, the median weekly MTX dosage was 17.5 mg (IQR 15–20; n = 35). Only 3 patients were nonresponders and those patients exhibited lower RBC MTXPG levels (mean ± SEM 27 ± 6 nmoles/liter) than did the responders (40 ± 18 nmoles/liter) (the significance level was not tested due to the low number of nonresponders). A lesser decrease (less improvement) in the physician's assessment of disease activity was associated with a higher weekly MTX dose administered (mean ± SEM generalized linear model estimate 0.029 ± 0.012; $P = 0.023$) and lower RBC MTXPG levels (generalized linear model estimate -0.0077 ± 0.025; $P = 0.002$). Similarly, a lesser decrease in the DAS28 (percentage change from baseline) was associated with a higher MTX dose administered (gen-
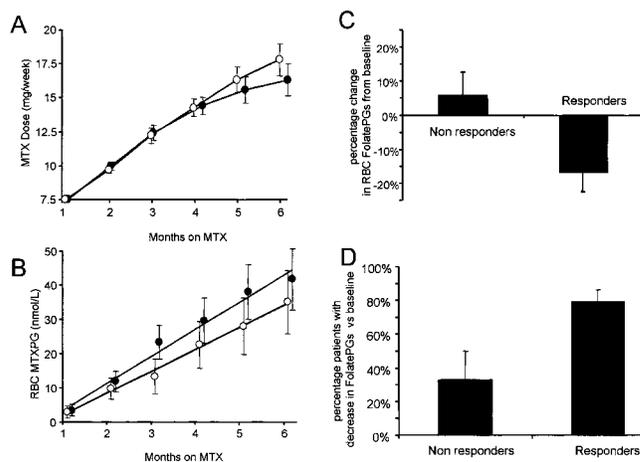
Figure 2. Erythrocyte methotrexate (MTX) and folate polyglutamate (PG) levels and MTX efficacy. **A,** MTX dosage administered to patients whose improvement in the Disease Activity Score in 28 joints (DAS28) at the month-6 visit was greater than the median improvement of 41% (●) and patients whose improvement was less than the median of 41% (○). **B,** Red blood cell (RBC) MTXPG levels in patients whose improvement in the month-6 DAS28 was greater than the median of 41% (●) and patients whose improvement was less than the median of 41% (○). **C,** Percentage decrease in RBC folate PG levels from baseline to month 4 in responders and nonresponders. **D,** Percentage of patients who exhibited a decrease in RBC folate PG levels from the initiation of therapy to month 4 ($P < 0.01$, responders versus nonresponders). Values in **A** and **B** are the mean and 95% confidence interval; values in **C** and **D** are the mean and SE.

eralized linear model estimate 0.011 ± 0.051; $P = 0.023$) and fewer RBC MTXPGs (generalized linear model estimate -0.003 ± 0.001; $P = 0.005$) (Figures 2A and B).

Finally, the change in DAS28 from baseline to the sixth study visit could be predicted using RBC MTXPG concentrations measured at the third study visit; patients with RBC MTXPGs <20 nmoles/liter at month 3 were more likely to show a change in the DAS28 below the group median (less improvement) (odds ratio [OR] 8.4 [95% confidence interval (95% CI) 1.4–52.3]; $P = 0.02$) compared with those with RBC MTXPGs >20 nmoles/liter. These observations remained unchanged after adjustment for concurrent medications (DMARDs, NSAIDs, and prednisone).

In 43 patients, the median RBC folate PG level at baseline (or in the first month following enrollment) was 1,222 nmoles/liter of RBCs (IQR 1,006–1,513). At the month-4 visit, the median RBC folate PG level in these 43 patients had decreased to 1,065 nmoles/liter (IQR 547–1,334) ($P = 0.002$). In 5 patients, the change in RBC folate PG levels was not evaluable because of insufficient blood volume. Patients who exhibited any decrease

Table 2. Allele and genotype frequencies for polymorphisms in the folate pathway*

Polymorphism (risk genotype)	Allele frequency, % (95% CI)	No. (%) with risk genotype
GGH −401C/T (GGH −401CC)	21 (12–30)	31 (65)
MTHFR C677T (MTHFR 677TT)	39 (29–48)	6 (12.5)
MTHFR A1298C (MTHFR 1298AC/CC)	33 (23–44)	25 (52)
ATIC C347G (ATIC 347GG)	30 (20–41)	7 (15)
MS A2756G (MS 2756AA)	20 (11–28)	31 (65)
TSER*2/*3 (TSER*2/*2)	46 (34–57)	13 (27)
MTRR A66G (MTRR 66GG)	60 (50–71)	18 (37)
RFC-1 G80A (RFC-1 80AA)	44 (34–53)	8 (17)
SHMT-1 C1420T (SHMT-1 1420CC)	25 (16–34)	27 (56)

* 95% CI = 95% confidence interval; GGH = γ-glutamyl hydrolase; MTHFR = methylenetetrahydrofolate reductase; ATIC = 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; MS = methionine synthase; TSER = thymidylate synthase enhancer region; MTRR = methionine synthase reductase; RFC-1 = reduced folate carrier 1; SHMT-1 = serine hydroxymethyl transferase 1.

in RBC folate PGs from the initiation of therapy to the month-4 visit were more likely to achieve a response to MTX (OR 7.7 [95% CI 1.4–40.8]; $P = 0.017$) compared with those with no decrease in RBC folate PGs (Figures 2C and D). Furthermore, a greater decrease in RBC folate PGs from the initiation of therapy to month 4 resulted in a greater decrease in the DAS28 ($R^2 = 0.200$, mean $\pm$ SEM estimate 0.32 ± 0.10) and in the physician assessment of disease activity ($R^2 = 0.201$, estimate 0.71 ± 0.22) ($P < 0.01$).

At the month-6 visit, the median RBC folate PG level was 994 nmoles/liter (IQR 828–1,290). Similar associations were observed. A greater decrease in RBC folate PGs from the initiation of therapy to month 6 resulted in a greater decrease in the DAS28 ($R^2 = 0.16$, mean $\pm$ SEM estimate 0.24 ± 0.10, $P = 0.022$) or in the physician's assessment of disease activity ($R^2 = 0.11$, estimate 0.48 ± 0.24, $P = 0.056$). Also, in a generalized linear model that included MTX dose with RBC MTX-PGs, lower RBC MTXPG levels resulted in a smaller decrease in RBC folate PGs (baseline to the month-6 visit, generalized linear model estimate -0.009 ± 0.003, $P = 0.002$).

A multivariate logistic regression analysis revealed that a lower likelihood of therapeutic response to MTX was associated with the MTHFR 677TT genotype (visit 4) (OR 22.2 [95% CI 1.2–42.2]); conversely, a greater likelihood of therapeutic response was associated with the SHMT-1 1420CC genotype (OR 7.4 [95% CI 1.0–56.4]). Thus, the MTHFR 677TT and SHMT-1 1420CT/TT genotypes were associated with a poor response, and patients who were carriers of either or both genotypes (22 patients; 36% nonresponders) were more likely (OR 5.6 [95% CI 1.0–31.2]) to have a poor response than those who had none of these genotypes (25 patients; 10% responders) ($P = 0.048$). Other genotypes were not associated with MTX efficacy ($P > 0.15$) (data not shown). At the month-6 visit, only 3 patients had not responded to MTX, and 2 of them carried the SHMT-1 1420CT/TT genotype. After adjustment for RBC MTXPG levels and MTX dose, the results remained unchanged, except that the TSER*2/*2 genotype resulted in a lower likelihood of response.

Finally, there was no difference in RBC folate PG levels between genotypes at the initiation of therapy (data not shown). However, at the month-4 visit, patients who were carriers of the SHMT-1 1420CC genotype (likely responders) had lower RBC folate PG levels (median 967 nmoles/liter [IQR 784–1,068]) compared

with those who were carriers of the SHMT-1 1420 CT/TT genotypes (likely nonresponders) (median 1,321 nmoles/liter [IQR 1,071–1,509]; $P < 0.01$).

Side effects. A total of 40 patients (83%) described some side effect at the time of at least 1 study visit, and the average percentage of each 4–6-week period in which side effects occurred (per patient) was 45% (95% CI 0–100). Side effects and percentages are shown in Table 1. The average percentage of each 4–6-week period in which gastrointestinal side effects occurred (per patient) was 32% (95% CI 0–94), and it was 28% (95% CI 0–67) for neurologic side effects. There was no significant change in the percentage of patients with gastrointestinal or neurologic side effects during the 6-month treatment period ($P > 0.55$) (data not shown). Four patients showed symptoms of hepatotoxicity (elevation of the AST above the upper normal limit). None of the patients showed symptoms of hematologic toxicity. The median erythrocyte MCV increased from 89 fl (IQR 87–92) to 93 fl (IQR 88–97) from baseline to month 4 (median increase 3.5%; $P < 0.001$), but none of the patients had an increased MCV >120 fl. At month 6, the median increase was 3.7% and only 1 patient had an MCV of 124 fl. A total of 4 patients experienced a severe side effect in 1 of the study visits (2 with severe lethargy and 2 with severe dyspepsia). Two patients required MTX dosage interruption (both at month 4; 1 had gastrointestinal and neurologic side effects and 1 had gastrointestinal side effects).

In a generalized linear model, RBC MTXPG and folate PG levels were not significantly associated with the occurrence or severity of side effects (data not shown). The higher MTX dose administered was, however, associated with an increased occurrence of neurologic side effects (mean $\pm$ SEM generalized linear model estimate 0.186 ± 0.090, $P = 0.038$) but not with gastrointestinal side effects ($P = 0.31$). A multivariate model that included the 9 risk genotypes revealed that the GGH -401CC, ATIC 347GG, MTHFR 1298AC/ CC, MTRR 66GG, and MS 2756AA genotypes explained 36% of the variability in the percentage of time with side effects during each 4–6-week period (Table 3). Other genotypes (TSER*2/*2, SHMT-1 1420CC, RFC-1 80AA, and MTHFR 677TT) did not contribute significantly to toxicity.

Similarly, 45% and 36% of the variability in the percentage of time with gastrointestinal and neurologic side effects, respectively, was explained by genetic determinants in the folate pathway. The model predicted that a given patient carrying all 5 risk genotypes would have a percentage period with side effects of 113%,

Table 3. Multivariate analysis of the percentage of each 4–6-week period in which side effects occurred (per patient)*

	Any side effects	Gastrointestinal side effects	Central nervous system side effects
No. of patients	48	48	48
Global R^2	0.360	0.452	0.358
Intercept	-7 ± 13	-29 ± 12	-15 ± 12
GGH -401CC vs. -401CT/TT	33 ± 9†	26 ± 8†	26 ± 8†
MTHFR 1298AC/CC vs. 1298AA	20 ± 9†	28 ± 8†	29 ± 11†
ATIC 347GG vs. 347CC/CG	24 ± 12†	25 ± 11†	29 ± 12†
MS 2756AA vs. 25756AG/GG	20 ± 9†	30 ± 8†	11 ± 9
MTRR 66GG vs. 66AA/AG	23 ± 10†	29 ± 8†	17 ± 9

* The multivariate equation is as follows: Period with side effects (%) = intercept + $\beta_1 \times$ GGH -401CC + $\beta_2 \times$ MTHFR 1298AC/CC + $\beta_3 \times$ ATIC 347GG + $\beta_4 \times$ MS 2756AA + $\beta_5 \times$ MTRR 66GG. The presence of a risk genotype (GGH -401CC, MTHFR 1298AC/CC, ATIC 347GG, MS 2756AA, or MTRR 66GG) is associated with a value of 1, while alternate genotypes are associated with a value of 0. Estimates (β_n) are expressed as percentages and are given for each risk genotype that explained some of the variability. Other risk genotypes in MTHFR 677TT, SHMT-1 1420CC, RFC-1 80AA, and TSER*2*2 were not associated with the occurrence of side effects ($P > 0.15$; regression estimate not shown). See Table 2 for definitions.
† $P \leq 0.05$.
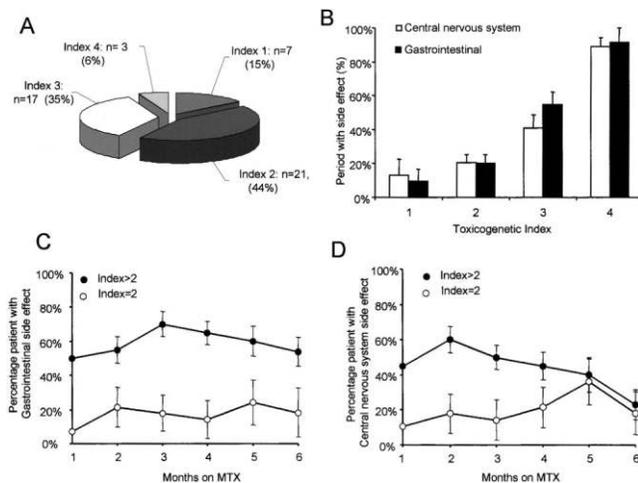
Figure 3. Toxicogenetic index and percentage of periods in which central nervous system and gastrointestinal side effects occurred. **A,** The toxicogenetic index was calculated as the sum of the γ-glutamyl hydrolase (GGH) -401CC, ATIC 347GG, MTHFR 1298AC/CC, MS 2756AA, and MTRR 66GG genotypes. The number and percentage of patients in each index category are shown. **B,** Association between an increased toxicogenetic index and an increased percentage of 4–6-week periods (per patient) during which central nervous system and gastrointestinal side effects occurred (for central nervous system side effects, $R^2 = 0.28$, mean $\pm$ SEM generalized linear model estimate = 0.21 ± 0.05, intercept = -0.18 ± 12, $P < 0.001$; for gastrointestinal side effects, $R^2 = 0.45$, estimate = 0.28 ± 0.05, intercept = -0.29 ± 11, $P < 0.001$). The percentage of periods with side effects per patient is shown for each index value. **C** and **D,** The percentage of patients with gastrointestinal (**C**) and central nervous system (**D**) side effects during each 4–6-week period and by index >2 versus ≤2. Values in **B–D** are the mean $\pm$ SEM. See Figure 1 for other definitions.

while, in contrast, a given patient carrying none of the risk genotypes would have a percentage period with side effects of -7% (intercept). Because none of the risk genotypes showed a single large effect, as illustrated by the low variability in the regression estimates (range 20–33%) (Table 3), the individual trait (i.e., side effect) resulted from the sum of the effects of all contributing genotypes. Therefore, the 5 risk genotypes (Table 3) were summed to derive a polygenic index (toxicogenetic index) for each patient (median 2; range 1–4).

An increased toxicogenetic index was associated with increased occurrence of gastrointestinal and neurologic side effects ($P < 0.001$) (Figure 3). The mean $\pm$ SEM percentage of each 4–6-week period in which side effects occurred per patient in those with an index of 1 (7 patients) was $23 \pm 12\%$, $38 \pm 6\%$ in those with an index of 2 (21 patients), $66 \pm 6\%$ in those with an index of 3 (17 patients), and 100% in those with an index of 4 (3 patients). A total of 34% of the variability in the percentage of the 4–6-week period with side effects was explained using the index, and each incremental index value resulted in a 25% increase in the percentage of the 4–6-week period with side effects ($R^2 = 0.34$, mean $\pm$ SEM generalized linear model estimate 0.249 ± 0.051, intercept 8.2 ± 12.7). Furthermore, patients with an index of ≥ 2 were more likely to report a side effect in 50% of the study visits compared with those with an index <2 (OR 13.9 [95% CI 2.6–75.4]; $P = 0.006$). These results remained unchanged after adjustment for con-

DISCUSSION

MTX is a slow-acting DMARD, and the time to achievement of a maximum therapeutic effect has been shown to be 6 months (24). However, recent evidence suggests that the time to reach an optimal MTX dosage is longer than was initially thought and that a significant delay in MTX dosage escalation may result in loss of effects (7). A large body of evidence suggests that the effects of MTX are mediated through polyglutamation to long-chain MTXPGs that inhibit de novo purine synthesis and promote the release of adenosine (4,10). We initiated this study with the hypothesis that MTXPG levels are associated with the therapeutic response, and measured MTXPG metabolites in erythrocytes, a convenient and accessible surrogate of hematopoietic cells, such as lymphocytes. Our analysis revealed that a poor response to MTX (based on the EULAR response criteria, change in the disease activity score, and the physician's assessment of disease activity) was associated with a low formation of RBC MTXPGs. Not surprisingly, a poor clinical response was associated with higher MTX doses administered as the clinician escalated the dosage to reduce disease activity. We thus confirm that an inadequate polyglutamation of MTX is associated with a poor response necessitating a MTX dosage escalation, as we have previously suggested (12).

These data suggest that monitoring MTX therapy using RBC MTXPG levels could be useful for optimizing therapy. First, the determination of MTXPGs could help guide the physician to prescribing a more aggressive increase in the MTX dosage in patients with a low polyglutamation rate. Second, since most patients usually receive MTX in combination with other DMARDs, the determination of RBC MTXPG levels could be useful for establishing whether MTX is being administered at an adequate dosage if a patient is responding poorly. A rational decision to increase the MTX dosage can be made in a patient without dose-limiting toxicity if the RBC MTXPG is low. Third, since a large proportion of patients failing conventional MTX therapy are treated with expensive biologic agents, the determination of RBC MTXPG levels could be useful for ascertaining whether MTX treatment is optimal before embarking on these other treatments. It is noteworthy to recall that 75% of a given MTX dose is excreted unchanged in the urine (within 48 hours of administration) (25) and that large interpatient variations in renal excretion of MTX will result in large interpatient variations in systemic exposure to MTX and active MTXPG levels. Also, it is important to recall that ~10% of patients excrete 99% of a given MTX dose in the urine (25) and that the appropriateness of MTX treatment and dosage escalation in those patients is highly questionable.

The depletion of erythrocyte folate PG levels appeared to be an important determinant of response, and our data suggest that the antifolate effects of MTX may contribute to the immunosuppressive and antiinflammatory effects of the drug. These observations are supported by in vitro findings, since folate deficiency inhibits the proliferation of human CD8+ T lymphocytes (26). If the association between decreases in folate PG levels and therapeutic response are indeed causal, it would follow that folic acid supplementation (which results in increased folate PG levels) (12) could partially antagonize the therapeutic response, as previously discussed (16). Whether the effect of folate supplementation on MTX efficacy is more pronounced in the US (27), where fortification of bread and cereals is now routine, as compared with European countries, where fortification with folate does not routinely occur, is not known, and additional studies are required.

It is increasingly recognized that complex traits, such as those associated with the response to drugs, are polygenic in nature rather than monogenic. In fact, an observable phenotypic trait is the total sum of various genetic components having low penetrance on their own. This polygenic nature of inheritance applies well to the field of antimetabolite-based therapies, since the integrity of the folate–purine–pyrimidine pathway is critical for cell survival, and only mutations that produce subtle alterations in a key enzymatic step may be transmitted across generations but are likely to exhibit minimal effects. We analyzed the association between polymorphisms in folate pathway enzymes and the therapeutic effects of MTX using a multivariate model that included these various low-penetrance genotypes. Our analysis indicates that a significant proportion of the variability in MTX efficacy/toxicity can be explained using this model and that a composite index adding these individual components maximizes phenotypic penetrance.

The observation that carriers of the MTHFR 677TT and SHMT-1 1420CT/TT genotypes were less likely to respond to MTX than those with the alternate genotypes is consistent with recent reports (28–30). SHMT-1 synthesizes 5,10-methylenetetrahydrofolate, the pivotal folate substrate for the formation of de novo purines by ATIC, of pyrimidines by TS, and of

5-methyltetrahydrofolate by MTHFR (Figure 1). Individuals with the 1420CT/TT genotype have higher RBC folate levels than those with the 1420CC genotype (31). We observed that genotypes potentially associated with increased 5,10-methylenetetrahydrofolate levels available for de novo purine synthesis, either through increased synthesis (as seen in those with the SHMT-1 1420CT/TT genotype) or decreased consumption by alternative routes (as seen in those with reduced levels of MTHFR 677TT) (Figure 1), are associated with a less robust response to MTX. Thus, we propose that genetic variants associated with increased 5,10-methylenetetrahydrofolate levels may increase ATIC activity and thus the level of MTXPGs necessary to achieve a satisfactory inhibition and the accumulation of AICAR that promotes the release of antiinflammatory adenosine (3).

In our study, the MTHFR C677TT polymorphism was not associated with toxicity. However, the MTHFR 1298AC/CC genotype was associated with an increased risk of toxicity, and those data are consistent with a recent report (32) but not with another (33). Other risk genotypes associated with an increased risk of toxicities were in the GGH promoter and ATIC. We also evaluated the contribution of risk genotypes in homocysteine remethylation–dependent enzymes, MS A2756G and MTRR A66G. MS catalyzes the remethylation of homocysteine to methionine in the presence of methylcobalamin, a cofactor synthesized by MTRR (Figure 1). Recently, an A2756G polymorphism in the open reading frame of MS was shown to result in increased homocysteine levels, decreased folate levels, and decreased cobalamin levels in patient carriers of the 2756A variant versus those with the 2756G variant (34–37). Conversely, an A66G polymorphism in MTRR was associated with decreased homocysteine levels, increased folate levels, and increased cobalamin levels in those with the 66A variant versus those with the 66G variant (34,38). Thus, both variants appear to have opposite contributions to homocysteine remethylation activity.

In our population of patients, those with the homozygous wild-type MS 2756AA or the homozygous mutant MTRR 66GG were more likely to experience gastrointestinal side effects (50% of the study visits) compared with those without these risk genotypes. These observations are entirely consistent with these previous studies (34–38), and we hypothesize that decreased MS activity (as seen in those with the MS 2756AA genotype) and decreased MTRR activity (as seen in those with the MTRR 66GG genotype) may

result in a low homocysteine remethylation status and increased risk of MTX toxicity. Conversely, the patients with a high homocysteine remethylation status would be protected against the development of gastrointestinal side effects.

Those associations strongly support the notion of a genetic basis for the variable response to antifolate therapy. Whether the polygenic index is predictive of effects in an independent cohort is not known, and confirmation will be necessary. Finally, the inconsistencies of the pharmacogenetic associations between studies clearly demonstrate that the contributions of these genetic components are, in all likelihood, highly dependent on folate homeostasis, treatment regimens, and possibly on other environmental factors, which should be better understood before implementing the "promising" (9) pharmacogenetic testing in routine clinical practice.

In conclusion, our study establishes that erythrocyte MTXPG concentrations are associated with MTX effects during a dosage escalation protocol in patients with RA who have not previously been treated with this antifolate. These results extend our previous observations (10,12) and provide a possible tool by which to guide clinical judgment on MTX dosing.

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