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PHARMACOGENETIC AND METABOLITE MEASUREMENTS ARE ASSOCIATED WITH CLINICAL STATUS IN RHEUMATOID ARTHRITIS PATIENTS TREATED WITH METHOTREXATE: RESULTS OF A MULTICENTERED CROSS SECTIONAL OBSERVATIONAL STUDY

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ABSTRACT

Objective: To investigate the contribution of red blood cell (RBC) methotrexate (MTX) polyglutamates (MTXPGs), RBC folate polyglutamates (folatePGs) and of a pharmacogenetic index to the clinical status of rheumatoid arthritis patients treated with MTX.

Methods: Adult patients treated with weekly MTX for more than three months were enrolled in a multi-centered cross-sectional observational study. Clinical status was assessed with a number of joint counts, a physician’s global assessment of disease activity and a modified health assessment questionnaire (mHAQ). RBC MTXPG and folatePG metabolite levels were measured by HPLC-fluorometry and radio-assay, respectively. A pharmacogenetic index cumulating low penetrance genetic polymorphisms in Reduced Folate Carrier (RFC-1 G80A), AICAR Transformylase (ATIC C347G) and Thymidylate Synthase (TSER *2/*3) was calculated. Statistical analyses consisted of a multivariate linear regression with clinical measures as dependent variables and metabolite levels with pharmacogenetic index as independent variables after adjustment for other covariates.

Results: In 226 patients enrolled at three sites, a multivariate analysis revealed that lower RBC MTXPG levels (median 40 nmol/L) and lower pharmacogenetic index (median 2) were associated with higher number of joint counts, higher disease activity and higher mHAQ (p<0.09). The multivariate analysis also established that higher RBC folatePG levels (median 1062 nmol/L) were associated with higher number of tender and swollen joints after adjustment for RBC MTXPG levels and the pharmacogenetic index (p<0.05).

Conclusion: These data suggest that pharmacogenetic and metabolite measurements may provide usefulness in optimizing MTX therapy. Prospective studies are warranted to investigate the predictive value of these markers to MTX efficacy.
INTRODUCTION

Several studies in controlled and uncontrolled clinical trials have established that methotrexate (MTX) is an effective disease-modifying anti-rheumatic drug (DMARD).\textsuperscript{1-3} Yet, there is a large inter-patient variability in the response to MTX, and the drug is inefficient at controlling disease activity and achieving remission in 30 to 40\% of patients\textsuperscript{4-7}. MTX is an anti-folate entering cells through the Reduced Folate Carrier (RFC-1)\textsuperscript{8} and activated to methotrexate polyglutamates (MTXPGs) by Folylpolyglutamate Synthase.\textsuperscript{9,10} This $\gamma$-linked sequential addition of glutamic acid residues enhances the intracellular retention of MTX and promotes the sustained inhibition of Amino-Imidazole Carboxamide Ribonucleotide (AICAR) Transformylase\textsuperscript{11} and Thymidylate Synthase (TS),\textsuperscript{12} the final steps in the $\textit{de novo}$ purine and pyrimidine biosynthesis, respectively. The resultant is an anti-proliferative effect and a release of the anti-inflammatory agent, adenosine.\textsuperscript{13,14}

The mechanisms underlying the large inter-patient variability in the therapeutic response to MTX in patients with rheumatoid arthritis are not precisely established, but several lines of evidence suggests that the genetic makeup of the patient may play a significant role.\textsuperscript{15,16} Other reasons may more simply include compliance issues and inadequate dosage. In fact, a recent study suggested that MTX dosage was sub-optimal in patients with rheumatoid arthritis,\textsuperscript{17} and a need for more rapid MTX dosage titration to individualize MTX dose-maximizing response was discussed.

It is now well established that patients with poor folate status have increased risk for MTX gastrointestinal and hematological related side effects,\textsuperscript{18,19} and that folic acid supplementation can decrease the risk for MTX’s related toxicity.\textsuperscript{20-22} However, as MTX may exert part of its pharmacological effects through folate depletion, it could be anticipated that folic acid supplementation would also decrease MTX efficacy. Although some studies have investigated the contribution of folate supplements to the effects of MTX,\textsuperscript{18,23} the data in the literature are controversial and the effects folate polyglutamate levels to MTX efficacy are not clearly established.

In the present study, we have investigated the contribution of pharmacogenetic markers and of metabolite measurements (RBC folatePGs and MTXPGs) to the clinical status of a large population of patients receiving MTX therapy.
PATIENTS AND METHODS

Study design

The study was cross-sectional at three investigational US sites (Rheumatology Consultants, Knoxville TN; Radiant Research, Daytona Beach FL; The Center for Rheumatology, Albany NY). To be eligible, patients (≥18 y) had to meet the revised criteria of the American Rheumatism Association for Rheumatoid Arthritis and to have received low-dose MTX therapy for at least three months. We designed the study with MTX as the sole DMARD to minimize potential confounding factors introduced by other drugs. However, low-dose corticosteroids (<10 mg/day) were allowed in the study. Folic acid supplementation for the prevention of MTX-induced side effects was also administered. Institutional review boards approved the study for each site, and patient consent was obtained for each patient. Patient demographics were collected at the time of enrollment in the study. The blood was drawn in EDTA containing tubes and shipped overnight to our remote location in San Diego, CA.

Clinical status assessment

Patient clinical and demographic characteristics were collected on case report forms at the time of the single study visit. Each attending physician and each patient were blinded to RBC MTXPGs, folatePGs and pharmacogenetic indices throughout the entire study. A reduced twenty-two joint count (including MCPs, PIPs, wrists and elbows) was employed. A physician’s global assessment of disease activity (10 cm visual analog scale) and a patient’s assessment of physical function using the modified-health assessment disability questionnaire (mHAQ) were also collected. In addition, a physician’s assessment of patient’s response to MTX using a 10 cm visual analogue scale was used. The physician’s assessment of patient’s response to MTX was scored from 0 (high response) to 10 (poor response).

Laboratory measurements

Red blood cell MTXPG concentrations (expressed as nmol/L RBCs) were measured using an HPLC-fluorometry procedure with a post-column photo-oxidation technique. Our preliminary analyses have shown that MTX tri-glutamate (MTXPG₃) is the predominant polyglutamate specie in RBC from patients with rheumatoid arthritis and is strongly predictive of the total long-chain MTXPG concentrations expressed as the sum of MTXPG₃ + MTXPG₄ + MTXG₅ (R²=0.92; n=226, data not shown). Therefore, RBC MTXPG₃ concentration was used as the marker of long-chain MTXPG concentration (MTXPG₃₋₅). Red blood cell folate polyglutamates (expressed as nmol/L RBCs) were measured using a radio-assay (Biorad, USA). Common polymorphisms in Reduced Folate Carrier (RFC-1/SLC191A1 G80A), AICAR Transformylase (ATIC C347G) and Thymidylate Synthase (TSER₂/₂: 28 bp variable number of tandem repeats in the promoter region) were measured as described.
Statistical analyses

The pharmacogenetic index is a cumulative composite index of individual genotypic components to maximize phenotype expression of low penetrance genetic polymorphisms. For ATIC C347G and TSER*2/*3 polymorphisms, a value of 0 was assigned to the ATIC 347CC or TSER*3/*3 genotype, a value of 1 was assigned to the ATIC 347CG or TSER*2/*3, genotype and a value of 2 was assigned to the ATIC 347GG or TSER*2/*2 genotype. For RFC-1 G80A, a value of 0 was assigned to the RFC-1 80GG or 80GA genotype and a value of 1 was assigned to the RFC-1 80AA genotype. Thus sum originating from each component (ATIC + TSER + RFC-1) was calculated and constitutes the pharmacogenetic index for the patient.

The analysis consisted of a multivariate linear regression (or univariate analysis, as appropriate), with clinical measures as dependent variables and RBC MTXPG levels, RBC folatePG levels and the pharmacogenetic index as independent variables. All regression estimates were adjusted for the presence of rheumatoid factors, the MTX dose administered, the duration of the disease and the concomitant administration of corticosteroids.

Using the physician’s assessment of patient’s response to methotrexate, the population was dichotomized into good responders (VAS<2cm) and poor responders to MTX (VAS> 2cm). Poor responders were compared to good responders using a multivariate logistic regression analysis with RBC MTXPG and pharmacogenetic index as independent variables. The probability of the event (being a poor responder) was derived from the logistic regression model. Odds ratio (OR), and probability (P) are given with a 95% confidence interval (CI). Analyses involving group comparison were performed using the Kruskal-Wallis test as appropriate.
RESULTS

Patient’s characteristics

A total of 226 patients (females, n=165, 73%; males, n=61, 27%) who were undergoing MTX therapy for more than 3 months were enrolled from December 2002 to November 2003 at three different US study sites (Tennessee site, 108 patients; Florida site, 53 patients; Albany site, 65 patients). The median weekly MTX dose administered was 15 mg, a total of 184 patients received folic acid supplementation (median 1mg/daily), and 107 patients were on concomitant low-dose corticosteroids. Using the physician’s assessment of patient’s response to MTX, the population of patients was dichotomized as 112 responders and 113 poor responders. Patient demographic data are summarized in Table I.

Median RBC MTXPG3 was 40 nmol/L (range 2-132 nmol/L; n=226). The allelic frequency for RFC-1 80A was 42% (CI 95%; 38-47%), it was 35% (CI 95%; 30-39%) for ATIC 347G and it was 49% (CI 95%; 44-53%) for TSER*2. The pharmacogenetic index ranged from 0 to 5 (median=2; Figure 1). The population of patients consisted of 221 caucasians and 5 black Americans. All polymorphisms were detected in these 5 patients (not shown). Higher MTX doses resulted in higher RBC MTXPG levels (univariate analysis, R²=0.14, p<0.001), and there was no association between the pharmacogenetic index and RBC MTXPG levels (univariate analysis, R²=0.001; p=0.9).

Association between RBC MTXPG levels, pharmacogenetic index and clinical status

A multivariate regression analysis revealed that RBC MTXPG levels and the pharmacogenetic index were associated independently with the number of swollen joints, tender joints, physician’s global assessment of disease activity and mHAQ. Results are presented in Table II. A poor clinical status (high number of tender and swollen joints, high disease activity, high mHAQ) was associated with low RBC MTXPG levels and low pharmacogenetic index (regression estimates<0; Table II). The association between the pharmacogenetic index (univariate analysis) to clinical status is presented in Figure 2.

A multivariate logistic regression including RBC MTXPG levels and the pharmacogenetic index revealed that MTXPGs below 60 nmol/L and low pharmacogenetic index were associated with increased likelihood for a physician’s assessment of patient’s response to MTX VAS>2cm (poor response; p<0.001 and p=0.034, respectively). The association between low RBC MTXPG levels, low pharmacogenetic index and the likelihood of poor response to MTX is presented in Figure 3.
Association between RBC folatePG levels and the clinical status

Because blood for folatePG determination was not available in the first 50 patients enrolled at the Tennessee site, and were missing (insufficient blood volume) in 5 patients enrolled elsewhere, we evaluated the contribution of folatePGs to clinical status in a subset of 171 patients. Median RBC folatePGs was 1062 nmol/L (range 282-3162 nmol/L; n=171). Higher folic acid doses administered resulted in higher RBC folatePG levels (p=0.009) (Figure 4A). After adjusting for MTX dose, duration of disease, presence of rheumatoid factors, administration of steroids, RBC MTXPG levels and the pharmacogenetic index, the multivariate analysis revealed that RBC folatePGs were not significantly associated with the physician’s global assessment of disease activity (p=0.18) or the mHAQ (p=0.79). However the multivariate analysis established that higher folatePG levels were associated with higher number of tender joints (global R=0.41; R²=0.166; estimate for RBC folatePGs=0.0020±0.0007; p=0.003) and higher number of swollen joints (global R= 0.36; R²=0.129; estimate for RBC folatePGs=0.0014±0.0006; p=0.028). The association between RBC folatePGs and the number of swollen and tender joints in a univariate analysis is illustrated in Figure 4B.
DISCUSSION

This is the first study to evaluate the contribution of pharmaco-genomic and pharmaco-metabolomic markers (RBC MTXPGs and folatePGs) to the effects of MTX in a large population of rheumatoid arthritis patients. We previously suggested that polyglutamation of MTX with common polymorphisms in the folate/purine/pyrimidine pathways were associated with MTX effects in patients enrolled in a single study site. In the present report, we have extended the enrollment to a larger cohort of patients in a total of three study sites, and have also evaluated the contribution of RBC folatePGs to the effects of MTX.

We designed the study with MTX as the sole DMARD to minimize potential confounding factors introduced by other drugs. Because our study was cross sectional, we employed a novel mean of assessing a patient's response to MTX treatment. We chose a visual analogue scale as it was felt that it would accurately reflect a clinician’s judgment of response in an appropriate dimension not captured by either of the standard outcome measures. This measure can be considered as a "real world" measure of the clinician's judgment of overall response in the common practice setting where ACR measures are not usually performed to evaluate treatment efficacy. The analysis revealed that low RBC MTXPG levels were associated with poor clinical status, and that patients with MTXPG levels below 60 nmol/L were 4 fold more likely to have a poor response to MTX. Although we cannot exclude the possibility that the maximum tolerated dose was achieved in poor responders having RBC MTXPG levels below 60 nmol/L, it is noteworthy to observe that only 23% of the patients in the cohort reached this threshold. Higher MTX doses resulted in higher MTXPG levels but higher MTX dosage tended to be associated with lower effects (poor clinical status). Given our cross sectional study design, there are two hypotheses to explain these results. First, MTX may have been increased during previous visits because the patient was not responding to MTX. These individuals would have had a level of RBC MTXPG which we hypothesize would have been low. Alternatively, some patients may have been responders at low MTX dosage with the formation of a satisfactory level of MTXPG commensurate with a good response.

We have to acknowledge that there are some limitations in our cross sectional observational study design, and some cautions should be exercised while interpreting the data. For example, the cohort of patients was on MTX for an average of 5 years and we cannot extrapolate and generalize our data to patients starting MTX therapy. In addition, none of the patients enrolled experienced toxicity requiring MTX withdrawal at the time of the visit, and therefore, MTXPG levels associated with toxicity are not known. However, this study lays the groundwork for a prospective longitudinal study aimed at confirming the value of RBC MTXPGs to establish whether the weekly dose of the drug is adequate to achieve the desired response. In fact, our preliminary analysis from such prospective study appear to confirm our hypothesis generating results and higher MTXPG levels appear to predict the ACR20 criteria during dose escalation. Moreover, recent study suggests that increasing MTX dose does not necessary produce greater...
effects, and it will be important to determine whether MTX polyglutamate formation is subjected to saturation mechanisms.

It is now well established that pharmacogenetics can provide usefulness in optimizing therapy. In the present study, we evaluated the contribution of common polymorphisms in the folate (RFC-1 G80A), purine (ATIC C347G) and pyrimidine pathways (TSER*2/*3) to MTX effects. All these polymorphisms were previously associated with alteration in the response to MTX or with alteration in folate pools.

Because the integrity of these pathways is critical for cellular homeostasis, inborn errors that severely impair expression of these keys enzymatic steps result in high penetrance phenotype during childhood. For example mutations that severely impair AICAR Transformylase activity are associated with devastating neurologically disorders, those affecting cellular folate uptake results in severe aplastic anemia, and to our knowledge, no deficiency in Thymidylate Synthase has ever been reported. Consequently, only mutations associated with subtle alterations in these key enzymatic steps may be transmitted across generations (common polymorphisms), and are likely to exhibit low marginal phenotypic expression. Therefore, we calculated a composite index cumulating individual genotypic components to maximize the pharmacogenetic contribution of these low penetrance polymorphisms.

The pharmacogenetic index contributed to MTX effects and lower index values resulted in diminished therapeutic efficacy. We speculate that lower indices are associated with subtle alterations in the folate, purine and pyrimidine homeostasis that result in poor sensitivity to MTX. Furthermore, both low RBC MTXPG levels and a low pharmacogenetic index were independently associated with poor clinical status and therefore, both markers may be useful to stratify MTX dose to maximize effects. Of course, additional polymorphisms in other genes are likely to contribute to the effects of MTX, and hence could increase the modest variance explained by the genetic index in the study.

The vast majority of patients enrolled in the study received folic acid supplementation to prevent MTX’s induced side effects. Because part of the effects of MTX may be mediated through depletion in folate cofactors, we hypothesized that folic acid supplementation could interact with MTX effects. Our analysis revealed that RBC folatePG levels influenced the effects of MTX, with higher RBC folatePGs contributing to higher number of tender and swollen joints. This first line of evidence suggests that folatePG levels may partially antagonize MTX effects. It would therefore appear that additional investigations into rational dosing of folic acid with MTX are appropriate.

In conclusion, these novel data suggest that a therapeutic drug monitoring of MTX therapy combining pharmacogenetic and intracellular metabolite measurements may be useful to optimize MTX therapy. Prospective studies will be necessary to confirm these findings and determine with certainty the predictive value of these markers.
ACKNOWLEDGEMENTS

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ETHICS APPROVAL:
Institutional review boards approved the study for each site, and patient consent was obtained for each patient.

COMPETING INTERESTS:

Thierry Dervieux, Diana Orentas Lein, Katie Smith are employed by Prometheus Laboratories and have shares in the company. Joel Kremer, Daniel Furst and Jacques Caldwell have received funds for clinical research from Prometheus Laboratories.
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FIGURE LEGEND

**Figure 1: Pharmacogenetic index**

The index ranged from 0 to 5 (median 2). Two patients carried an index of 5 which corresponded to the presence of the three homozygous variant genotypes (value of 2 for ATIC 347GG + value of 2 for TSER*2/*2 + value of 1 for RFC-1 80AA). 15 patients carried an index of 0, which corresponded to the presence of ATIC 347CC (value of 0), TSER*3/*3 (value of 0) and the presence of either RFC-1 80GG or GA genotype (value of 0 for wild type and heterozygous). The number of patients (% of total population) is given for each index.

**Figure 2: Association between the pharmacogenetic index and the clinical status**

In a univariate analysis, lower pharmacogenetic index was associated with higher number of tender joints (p=0.002; panel A), higher number of swollen joints (p=0.003; panel B), higher physician’s global assessment of disease activity VAS (p=0.032; panel C) and higher mHAQ (p=0.047; panel D). Results are expressed as mean±SEM. The linear regression line is given.

**Figure 3: Association between MTXPGs, pharmacogenetic index and the physician’s assessment of patient’s response to MTX.**

Patients having RBC MTXPG levels below 60 nmol/L were 4.4-fold (CI 95% 2.0-8.5; p=0.0001) more likely to present a physician’s assessment of patient’s response to MTX VAS>2cm (poor response). Lower pharmacogenetic index was also associated with a higher likelihood of physician’s assessment of patient’s response to MTX VAS>2cm (p=0.034). The Figure summarizes the descriptive analysis of the data. Probability (CI 95%) is given.

**Figure 4: Association between RBC folatePG concentrations and the number of tender and swollen joints**

Panel A: Higher daily folic acid doses resulted in higher RBC folatePGs (p=0.009).

Panel B: In a univariate linear regression, higher folatePGs were associated with higher number of tender (p=0.002) and swollen joints (p=0.006). The number of tender and swollen joints calculated from the univariate regression is given for folatePGs in the 10th (622 nmol/L), 25th (738 nmol/L), 50th (1062 nmol/L), 75th (1447 nmol/L) and 90th percentile (1736 nmol/L) of the patient population. The Figure summarizes the descriptive analysis of the data. Bars represent values with CI 95%.
### Table I: Clinical characteristics of the 226 patients enrolled in the study.

Results are expressed as median (inter-quartile range) or number (%) as appropriate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>66 (57-74)</td>
</tr>
<tr>
<td>Number of females</td>
<td>165 (73%)</td>
</tr>
<tr>
<td>Duration of disease (y)</td>
<td>8.6 (4.2-17.9)</td>
</tr>
<tr>
<td>Number of months under MTX</td>
<td>51 (19-97)</td>
</tr>
<tr>
<td>Presence of rheumatoid factors (IgM)</td>
<td>156 (69%)</td>
</tr>
<tr>
<td>Administration of low dose corticosteroids</td>
<td>107 (47%)</td>
</tr>
<tr>
<td>Weekly MTX dose (mg)</td>
<td>15 (10-17.5)</td>
</tr>
<tr>
<td>Folic acid supplementation</td>
<td>184 (81%)</td>
</tr>
<tr>
<td>Number of tender joints</td>
<td>1.0 (0-5.0)</td>
</tr>
<tr>
<td>Number of swollen joints</td>
<td>3.0 (1.0-6.0)</td>
</tr>
<tr>
<td>Physician’s global assessment of disease activity 10 cm VAS</td>
<td>2.5 (1.2-4.3)</td>
</tr>
</tbody>
</table>
Table II: Multivariate linear regression of clinical measures with RBC MTXPG levels and pharmacogenetic measurements in the 226 patients.

The independent variables were RBC MTXPGs (in nmol/L) and pharmacogenetic index. Covariates included in the analysis were the presence of rheumatoid factors, the duration of the disease, the concomitant administration of low dose corticosteroids and the MTX dose administered. Estimates ±SE with p value are given for MTX dose, RBC MTXPGs and pharmacogenetic index. Global R and R² are also given.

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>number of tender joints</th>
<th>number of swollen joints</th>
<th>physician’s global assessment of disease activity 10 cm VAS</th>
<th>mHAQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global R (R²)</td>
<td>0.33 (0.11)</td>
<td>0.28 (0.08)</td>
<td>0.35 (0.12)</td>
<td>0.30 (0.09)</td>
</tr>
<tr>
<td>MTX dose (mg/week)</td>
<td>0.263±0.076 (p&lt;0.001)</td>
<td>0.151±0.068 (p=0.027)</td>
<td>0.113±0.031 (p&lt;0.001)</td>
<td>0.024±0.002 (p=0.002)</td>
</tr>
<tr>
<td>RBC MTXPGs (nmol/L)</td>
<td>-0.050±0.015 (p=0.001)</td>
<td>-0.022±0.013 (p=0.011)</td>
<td>-0.0257±0.006 (p&lt;0.001)</td>
<td>-0.004±0.001 (p=0.006)</td>
</tr>
<tr>
<td>Pharmacogenetic index</td>
<td>-0.981±0.336 (p=0.003)</td>
<td>-0.877±0.300 (p=0.003)</td>
<td>-0.260±0.136 (p=0.049)</td>
<td>-0.063±0.034 (p=0.084)</td>
</tr>
</tbody>
</table>
RBC MTXPG < 60 nmol/L
RBC MTXPG ≥ 60 nmol/L

Pharmacogenetic Index

Probability of physician's assessment of patient's response to MTX VAS ≥ 2 cm

0% 20% 40% 60% 80% 100%

Pharmacogenetic Index

0 1 2 3 4 5
A

Folic acid dose (mg/day)

RBC folatePGs (nmol/L)

B

FolatePGs (percentile)

number of Joints

A

Tender

Swollen

B

10th 25th 50th 75th 90th