Published in final edited form as:
Curr Rheumatol Rep. 2010 October ; 12(5): 342–347. doi:10.1007/s11926-010-0120-3.

Measurement of Erythrocyte Methotrexate Polyglutamate Levels: Ready for Clinical Use in Rheumatoid Arthritis?

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Abstract

Methotrexate (MTX) is one of the most commonly prescribed and most effective drugs for the treatment of rheumatoid arthritis (RA). Given the partial response of many patients and the side effect profile of the drug, there is considerable interest in identification of biomarkers to guide MTX therapy in RA. Upon entering cells, MTX is polyglutamated. Measuring methotrexate polyglutamates (MTX PGs) levels in circulating red blood cells (RBC) has been proposed as an objective measure that can help to optimize MTX therapy in RA. There is conflicting data with regard to the clinical utility of measurement of MTX PGs measurements as a predictor of the efficacy or toxicity of low-dose MTX effects in RA. Should large, randomized clinical trials of this assay show consistent, reproducible, long-term correlations between MTX PG levels and efficacy and toxicity, this test could become a prominent tool for clinicians to optimize the use of MTX in RA.

Keywords

Rheumatoid Arthritis; Methotrexate; Polyglutamates

Background

During the past several decades, low dose MTX, a folate antagonist, has gained acceptance as the anchor disease modifying anti-rheumatic drug (DMARD) against which newer treatments for RA are judged. Its widespread use in the treatment of inflammatory arthritis and other autoimmune diseases is justified by its proven long term efficacy and well characterized side effect profile(1-3). The dosage required for optimal efficacy and lowest toxicity among individual RA patients is variable and unpredictable. Therefore, doses usually start low (7.5 - 15 mg once a week) and are progressively increased to 20-25 mg per

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week based on disease response and/or toxicity. Most patients show improvement at these doses, but incomplete responses are common, resulting in the need to add otheragents. Recent data have shown that early aggressive treatment of RA is cost effective (4) and associated with improved outcomes, both functional and structural (5, 6). Thus, a *priori* knowledge of a patient's response to treatment would allow for a shortened interval from the initial diagnosis to adequate control of the disease, resulting in improved patient outcomes.

The use of MTX is limited by unpredictable various adverse effects, including gastrointestinal disturbances, mucositis, fatigue, alopecia, elevated serum transaminase levels, and bone marrow toxicity (3, 7, 8). To monitor for the development of these adverse effects, patients require frequent blood tests, which are cumbersome and the overall cost is significant when summed over many years. Over the last decade, quantification of intracellular RBC MTX PG has been suggested as a biomarker to guide methotrexate therapy (9).

Pharmacokinetics of low-dose methotrexate

After oral or parenteral administration, the serum concentration of MTX falls rapidly (10). Methotrexate in its monoglutamate state (MTX PG_1) is taken up by cells via the reduced folate carrier and is metabolized by folylpolyglutamate synthetase to MTX polyglutamates (MTX PGs) (11, 12). The products of glutamation are labeled MTX PG_n, where n represents the number of glutamate residues. The addition of up to 6 glutamate residues has been reported (13), but cumulatively MTX PG_1 (native form) to MTX PG_5 (methotrexate pentaglutamate) have been reported to account for 99.6% of total intracellular MTX PG (13). The most prevalent type of polyglutamate species is MTX PG_3 (14-18). Methotrexate PGs are trapped intracellularly, which is essential for MTX inhibition of multiple enzymes, including dihydrofolate reductase, thymidylate synthase, and 5-aminoimidazole 4-carboxamide ribonucleotide transformylase (12, 19). Inhibition of these enzymes results in inhibition of purine synthesis and accumulation of adenosine, which has anti-inflammatory effects (20). Gamma glutamyl hydrolase catalyzes the removal of terminal glutamic moieties from MTX PGs, returning MTX to its monoglutamate form, which then can efflux from the cell through multidrug resistance-associated proteins (19). MTX PGs can be measured by high performance liquid chromatography in RBCs and their levels in these cells are thought to be representative of concentrations in other cells, such as lymphocytes, that are important in the pathogenesis of RA (14).

Monitoring MTX therapy by measuring MTX PGs

The therapeutic effects of MTX in RA depend on its conversion MTX PGs (19), so measuring intracellular MTX PGs has been proposed as an objective method to guide MTX therapy (21). Angelis-Stoforidis et al measured MTX PGs in circulating RBCs, mononuclear cells and neutrophils in 65 patients with RA receiving weekly low-dose MTX (dose range 2.5-37.5 mg) for at least 2 months (22). Each patient was classified as a responder, partial or non- responder based on a physician's global clinical assessment which included history, joint examination and a comparative temporal assessment. RBC MTX PGs levels were higher in treatment responders (60.7 ± 18.9 nmoles/liter) and partial responders (50.8 ± 23.3 nmoles/liter) compared to non-responders (21.5 ± 10.5; $p = 0.0001$). Since the MTX dose was higher in non-responders than in responders, the difference in MTX PGs concentration could not be explained on the basis of the dose. RBC MTX PG levels tended to be higher in patients with side effects, but this difference was not statistically significant ($p = 0.15$).

Similar results were reported in a series of studies by Dervieux et al (17). In the first study, RBC MTX PG concentrations were measured at a single time point in a population of 108 RA patients on low-dose MTX therapy for greater than 3 months (median 65 months, range

3-266 months). In this study, RBC MTX PG_3 was used as marker of long-chain MTX PG (MTX PG_{3-5}) concentrations, as it is strongly predictive of total long-chain RBC MTX PG levels. The median RBC MTX PG_3 in all study subjects was 40 nmoles/liter (range <5-131 nmoles/liter). After adjusting for use of corticosteroids and folic acid, higher concentrations of RBC long chain MTX PGs were associated with increased therapeutic response to MTX as determined by lower total joint count (p=0.04), total swollen joint count (p=0.021), and physician's global assessment (p=0.0003). Long-chain MTX PG levels were not associated with patient's global assessment of disease activity, modified health assessment questionnaire (mHAQ) or erythrocyte sedimentation rate (ESR). In addition, higher MTX doses correlated with higher RBC MTX PG concentrations (R^2=0.078, p=0.003) and patients with an RBC MTX PGs level > 60 nmol/L had a 14-fold (95% confidence intervals (CI), 3.6-53.8, p<0.001) higher likelihood of having a VAS score of ≤ 2 cm on a visual analog scale [range 0 (high response) to 10 (poor response)] for the physician's assessment of patient's response to treatment.

The same group of authors confirmed their initial findings in a larger multicenter cross-sectional observational study (23). RBC MTX PGs and RBC folate polyglutamate levels were measured in 226 RA patients with a median disease duration of 8.6 years (interquartile range 4.2-17.9 years) who were on MTX for >3 months (median 51 months, interquartile range 19-97 months). After adjusting for multiple factors including presence of rheumatoid factor (RF), disease duration, use of corticosteroids, and MTX dose, the RBC MTX PG_3 levels correlated significantly with markers of disease activity. Subjects with MTX PGs concentration < 60 nmol/L were 4.4-fold (95% CI 2.0-8.5, p =0.0001) more likely to have a poor response to MTX as defined by physician's assessment of patient's response to MTX. Moreover, higher RBC folate PG levels (median 1,062 nmol/L, range 282-3,162 nmol/l) were associated with higher folic acid doses (p=0.009). In this paper, the investigators described a pharmacogenetic index defined as the sum of homozygous variant genotypes of common polymorphisms in the reduced folate carrier (*RFC-1* G80A), AICAR transformylase (*ATIC* C347G) and thymidylate synthase (*TSER* *2/*3: 28 bp variable number of tandem repeats in the promoter region). In multivariable analysis, after accounting for MTX PG levels and the pharmacogenetic index, higher folate PG levels were associated with a higher number of tender and swollen joints (p<0.05) suggesting that folate levels may partially mitigate MTX effects. More recent data, however, suggest that folic acid supplementation may inhibit *in vivo* aldehyde oxidase (and therefore, the catabolism of MTX to 7-OH-MTX) and thus potentiate the efficacy of MTX (24).

In another study from Dervieux et al., the contribution of MTX PGs and folate PGs to the therapeutic effects of MTX was evaluated in 48 MTX-naïve RA (median disease duration 1 year, interquartile range 0.3-5 years) patients enrolled in a prospective longitudinal study and followed for 6 months (9). At 6 months, the median weekly dose of MTX was 17.5 mg (interquartile range 15-20) and the median decrease in the Disease Activity Score for 28 joints (DAS28) was 2.0 in 35 patients that completed 6 consecutive visits. Patients with a lesser decrease in the DAS28 (less improvement) had lower RBC MTX PG levels (P < 0.05), despite the higher MTX dose (P < 0.05). Patient's response to MTX therapy at 6 months, as measured by the decrease in DAS28, could be predicted by the RBC long chain MTX PG measurements at 3 months after the start of MTX treatment. Patients with RBC long chain MTX PGs <20 nmoles/liter at month 3 showed less improvement in DAS28 compared with those with RBC MTX PGs>20 nmoles/liter, even after adjusting for concurrent medications, i.e. other DMARDs, NSAIDs and prednisone. In addition, similar to findings in previous studies, (22) RBC MTX PG and folate PG levels did not correlate with the occurrence or severity of MTX side effects.

In another longitudinal prospective study which included 40 MTX-naïve RA patients and 36 patients taking long term MTX, Hornung et al. investigated the use of RBC MTX concentrations in RA treatment as well as the correlation between plasma MTX concentration and intracellular MTX concentrations (25). The RBC MTX concentrations reached a steady state level in the first 6-8 weeks. The weekly dose of MTX and steady state RBC MTX levels were weakly correlated ($r^2 = 0.16$). RBC MTX content (measured by a radiochemical-ligand binding assay) in RA patients commencing treatment with MTX was higher in responders (mean ± standard deviation (SD) 25.74 ± 12.99) compared to nonresponders (17.44 ± 7.51) (p 0.013).

In contrast to the findings from Dervieux et al., a recent study from a different group of investigators did not show a relationship between MTX PG concentration and disease control in patients with RA receiving long-term MTX (18). They reported a cross-sectional study that included 192 RA patients receiving MTX for at least 3 months. The characteristics of the RA patient study group were: 73% women, mean RA duration 10.5 years, 81% RF positive, 76% anti-CCP antibody positive, 63% with radiographic erosions. The median dose of MTX was 15 mg/week, with a range of 5-25 mg/week. The MTX dose was significantly higher in patients with higher disease activity as evidenced by swollen joint counts, DAS28, physician's global assessment score, physician-rated response to MTX, patient's global assessment score, clinical disease activity index (CDAI), and simplified disease activity index (SDAI). There was a significant association between the MTX dosage and RBC MTX PG_3, MTX PG_4, MTX PG_5, MTX PG_{1-5} and MTX PG_{3-5} concentrations ($p<0.0001$ for all). MTX PG_4, MTX PG_5, MTX PG_{1-5} and MTX PG_{3-5} levels were higher in the group with high disease activity (DAS28 ≥ 3.2) compared with the group with low disease activity (DAS28 < 3.2). MTX PG_5 concentrations correlated with disease activity measures after adjusting for age, sex, RA duration, smoking status, anti-CCP antibody status, other medications (corticosteroids, NSAIDs, DMARDs), and folate status. After correction for age, estimated glomerular filtration rate (GFR) and MTX dosage, there was no significant difference in MTX PGs between the group with high disease activity and the group with low disease activity; RBC MTX PG_5 concentrations remained significantly higher in the group with high disease activity ($P = 0.02$). MTX PG_5 was undetectable in 25% of the group with low disease activity compared with 19% of the group with high disease activity ($P = 0.31$). The investigators found that the RBC folate concentration was higher in the patients with high disease activity compared with the group with low disease activity (mean ± SEM 786.9 ± 31.2 nmoles/liter versus 664.2 ± 27.4 nmoles/liter; $P = 0.002$). In this study, there was no association between the MTX dosage and the presence of any adverse effect. Furthermore, there was no correlation between MTX PG concentration and adverse effects, similar to findings in other studies (9, 22).

Pharmacokinetics of MTX PGs

Given the proposed role of MTX PGs as surrogate markers of treatment response to MTX, knowledge of the pharmacokinetics of MTX PGs species is required for optimal timing of blood sampling. In a recent study, Dalrymple et al. sought to define the time to steady state and the half-life of accumulation of RBC MTX PG_{1-5} in 10 RA patients starting oral MTX. They also assessed the length of time for RBC MTX PG_{1-5} to become undetectable and the half-life of elimination of RBC MTX PG_{1-5} in 10 patients discontinuing oral MTX (14). The patients started oral MTX at a median weekly dosage of 10 mg, which was titrated based on clinical response to a median weekly dosage of 15 mg. All patients received folic acid supplementation at a dosage of 5 mg/week, administered 3-4 days after the dose of MTX. The median times to reach steady state in RBCs (defined as 90% of the maximum concentration) were 6.2, 10.6, 41.2, 149, and 139.8 weeks, respectively, for MTX PG_1, MTX PG_2, MTX PG_3, MTX PG_4, and MTX PG_5. The median half-life of accumulation for

RBC MTX PG_{1-5} ranged from 1.9 weeks to 45.2 weeks. Among patients discontinuing MTX, RBC MTX PGs became undetectable in a median time of 4.5, 5.5, 10, 6, and 4 weeks, respectively, for MTX PG_1, MTX PG_2, MTX PG_3, MTX PG_4, and MTX PG_5. The median half-life of elimination for RBC MTX PG_{1-5} ranged from 1.2 weeks to 4.3 weeks. The authors found a significant inter-patient variability in the measured RBC MTX PG levels which was not explained by age, but which correlated with impaired renal function. While this study was small, the variability in time to steady state among the patients calls into question the clinical usefulness of RBC MTX PG_{1-5} as a biomarker for treatment response.

Factors that influence MTX PG concentrations

There is a wide inter-patient variability of RBC MTX PG concentrations in patients receiving low dose MTX therapy (9, 17, 26). Several studies have focused on the genetic factors that contribute to this interpatient variability, specifically common variations in genes encoding enzymes in the folate pathways. As mentioned above, Dervieux et al. (27)evaluated whether polymorphisms in reduced folate carrier (*RFC-1/SLC19A1* G80A) and [gamma]-glutamyl-hydrolase (*GGH*–401C/T) affect MTX PG levels in 226 patients with RA treated with weekly low-dose MTX (median 15 mg, range 5-25mg) for more than 3 months(27). Median total MTX PG was 102 nmoles/liter (range <5 to 358) and median long-chain MTX PG_{3-5} was 56 nmoles/liter (range <5 to 224). The presence of the *RFC-1/SLC19A1* 80AA genotype was associated with increased MTX PG levels (p=0.033) and the presence of the *GGH* 401TT genotype was associated with decreased MTX PG levels (p=0.029). Patients with the *GGH* 401TT genotype were 4.8-fold (OR CI 95% 1.8–13.0; $P = 0.007$) more likely to have MTX $PG_{3–5}$ levels below the group median compared to patients with the *GGH* 401CC or CT genotype. In contrast, patients with the *RFC-1/SLC19A1* 80AA genotype were 3.4-fold more likely to have MTX $PG_{3–5}$ levels above the group median compared to those with the 80GG or 80GA genotype (OR CI 95% 1.4–8.4; $P < 0.001$). In a study of non-RA patients treated with high dose MTX for acute lymphoblastic leukemia, a functional *GGH* C452T resulted in accumulation of RBC MTX PG (28).

Stamp et al. evaluated the non genetic factors that influence MTX PG concentrations in patients receiving long term stable low dose of MTX (26). Their analysis included 192 patients taking oral MTX for at least 3 months, with a stable dosage for at least 1 month prior to study entry. Increased age, lower estimated GFR, higher MTX dose, longer duration of treatment, and use of prednisone were associated with significantly higher MTX PGs concentrations in univariate analysis. Sex, autoantibody status, RBC folate level and body mass index (BMI) had no significant effect on MTX PG levels. Smoking was associated with lower concentrations of MTX PG_3, MTX PG_{3-5}, and MTX PG_{1-5}. Concomitant use of other DMARDs was associated with lower MTX PG_2 levels, and treatment with non-steroidal anti-inflammatory drugs was associated with lower MTX PG_3 and MTX PG_{1-5} concentrations. Multivariate regression analysis revealed that age, MTX dosage, and estimated GFR were the major determinants of MTX PG concentrations.

The influence of route of administration of MTX has recently been studied, albeit in children. Becker et al. evaluated the predictors of RBC MTX PG variability in a cohort of 99 juvenile inflammatory arthritis (JIA) patients who were receiving stable doses of oral (33%) or parenteral (67%) MTX for at least 3 months (13). At the time of the RBC MTX PG measurements, 45.5% patients were concurrently taking folic acid in doses ranging from 0.4 to 5 mg per day. Their results demonstrated a 40-fold inter-individual variability in total intracellular MTX PG concentration, mean 85.8 ± 48.4 nmoles/liter (range 4.8-218.5 nmoles/liter). There was also a significant variability (i.e. 40-100 fold) in individual MTX PG levels. Route of administration (p=0.0002), age (p=0.004), dose (p<0.0001), duration of MTX therapy (p=0.0003), and NSAID use (p=0.07) were associated with total MTX PG

concentrations in multivariable analysis. There was no association between gender, use of anti-TNF agents, or folic acid dose with total MTX PG concentrations. Multivariable analysis revealed that MTX dose (P=0.015), duration of MTX therapy (P=0.005), and route of administration (P=0.004) remained significant predictors of total MTX PG concentrations (R^2= 0.36). Total MTX PG concentrations were significantly greater in patients on subcutaneous MTX versus oral MTX (mean 98.5 ± 50.5 nmol/L subcutaneous, compared to 61.1 ± 32.5 nmol/L oral, p=0.0002), but this difference did not remain significant after correcting for MTX dose. There were differences however in the type of MTX PGs found between the two groups where higher concentrations of MTX PG_{1+2} were observed in patients receiving oral MTX (p=0.003) while higher concentrations of MTX PG_{3-5} were noted in patients receiving MTX subcutaneously (p<0.0001), even after correcting for dose.

A clinically available RBC MTX PG assay

Largely on the basis of research on RBC MTX PG conducted by Dervieux and others, an RBC MTX PG_3 assay is now clinically available under the name Avise PG^{SM} (Cypress Biosciences, Inc., San Diego, CA). The assay requires that 5 ml of whole blood from RA patients who have been on MTX therapy for at least 3 months duration be shipped by overnight courier in a coolant equipped kit to the company's laboratory, which is certified to perform high complexity clinical laboratory testing under CLIA (Clinical Laboratory Improvement Amendments). The methodology includes a proprietary liquid chromatographic method with post column photo-oxidation technique (Cypress Biosciences, Inc., San Diego, CA).

Laboratory test result quality is highly dependent upon proper specimen collection and handling, and samples that are grossly hemolyzed or lipemic are not suitable for the assay (29). Results of the Avise PG^{SM} test are reported as therapeutic (>60 nmol/L); intermediate (20-60 nmol/L); or subtherapeutic (< 20 nmol/L). As stated on the Cypress results form, the results should be interpreted in the context of additional clinical findings, and it is the responsibility of the physician as to how this information is used to guide patient care. In their analysis, Stamp et al. (18) classified RA patients according their DAS28 and RBC MTX PG_{3-5} levels into four categories: Responders (low DAS28 and low MTX PG levels); possibly overtreated (low DAS28 and high MTX PG levels); possibly undertreated (high DAS28 and low MTX PG levels); and resistant (high DAS28 and high MTX PG levels).

Additional questions and future studies

The utility measurement of RBC MTX PG levels as a biochemical marker of treatment response to MTX in RA remains an unresolved clinical issue. Discrepancies in findings reported by different research groups need to be evaluated in additional RA patients, as different PG levels have been reported to be clinically meaningful. For example, Stamp et al. unexpectedly found a positive correlation between MTX PG_5 and multiple clinical factors, including higher disease activity measures, suggesting that there is a group of patients in whom disease remains active despite higher MTX dosages. In contrast, Dervieux and colleagues have found that higher MTX PG_3 levels correlate with less severe disease activity and ostensibly better response to MTX therapy. The role of MTX PG levels as markers of toxicity should also be explored further.

As a next step, a large, longitudinal, prospective observational study of patients with RA beginning MTX should be performed. Such a study would help to resolve reported discordant associations of high MTX PG levels, allow the assessment of stability of MTX PGs over time, and facilitate exploration of associations in different racial/ethnic groups such as African-Americans. In addition, the MTX PG results could be correlated with a variety of other parameters, including radiographic severity and its progression; MTX

toxicity; and genetic variations in genes encoding enzymes and other proteins involved in the folate and adenosine pathways. Ultimately, a randomized trial of using the MTX PG assay to tailor the dose versus not using the assay (standard care) could be performed using efficacy and toxicity of MTX as main outcomes, with other measures, such as radiographic severity or use of add-on biologic agents as secondary outcomes.

Conclusions

The value of RBC MTX polyglutamates as a predictor of response and adverse events to low dose MTX therapy in patients with RA remains unclear because of the conflicting data in the literature, and many important questions remain unanswered. Large, longitudinal prospective studies are needed to examine the relationship between MTX polyglutamate levels, folate polyglutamate concentrations, and treatment response and side effects of low dose MTX therapy.

Acknowledgments

Supported by NIH 2P60 AR048095-06 (UAB Multidisciplinary Clinical Research Center, RP Kimberly, PI; MCRC Project 1, DK Arnett, PI).

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