A short-chain methotrexate polyglutamate as outcome parameter in rheumatoid arthritis patients receiving methotrexate

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Abstract

Objective

Methotrexate (MTX) is a cornerstone in the treatment of rheumatoid arthritis (RA). Although in general MTX is very effective, the major drawback is the large inter-patient variability in clinical response. The circulating levels of MTX polyglutamates (MTXPGs) are supposed to correlate with clinical efficacy, therefore having a potential role in drug monitoring. However, there is a controversial discussion about the importance of methotrexate polyglutamates as outcome parameters in the therapy of rheumatoid arthritis. The aim of the present study was to investigate the formation and pharmacokinetics of MTXPGs and to correlate their concentration with clinical response in MTX-naïve patients.

Methods

The pharmacokinetics of erythrocyte MTXPGs was determined in samples of nineteen MTX-naïve patients by high pressure liquid chromatography (HPLC) using post-column photo-oxidation and fluorimetric detection.
The relationship between erythrocyte concentrations of MTXPGs and the primary outcome parameter DAS-28 was assessed using the Spearman's correlation coefficient.

Results

The short-chain polyglutamate MTXPG2 revealed to be a potential marker for clinical outcome in rheumatoid arthritis with a statistically significant positive correlation of MTXPG2 C_{max} levels and improvement in DAS-28 (+0.518, p=0.023) over 16 weeks. Furthermore, C_{max} levels of MTXPG2 negatively correlated with basophils (-0.478, p=0.038) and eosinophils (-0.531, p=0.019), both pro-inflammatory cells involved in the disease.

Conclusion

MTXPG2 seems to be a potential indicator for clinical response and may serve as a marker for drug monitoring.

Key words
methotrexate, rheumatoid arthritis, methotrexate polyglutamates, outcome

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Introduction

Disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate (MTX) slow down the natural course of rheumatoid arthritis (RA) by reducing joint damage including pain and retarding loss of function. DMARDs are the basis of rheumatoid arthritis therapy and are adequate in many patients, but they are discontinued because of inefficacy in 25% of patients or because of adverse events in 20% of patients (1, 2).

Nevertheless, due to its positive benefit-risk ratio methotrexate remains the first-line drug in the treatment of rheumatoid arthritis not responsive to non-steroidal anti-inflammatory drugs (NSAIDs) alone and is still in focus of research.

Methotrexate was originally developed in the early 1950s as a folate antagonist for the treatment of cancer. Since its common use in rheumatology started after the publication of four controlled studies in 1985 (3), methotrexate is a mainstay in the therapy of rheumatoid arthritis and the most widely used DMARD worldwide – the "anchor drug" in rheumatoid arthritis (4). An important benefit of this drug is to individually adapt doses in accordance of the patient´s disease status (5). If introduced early in the course of disease, methotrexate can prevent or slow down disease progression. Consequently, patients have significantly fewer new erosions and higher levels of radiographic stabilisation in contrast to patients treated with other DMARDs (6).

Although MTX is generally very effective, the major drawback is the large inter-patient variability in clinical response. According to the criteria of the American College of Rheumatology (ACR)-20, it is estimated that clinical response lies between 46 and 65% (7, 8). Serum levels are not suitable for drug monitoring, because the drug is eliminated from plasma within 24 hours (9) and methotrexate is efficacious for one week due to intracellular storage.

In contrast, the circulating levels of MTX polyglutamates (MTXPGs), which are formed intracellulary, are supposed to correlate with clinical efficacy. Both methotrexate and its main metabolite 7-hydroxy-methotrexate (7-OH-MTX) are converted to polyglutamyl derivates by the enzyme folylpolyglutamate synthetase (FPGS). Polyglutamation can be reversed by the enzyme gamma-glutamyl hydrolase (GGH), which facilitates MTX efflux from the cell by catalysing the removal of gamma-linked polyglutamates.

Polyglutamated MTX, which can have up to seven glutamic acid moieties (MTXPG2-7), has several important functions. It not only retains MTX within the cell (10) but it also inhibits dihydrofolate reductase (DHFR), an enzyme which reduces dihydrofolate (DHF) to tetrahydrofolate (THF). THF is the precursor of the biologically active folate cofactor 5-methyl THF, which is required for the generation of methionine from homocysteine and for the synthesis of polyamines (11). Furthermore, polyglutamated MTX inhibits thymidylate synthase (TYMS), which converts deoxyuridylate to deoxythymidylate in the *de novo* pyrimidine biosynthetic pathway (12). Although the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) in the folic acid pathway is not a direct target of MTX, it is influenced by the drug due to its effects on the intracellular folate pool. Consequently, the concentration of MTX polyglutamates in erythrocytes is supposed to correlate with therapeutic efficacy (13, 14).

More detailed information on the role of methotrexate in rheumatic diseases is provided in the following supplement: *Clin Exp Rheumatol* 2010; 28 (Suppl. 61): S15-S18.

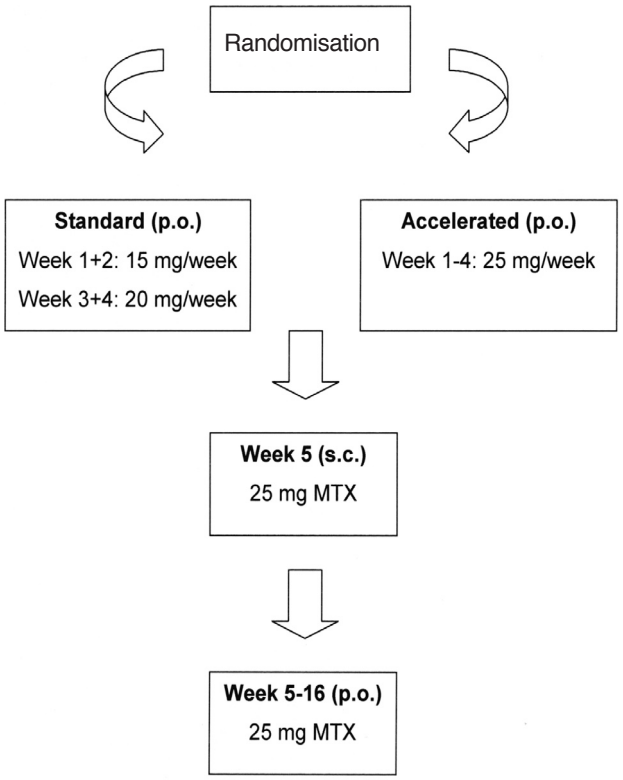
On the basis of recent research reports, it is a controversial issue if methotrexate polyglutamates play a role as outcome parameters in the therapy of rheumatoid arthritis. On the one hand, Dervieux and colleagues stated that the concentrations of long-chain erythrocyte polyglutamates with three or more glutamic residues are associated with therapeutic response (13). On the other hand, Stamp *et al.* showed that polyglutamate concentrations do not correlate with disease activity (19). However, the measurement of erythrocyte blank values before starting MTX-based therapy has not been included in previous studies. Therefore, important information on polyglutama-

tion *per se* is unavailable, which makes it difficult to give a clear statement on metabolite formation.
Thus, the aims of the present project were firstly to investigate the formation and the pharmacokinetics of MTX polyglutamates and secondly to correlate their concentration with clinical response in MTX-naïve patients.

Patients and methods

Patients and study protocol
After obtaining approval from the local ethics committee (Ethikkommission der Stadt Wien) and informed consent from each patient in writing, the randomised, double-blind, controlled clinical trial was performed in accordance with Good Clinical Practice guidelines and ethical principles that have their origin in the Declaration of Helsinki.
As previously described in this journal, MTX-naïve and newly diagnosed RA patients who fulfilled the American College of Rheumatology criteria for rheumatoid arthritis were enrolled at the Medical Department for Rheumatology at the Kaiser-Franz-Josef Hospital in Vienna between July 2008 and July 2009.
Patients with a former MTX use, pulmonary or infectious (HIV, hepatitis B and C) diseases, low disease activity (DAS-28 ≤3.2) or contraindications for MTX were excluded from participation.
The present study was originally designed to compare the pharmacokinetics as well as the clinical response of two different MTX starting doses. Using a stratified randomisation, patients were assigned to start either with 15 mg or 25 mg MTX, administered orally. This route of administration was chosen because in Austria MTX is generally given as an oral therapy. To get a 100% reference level for bioavailability, a subcutaneous dose of 25 mg MTX was administered to all patients at week 5. A weekly oral dose of 25 mg MTX was maintained until week 16 (Fig. 1). Concomitant therapy with non-steroidal anti-inflammatory drugs (NSAIDs – diclofencac, ibuprofen, celecoxib and naproxen) and corticosteroids (prednisolone ≤10 mg per day) was allowed because of ethical reasons and to obviate disease progression. Corticosteroid therapy was started shortly before or even together with MTX to bridge the time until response to MTX was achieved. If justified, the prednisolone dose was reduced in dependence on patient's disease status. Combination therapy with other DMARDs was not permitted during the study. To prevent food-drug-interactions, patients were instructed to take MTX in the fasting state. In addition, 5 mg of folic acid were administered two days after MTX intake to increase the tolerability of methotrexate. Overall, patients were observed for 16 weeks.

Fig. 1. Dosing regimens.



Clinical assessment
Disease activity was assessed using the Disease Activity Score in 28 joints (DAS-28 4v, erythrocyte sedimentation rate (ESR)), the swollen joint count (SJC), the tender joint count (TJC), the duration of morning stiffness (min), PGA (Patient's global assessment), EGA (Evaluator's global assessment) and a modified version of the Health Assessment Questionnaire (HAQ). The intensity of pain and fatigue was measured using a visual analogue scale (VAS, 0-100 mm).
To avoid inter-observer variability, joint counts were performed by a single trained person. Demographic and clinical data were recorded using standardised case record forms (CRFs). In addition, patients received a questionnaire to self-monitor a potential improvement in disease activity and possible adverse events.

Laboratory assessment
Laboratory measurement included a complete blood cell count and liver and kidney function tests. In addition, inflammatory parameters like the ESR and the C-reactive protein (CRP) as well as rheumatoid factors were determined.

Measurement of MTX polyglutamates
For the pharmacokinetic analysis, EDTA whole blood samples were collected immediately before and 1.5, 4, 48, 96 and 168 hours after drug administration at week 1, 5 and 10. These blood collection times were chosen based on published pharmacokinetic data of MTX (20, 21). The C_{max} (maximum plasma concentration of MTX) in plasma is achieved 1.5 hours after drug administration; after 4 hours no further increases in plasma concentrations can be observed. The formation of methotrexate polyglutamates starts

approximately 48 hours after drug intake. A final blood sample was taken at week 16.

To separate red blood cells (RBCs, erythrocytes) from plasma, samples were centrifuged for 20 minutes (500 x g at +4°C). RBCs were washed twice in 3 volumes of Hank's balanced salt solution (HBSS). After vortexing for 15 seconds, RBCs were centrifuged for 10 min at 200 x g each time. The supernatant was discarded. In the next step, packed RBCs were haemolysed with 12 ml of distilled water and vortexed for 15 seconds. Samples were aliquoted in 1 ml tubes and boiled for 10 min at 100°C using a heating block (Eppendorf). After boiling to denature proteins, cells were immediately put on ice for 15 min and finally centrifuged for 15 min (1800 x g at 4°C). The precipitate was discarded; the supernatant was aliquoted in 1 ml tubes and stored at -80°C until analysis.

Erythrocyte methotrexate polyglutamates (MTXPG1-7) were analysed by a high-pressure liquid chromatography (HPLC) method using post-column photo-oxidation followed by fluorimetric detection, as previously described by Dervieux and co-workers (13).

Studies were performed on an Agilent 1100 HPLC Chemstation system consisting of a binary pump, an autoinjector, a fluorimetric detector and a system controller. For post-column photo-oxidation, a photochemical reactor unit equipped with a 254 nm low-pressure mercury UV-lamp and a 1/16-inch (o.d.) teflon tubing (0.25 mm i.d.) (Aura Industries, New York, USA) was implemented between the analytical column and the fluorimetric detector. For chromatographic separation, a LiChroCART® 250-4 LiChrospher® 100 RP-18 endcapped column (5 μm particle size, Merck) protected by a LiChroCART® 4-4 LiChrospher® 100 RP-18 endcapped guard column (5 µm, Merck) was used.

Mobile phase 1 consisted of 10 mM ammonium acetate (pH 6.50), containing 2% of hydrogen peroxide (30% in water); mobile phase 2 of 100% acetonitrile (ACN). After sterile filtration, mobile phases were used at a flow rate of 1.0 ml per min.

To optimise separation, a 17-min linear gradient from 0% to 17% ACN (mobile phase 2) was used. After 17 min, the mobile phase was returned to 100% mobile phase 1 until the stop time of 22 min. Before starting the next analysis cycle, the system was re-equilibrated for 15 min (post-time).

Using fluorimetric detection, MTXPGs were measured at an excitation wavelength set at 274 nm and an emission wavelength set at 470 nm. The fluorimetric signals of MTXPGs were recorded between 7.0 and 14.1 min (correlation coefficients ≥0.996, limit of detection: 0.37 nM for MTXPG1, 0.52 nM for MTXPG2, 2 nM for MTXPG3, 5 nM for MTXPG4, 13 nM for MTXPG5, 28 nM for MTXPG6 and 48 nM for MTXPG7).

The chromatography was performed at ambient temperature. All results were normalised to a blood sample volume of 6 ml to obtain standardised conditions for pharmacokinetic analysis.

Sample size calculation and statistical analysis

The sample size calculation was done to compare two different MTX starting dose regimens, based on a study investigating the pharmacokinetics of 25 mg of methotrexate (22) assuming a 67% higher MTX level in the accelerated dose regime and a dose-proportional bioavailability. We calculated that 9 patients in each dosing group will be sufficient to detect the expected difference in the pharmacokinetics of methotrexate (α=0.05, β=0.80).

The relationship between erythrocyte concentrations of methotrexate polyglutamates and the primary outcome parameter DAS-28 was assessed using the Spearman's correlation coefficient. Secondary outcome parameters were the HAQ-Score, CRP, ESR, VAS pain and VAS fatigue in addition to pharmacokinetic parameters such as the Area under the Curve (AUC), the maximum plasma concentration (C_{max}) and the time to maximum concentration (T_{max}).

Clinical and laboratory parameters before starting MTX and after 16 weeks were compared using the Wilcoxon-test for paired samples (p<0.05).

Results

Demographics

Of the nineteen recruited patients, ten patients received a standard starting dose and nine patients were randomised to the accelerated dosing group. The median age was 56 years. 68% of patients were female and 32% were male. Among the study population, 42% were rheumatoid factor positive. At study entry, rheumatoid arthritis patients had on average 5.9 swollen joints, 8.9 tender joints and scored 52.4 by VAS for joint pain and 44.5 by VAS for fatigue. Mean DAS-28 4v (ESR) was 4.7 and mean HAQ-Score was 1.5.

No statistically significant differences in baseline demographic and clinical characteristics were observed between the two patient groups receiving different dosing schemes (Mann-Whitney U-test for independent samples, p=0.05).

Efficacy of methotrexate-based therapy

Measurement of laboratory and clinical parameters at the end of the study at week 16 demonstrated that MTX significantly reduced DAS-28, HAQ-Score, PGA, EGA, VAS pain, VAS fatigue, the number of swollen and tender joints as well as the duration of morning stiffness and rheumatoid factors (Wilcoxon test for paired samples, p=0.05). ESR was statistically significantly reduced using Student's t-test (p=0.039, CI 95%), but not when using the Wilcoxon test for paired samples (p=0.070). Although the mean C-reactive protein concentrations were decreased by the use of methotrexate, this was not of statistical significance (p=0.076), possibly due to the small sample size.

The present study further confirms the favourable benefit-risk ratio of methotrexate in rheumatoid arthritis. No serious adverse events were noted. Side effects like nausea were reported by approximately half of patients at the beginning of MTX therapy, but were self-limiting.

Measurement of erythrocyte methotrexate polyglutamates using high pressure liquid chromatography

Figure 2 illustrates a typical chromatogram of MTXPG1-7 calibrators (20 nM)

in water. Potential limits of the pharmacokinetic analysis were interferences at the chromatographic positions of MTXPG4 and MTXPG5. Because no statistically significant changes in the concentrations of these polyglutamates were observed during MTX therapy (data not shown), MTXPG4 and MTXPG5 were not included in data analysis. The interferences may be explained by the fact that long-chained MTX-polyglutamates are structural analogs of folic acid and therefore the system possibly was not selective enough to differentiate between natural occurring folates and MTX-polyglutamates. MTXPG6 and MTXPG7 were not observed in the chosen population of rheumatoid arthritis patients, which is in accordance to other clinical trials (13).

Pharmacokinetics of erythrocyte methotrexate polyglutamates

The main pharmacokinetic parameters (C_{max}, T_{max}, AUC and Half-life) of MTXPG1-3 at week 1, 5 and 10 are presented in Table I.

Correlation analysis of erythrocyte polyglutamate concentrations and disease activity measurement

To analyse the relationship of erythrocyte methotrexate polyglutamate levels and laboratory as well as clinical parameters, a correlation analysis calculating the Spearman's coefficient was performed.

MTXPG1. At week 5, C_{max} levels of MTXPG1 (nM) negatively correlated with monocyte counts (-0.507, p=0.027) which play an essential role as inflammatory parameters in rheumatoid arthritis. Further, a negative correlation of C_{max} levels of MTXPG1 (nM) and the duration of morning stiffness (min) was observed at week 16 (-0.616, $p \leq 0.05$), showing a positive impact of MTXPG1 on clinical parameters.

MTXPG2. At week 5, MTXPG2 C_{max} levels positively correlated with improvement in DAS-28 (+0.518, p=0.023) over 16 weeks. Moreover, the Half-life (h) of MTXPG2 negatively correlated with the duration of morning stiffness (min) (-0.975, p=0.005).

In addition, a negative correlation between C_{max} levels of MTXPG2 and eosinophils was observed (-0.559, p=0.013). Because eosinophils play a role in fibrin removal, eosinophilia is a typical characteristic for inflammation and rheumatoid arthritis. Additionally at week 10, C_{max} levels of MTXPG2 negatively correlated with basophils (-0.478, p=0.038) and eosinophils (-0.531, p=0.019) – both important inflammatory cells in rheumatoid arthritis. This observation reveals the importance of MTXPG2 as a marker for response of methotrexate in rheumatoid arthritis. The results of the correlation analysis of MTXPG2 with different clinical and laboratory parameters at week 5 and 10 are presented in Table II.

MTXPG3. MTXPG3 AUC levels (nM*h) as well as C_{max} levels (nM) negatively correlated with the duration of morning stiffness (min) (both -0,567, p=0,011). However, higher levels of MTXPG3 do not seem to serve as an appropriate marker for clinical outcome, because there was a statistically significant positive correlation of the C_{max} levels of MTXPG3 and ESR (+0.505, p=0.027) as well as CRP (+0.627, p=0.004) (data not shown).

Figure 3 shows a scatter-plot of the positive correlation between C_{max} levels of MTXPG2 (nM) and improvement in DAS-28 at week 5. In Figures 4 and 5, the negative correlations of C_{max} levels of MTXPG2 with basophils and eosinophils at week 10 are exemplified.

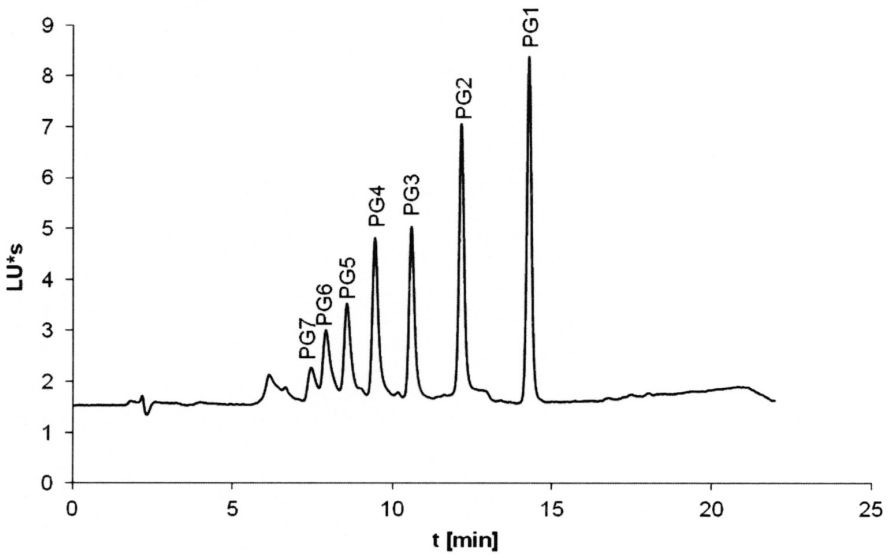


Fig. 2. Chromatogram of MTXPG1-7 calibrators (20 nM) in water.

Table I. Pharmacokinetics of MTXPG1-3: results are expressed as the mean (± SD).

	AUC (nM*h)	T_{max} (h)	C_{max} (nM)	Half-life (h)
MTXPG1				
Week 1	2964 ± (5578)	12.11 ± (37.77)	17.42 ± (8.37)	347 ± (609)
Week 5	12734 ± (11683)	3.61 ± (0.94)	40.92 ± (12.21)	649 ± (611)
Week 10	53001 ± (1.2E5)	3.74 ± (0.79)	41.87 ± (10.57)	1884 ± (3407)
MTXPG2				
Week 1	474 ± (457)	132.50 ± (66.85)	11.06 ± (12.51)	-
Week 5	49145 ± (1.4E5)	111.61 ± (78.62)	15.92 ± (3.99)	7705 ± (10374)
Week 10	8260 ± (14340)	75.47 ± (78.22)	22.06 ± (5.61)	1452 ± (856)
MTXPG3				
Week 1	9925 ± (12607)	84.58 ± (87.13)	45.43 ± (37.69)	324 ± (234)
Week 5	15655 ± (23258)	105.21 ± (78.77)	48.36 ± (34.76)	487 ± (95)
Week 10	90655 ± (1.9E5)	55.89 ± (65.23)	68.67 ± (36.45)	1802 ± (1727)

Discussion

Although in general MTX is very effective, the major drawback is the large inter-patient variability in clinical response, which is estimated to be between 46 and 65% (7, 8). To select patients not responding to MTX in time

Table II. Correlation analysis of MTXPG2 with clinical parameters using Spearman´s correlation coefficient.

MTXPG2	Correlation partner	Correlation	Significance (two-sided)
C_{max} (nM) Week 5	Improvement in DAS-28	+ 0.518	0.023
	Leucocytes (/nl)	+ 0.721	<0.001
	Neutrophils (/nl)	+ 0.669	0.002
	Eosinophils (%)	- 0.559	0.013
C_{max} (nM) Week 10	Eosinophils (%)	- 0.531	0.019
	Basophils (%)	- 0.478	0.038
	Improvement in DAS-28	+ 0.475	0.040
Half-life (h) Week 5	Duration of Morning Stiffness (min)	-0.975	0.005

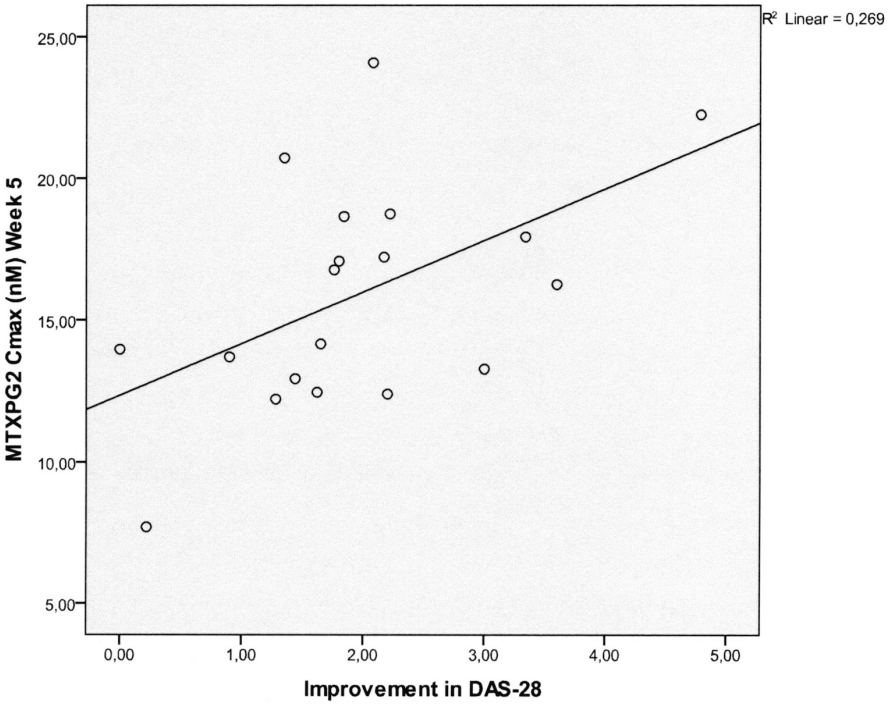


Fig. 3. Correlation of MTXPG2 (C_{max}, nM) at week 5 and improvement in DAS-28 over 16 weeks.

to prevent disease progression and joint destruction, an appropriate drug monitoring would be required. Since the drug is efficacious for at least one week while being eliminated from plasma within 24 hours, serum MTX levels are not suitable for drug monitoring. In contrast, concentrations of erythrocyte MTXPGs are supposed to correlate with clinical response. Nevertheless, on the basis of recent research there is a controversial discussion on the role of MTXPGs in drug monitoring (13, 14, 19). However, previous studies did not include the measurement of blank erythrocyte values before starting MTX, with the consequence that important information on basal polyglutamation is not available. To our knowledge, this is the first pharmacokinetic study including the measurement of erythrocyte blank values before starting MTX-based therapy in rheumatoid arthritis patients. Our results on polyglutamation of methotrexate now challenge the existing opinion that only long-chain polyglutamates with more than three glutamic residues correlate with clinical response in rheumatoid arthritis.

Contrary to existing theories (13, 14, 19), we found MTXPG2, a short-chain polyglutamate, to statistically significantly correlate with the DAS-28 4v (ESR), which incorporates laboratory as well as subjective parameters and is an accepted tool for monitoring disease progression. Furthermore, a negative correlation of MTXPG2 and the duration of morning stiffness was observed. In addition, C_{max} of MTXPG2 negatively correlated with circulating levels of eosinophils. Although cases of massive eosinophilia are rare in rheumatoid arthritis, it occurs as part of the disease process (23). Eosinophils are not a notable feature of the synovial membrane infiltrate or cellular joint exudate, but they indirectly participate in the inflammatory reaction of rheumatoid arthritis. This is reflected by high serum levels of the eosinophil cationic protein, an eosinophil specific granule protein. Higher levels of the eosinophil cationic protein appear to be associated with a more aggressive course of rheumatoid arthritis (24). Moreover, a negative correlation of basophils and C_{max} levels of MTXPG2 was observed. Although research results do not indicate a substantial role of basophils in rheumatoid arthritis, there is some evidence that basophils are increased in inflammatory diseases, for example in children with active polyarticular arthritis (25).

Altogether, the correlation of MTXPG2 with laboratory as well as clinical parameters suggests its relevance for drug monitoring.

Compared to previous studies (13, 14), we can not confirm that higher levels of the longer-chain polyglutamate MTXPG3 correspond to a more favourable clinical outcome. In contrast, in our study a statistically significant positive correlation of the C_{max} levels of MTXPG3 and ESR as well as CRP was observed. Firstly, this advocates that primarily the concentration of short-chain polyglutamates is suitable for predicting clinical outcome when using methotrexate. Secondly, our results may be explained by the fact that the influence of MTX therapy on CRP and ESR did not reach statistical significance, probably due to the proportionally small sample size.

In addition to HPLC interferences, other potential limitations of our study are the proportionally small sample size and that patients were allowed to take concomitant medications like corticosteroids and NSAIDs, which may influence the pharmacokinetics of methotrexate.

Another consideration is that information on intracellular folate concentra-

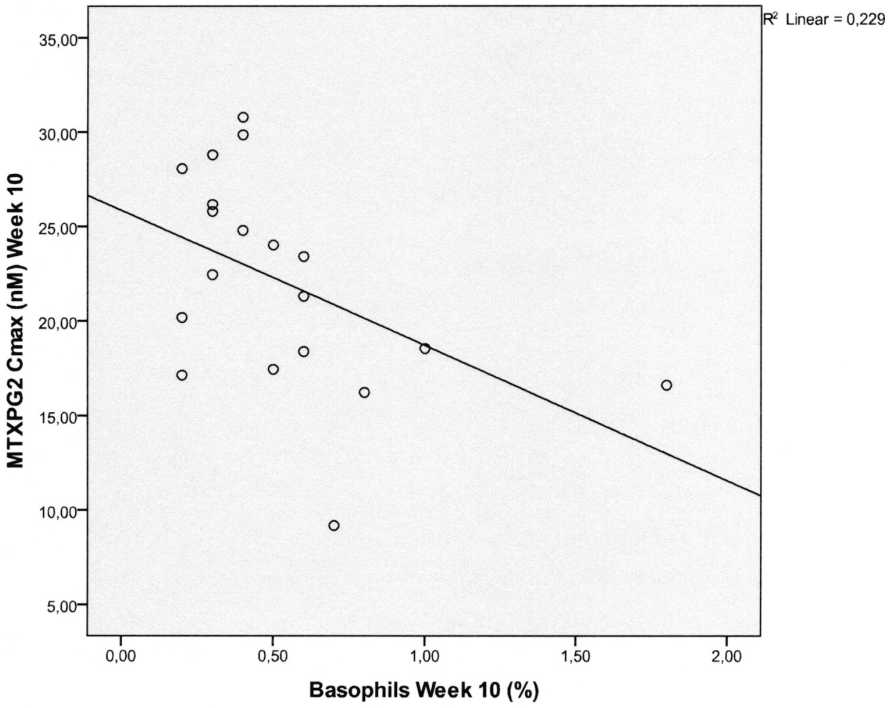


Fig. 4. Correlation of MTXPG2 (C_{max}, nM) and basophils (%) at week 10.

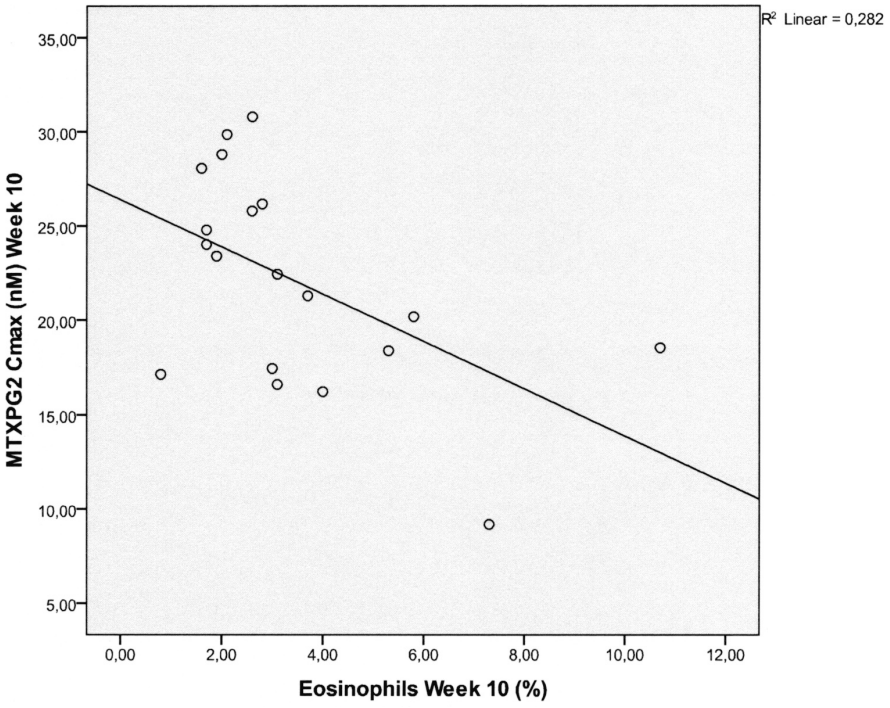


Fig. 5. Correlation of MTXPG2 (C_{max}, nM) and eosinophils (%) at week 10.

tions is lacking. Although we measured serum folate levels and no correlation with erythrocyte polyglutamate levels was found, erythrocyte folates were not determined in our study. It is well known that high concentrations of intracellular folates can result in a decrease in MTX polylgutamation. Because erythrocyte folate levels have been shown to be related to disease activity and clinical response, this point should be evaluated in future investigations.

Conclusions

To improve MTX therapy and select patients not responding to the drug in time, the measurement of MTX erythrocyte concentrations is an appropriate tool. In particular, the short-chain polyglutamate MTXPG2 seems to be a potential predictor for clinical response and may serve as a marker for drug monitoring.

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