Therapeutic Drug-Monitoring of Methotrexate-Polyglutamates in Rheumatoid Arthritis

Therapeutic Drug-Monitoring of Methotrexate-Polyglutamates in Rheumatoid Arthritis

Therapeutische bloedspiegel bepaling van methotrexaat-polyglutamaten bij reumatoïde artritis

Proefschrift

Ter verkrijging van de graad doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.A.P. Pols
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
18 juni 2014 om 13:30

door

Ethan den Boer

Geboren te Rotterdam

ERASMUS UNIVERSITEIT ROTTERDAM

Promotie commissie

Promotor: Prof.dr. J. Lindemans

Overige leden: Prof.dr. I.P. Kema
                         Prof.dr. P.L.C.M. van Riel
                         Prof.dr. A.G. Vulto

Copromotoren: dr. R. de Jonge
                         dr. T.M. Luider

The anthropologists got it wrong when they named our species Homo sapiens ('wise man'). In any case it's an arrogant and bigheaded thing to say, wisdom being one of our least evident features. In reality, we are Pan narrans, the storytelling chimpanzee.

— Terry Pratchett, Ian Stewart and Jack Cohen - *The Science of Discworld II: The Globe*

Table of contents

Chapter 1

Introduction

Reumatoid Arthritis

Rheumatoid Arthritis (RA) is a chronic autoimmune disease, characterized by the swelling of joints, uncontrolled proliferation of synovial tissue and multisystem co-morbidities. RA mainly affects the joints of the hands, feet, knees, wrist and elbows, with joint damage occurring early in the disease course. RA affects an estimated 1% of the general population and women have a higher risk of developing RA than men. Despite the fact that treatment strategy has changed considerably over the years, reflected by a much improved disease outcome, there is still no cure for RA.

Early initiation of therapy is effective in prevention of joint damage and results in milder medication regimes while maintaining disease remission. [1-4] Early in the disease, the inflammation is less self-perpetuating and easier to suppress, therefore it is important to start treatment as early as possible in order to optimize outcome, minimize medical costs, improve quality of life, and improve medical decision making.

MTX as cornerstone in RA treatment

Most newly diagnosed chronic inflammatory arthritis patients are prescribed methotrexate (MTX) monotherapy with glucocorticoid bridging therapy during the first months as recommended by the guidelines of the European League Against Rheumatism (EULAR). [4-6] Although the exact workings of MTX in RA are still unknown, MTX is the 'gold standard' in the treatment of various forms of chronic arthritis because it is an effective, safe and inexpensive drug. [6, 7]

MTX is administered in a fixed (non-personalized) dose, which can be increased when response is insufficient. Optimal treatment dosage is very different from patient to patient and a significant number of patients do not achieve sufficient remission (20-40%) or develop adverse events (30%), leading some patients to stop treatment. [8] The EULAR guidelines for treatment recommend to strive for remission in the first three months of treatment. [4-6] In order to achieve this, patients are monitored quite closely and every 1-3 months disease activity is measured. If the target remission in disease activity is not achieved, treatment is intensified by increasing the dose or adding co-medication such as biologicals. This leads to an unpredictable search for the right treatment and dose of MTX until it is determined by trial and error.

Therapeutic drug monitoring and individualized treatment

Individualized MTX therapy could result in better and faster disease control (lower disease activity and less erosive damage) without the need to titrate MTX over the course of treatment. This could potentially result in a higher quality of life with less adverse events leading to improved drug compliance.

A method for the prediction or early detection of non-response is not yet available, although some models have been proposed. [9-11] Reliable instruments to predict MTX

response/adverse-events and to adjust MTX dosing (individualized therapy) in daily routine patient care are lacking.

Therapeutic drug monitoring (TDM) of intracellular MTX concentrations may help to predict response to treatment and adverse events. Generally plasma levels of a drug are used for TDM and MTX levels can be easily measured in plasma, which is used for TDM in high-dose treatment of acute lymphoblastic leukemia (ALL). However, during low-dose MTX treatment such as in RA there is no accumulation in plasma because of the short half-life of <12 hours. MTX is rapidly cleared from the plasma and is unrelated to response. Therefore, MTX plasma levels are not a reliable tool for TDM in RA. [12-15]

In recent years, multiple pharmacogenetic studies found associations between several polymorphisms in the purine and folate metabolism and MTX response. [10, 16-19] These studies have shown that polymorphisms in folate pathway genes contribute significantly in predicting response to MTX. In addition, we have recently shown that the erythrocyte folate levels also predict response to MTX. [20] Next to these factors, the MTX concentration in erythrocytes has been associated with response in multiple studies. [12, 14, 16, 17, 21, 22] The intracellular MTX levels even override the contribution of certain genetic polymorphisms to predict MTX efficacy, [10, 16] making intracellular MTX an interesting target for TDM. [12, 16, 17, 21, 23-28] A cut-off value for response has been proposed by one research group, they found that patients with less then 60 nmol of MTX-PG3 per liter of erythrocytes were 4.4-fold more likely to have a poor response to MTX. [16]

Although evidence exists for the presence of a correlation between erythrocyte MTX levels and response, some studies have not found a correlation between erythrocyte MTX and response. [29, 30] However, most studies that investigated the relation between erythrocyte MTX levels and response were cross-sectional in nature and included patients that had been treated with MTX for extended period of time. This could lead to a selection bias for well tolerating, but low/moderately-reacting patients thereby influence the results.

Pharmacodynamics/metabolism of MTX

MTX is generally administered weekly by orally administered tablets, but can be given subcutaneous (or intramuscular) when response is insufficient, when patients do not tolerate oral tablets or when compliance is low. Orally administered MTX is rapidly absorbed in the small intestine and in RA maximum plasma concentrations are reached by 1.5 hours under fasting conditions. [31-33] Parenteral administration leads to more rapid absorption and higher plasma levels compared to oral intake. In plasma, the major metabolite of MTX is 7-OH MTX, which is thought to be formed in the liver and excreted in the bile. The other metabolite that is found in plasma is 4-amino-4-deoxy-N-methylpteroic acid (DAMPA), which is most likely formed by gut-bacteria.

Methotrexate is structurally similar to folic acid (Figure 1) and is therefore thought to share most of the uptake and metabolism routes. [25, 34] MTX is transported into the cell primarily by the reduced folate carrier (RFC). While circulating MTX contains one glutamate

moiety (MTX-PG1), intracellular MTX consists of multiple metabolites with a varying amount of glutamate groups (Figure 1). Once in the cell, extra glutamate moieties are added in a ATP dependent process by folylpolyglutamate synthase (FPGS) to MTX polyglutamates (MTX-PGs) by γ-linked sequential addition of glutamic acid residues. [35] In a competing reaction, the MTX-PGs are deconjugated by γ-glutamyl hydrolase (GGH), returning MTX to its monoglutamate form, which is pumped out of the cell by the ATP-binding cassette (ABC) family of transporters. The balance between import, export, polyglutamation and deconjugation leads to a variety of MTX-PG chain-lengths of up to 7 glutamates (MTX-PG2-7, Figure 2). The concentration and distribution of these MTX-PGs is dependent on multiple factors such as MTX dosage, route of administration and age. [13, 23, 27, 36, 37] Multiple SNPs in the folate pathway have also been shown to be associated with MTX-PG status. [12, 38, 39]
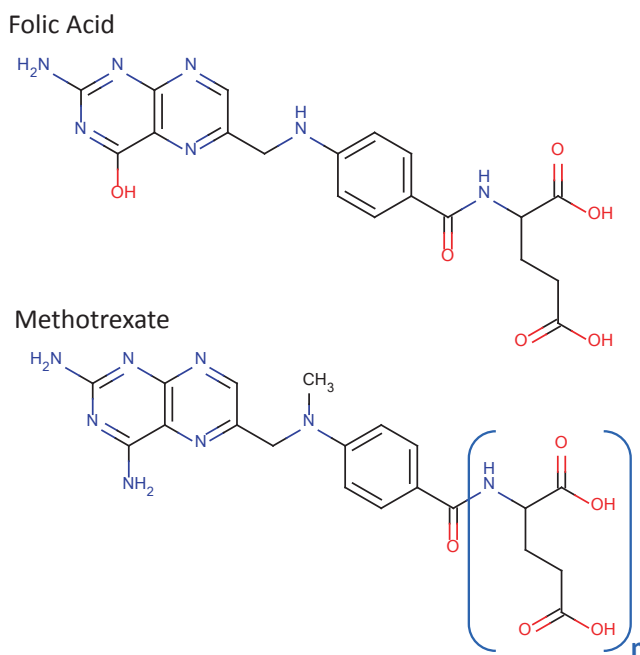
Figure 1. Molecular structures of folate and methotrexate. The glutamate group of methotrexate is indicated between brackets, up to 6 additional (n=7) glutamate groups are added during polyglutamation in the cell.

Polyglutamation is important for MTX treatment as it is responsible for increasing the size of the molecule, making it much more anionic. As a result MTX-PGs cannot diffuse out of the cell. [40] Also, the efflux of MTX is severely hindered after polyglutamation. In fact, with increasing polylgutamation and a longer MTX-PG chain, the efflux rapidly decreases. While MTX-PG1 is actively pumped out of the cell by the ABCC1 to ABCC4 efflux transporters, MTX-PG2 and MTX-PG3 are only transported by the ABCC5 and ABCG2 efflux transporters and there is no evidence of MTX-PG4-7 being transported out of the cell (Figure 2). The results hereof is a build-up of MTX-PGs in the cell over time. [40]

Adapted from Dalrymple et al. Arthritis Rheum. 2008 Nov;58(11):3299-308
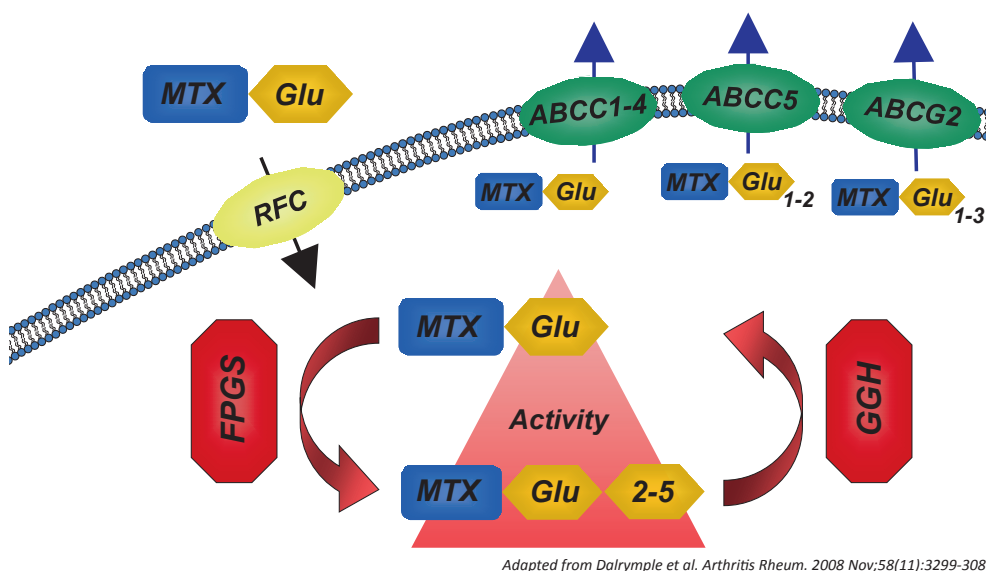
Figure 2. MTX-PG metabolism in the cell. After entering the cell via the reduced folate carrier (RFC), MTX is polyglutamates by FPGS, increasing activity and leading to retention of intracellular MTX as MTX-PGs are not a suitably substrate for the efflux proteins. In a competing reaction, MTX-PGs are de-glutamated by GGH, leading to a dynamic equilibrium that is dependent on the import, export and (de-)polyglutamation of MTX.

MTX: Methotrexate; Glu: glutamate group; RFC: reduced folate carrier; ABCC1-5/ ABCG2: ATP-binding cassette (ABC) transporters; FPGS: Folylpolyglutamate synthase; GGH: gamma-glutamyl hydrolase.

The importance of MTX-PGs

The polyglutamated forms of MTX also have significantly higher affinity for its targets enzymes compared to native MTX and efficacy of MTX is thought to increase with the polyglutamation levels. [10, 12, 23, 28, 41, 42] In low-dose MTX treatment, the setting for RA treatment, the pentaglutamate (MTX-PG5) is the highest order of glutamylation detected, while the triglutamate form (MTX-PG3) of MTX predominates. [13, 22, 27, 36, 43, 44]

In line with the highly variable clinical response to MTX, the time to reach steady-state erythrocyte MTX levels is highly variable between patients as are the steady state erythrocyte MTX levels. [13, 14, 37] This link between inter-patient MTX response variability and the large inter-individual variation in the rate and extent of erythrocyte MTX accumulation suggests that erythrocyte MTX measurement may be a valuable tool for the clinician to individualize MTX treatment in an early phase in order to achieve faster disease remission and less erosive damage.

In low dose MTX, the median time to reach steady-state erythrocyte MTX levels is highly variable between patients and increases with the number of PGs attached to MTX. [13, 37] For example, MTX-PG3 has a median time to reach steady-state of 41.2 weeks (range 19.8-66.7 weeks) compared to 139.8 weeks (range 15.5-264.0 weeks) for MTX-PG5.

[13] Steady-state levels also are highly variable between patients: total erythrocyte MTX-PG concentration varied between 90.9 and 351.5 nmol/$8*10^{12}$ erythrocytes. [13, 14, 23, 27, 43] The mechanisms and determinants of the highly variable intracellular MTX-PG levels are still not completely known, as most studies focus on the prediction of response/adverse events. Previous research has shown that increased age, higher dose, route of administration and decreased renal function are associated with higher erythrocyte MTX-PG levels.27, [36, 37] A relation betwee multiple single nucleotide polymorphisms (SNP) in MTX pathway genes with MTX-PG levels has also been suggested. [24, 28] However, these studies used cross-sectional cohorts with a wide range of treatment duration between patients making it difficult to interpret the results or extrapolate them to a currently treated patient. Also, none of the studies included an independent cohort to validate their findings.

Measurement of MTX-PGs

The physiological action of Methotrexate (MTX) is most likely exerted by the white blood cells. However, when determining MTX-PGs in the cells, erythrocytes are mainly chosen. Erythrocytes are a more accessible, cost-effective and patient-friendly resource because of the high amounts of erythrocytes in the blood and the ease of isolation, facilitating clinical implementation. As erythrocytes lack the cellular machinery for the polyglutamation of MTX, the erythrocyte MTX-PG levels are considered to be a representation of the incorporation of MTX-PGs in the progenitor cell and are thought to function as a surrogate marker for the white blood cell levels. [16, 45]

In contrast to plasma levels, intracellular levels are difficult to measure because the intracellular MTX content consists of multiple MTX-PG species and specialty equipment like HPLC or LC-MS/MS is needed. Of the methods aimed towards the determination of intracellular methotrexate, a large part is devoted to the measurement of total intracellular MTX instead of the separate MTX-PGs.

Total intracellular MTX can be assessed by fluorescence polarization immunoassay (FPIA), when adapted to measure in hemolysate. [46, 47] This utilises the cross reactivity between MTX species to measure all MTX-PGs. A specific problem that arises with immunoassay-based methods is the high cross reactivity of the assays. While this enables the measurement of the intracellular MTX-PGs, it also causes interference from other MTX metabolites such as DAMPA and 7-OH-MTX. [48, 49] Other methods generally break-down the intracellular MTX-PGs by enzymatic hydrolysis to MTX, which is then measured. [26, 50, 51] This has as the benefit that it shortens run-time and simplifies interpretation. However, total intracellular MTX levels might be influenced by high amounts of MTX present in the plasma, as MTX can diffuse over the erythrocyte membrane. [52] This makes it very important to know when patients take their medication, in order to plan the blood sampling outside the window of high plasma MTX.

Intracellular MTX-PG concentrations can be determined by analytical techniques such as Radio ligand binding with fractioning of the eluate, [53] capillary-zone electrophoresis

[54] and high-performance liquid chromatography with post-column photo-oxidation. [43] However, these techniques are laborious, can be influenced by interference from endogenous folates, [44] and use equipment not generally available at a clinical lab. Therefore, they are less suitable for routine measurement in patients.

Recently, two MS based methods have been described for the measurement of intracellular MTX-PGs. However, one method was designed for measuring in the Caco-2 cell-line, and both sample preparation and chromatography is not convertable to erythrocytes. [55] The other method consists of an ion-pairing liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay. [44, 56] In multi-assay mass spectrometry (MS), the use of ion-pairing can lead to interference with the other assays and needs extensive clean-up between runs, which is not ideal for routine clinical application. Most importantly though, neither of these methods used stable-isotope-labelled internal standards for each MTX-PG, and are therefore not able to compensate for recovery and matrix-effects for the individual MTX-PG.

Aims of this thesis

The aims of this thesis were:

1)   To develop a quantitative method for the measurement of erythrocyte MTX-PG.
2)   To examine which clinical, genetic, socio-demographic, and biochemical factors influence the erythrocyte MTX-PG levels in patients treated with low-dose MTX.
3)   To examine the prospective accumulation of MTX-PGs in erythrocytes during low-dose pulse MTX treatment and its association with response.

Outline of the thesis

In *Chapter 2* we describe the development of a new bioanalytical assay to measure erythrocyte MTX-PGs in using a novel high-throughput rapid mass-spectrometric technology, which merges a high-repetition matrix-assisted laser desorption/ionization (MALDI) source with a triple quadruple mass analyzer.

Because the MALDI-MS/MS is a very specific and non-standard technology, it might not be possible for the routine clinical lab to obtain such a machine and a more 'standard' method would be useful. Therefore in *Chapter 3* we show the development and clinical validation of a new LC-MS/MS based assay for the determination of the erythrocyte MTX-PGs, and the subsequent application of the method in 50 samples from MTX treated patients.

Although plasma levels are cleared within 24 hours and do not correlate with response, being able to precisely measure the plasma levels may be important for pharmacokinetic studies and other disease types. An interference free method to measure plasma MTX is also important during glucarpidase treatment to rescue patients from toxic MTX levels. [57-59] Therefore we have adapted the method described in chapter 3 for the

measurement of MTX in plasma. ***Chapter 4*** describes the development and validation of a new LC-MS/MS based method for the determination of MTX in plasma, and the subsequent comparison to the in-house immune-assay.

As not every routine clinical lab will have an LC-MS/MS system available for the measurement of MTX-PGs, an easy and accessible method for the determination of total MTX-PGs in erythrocytes using a routine immune-analyser is described in ***Chapter 5***.

The factors that influence the erythrocyte MTX-PGs levels are not clear yet, therefore in ***Chapter 6*** we apply this method in two independent prospective study populations of MTX treated RA patients in order to determine the biological, genetic and behavioral factors that determine the intracellular levels of MTX-PGs.

Because there is little information available about the effect of different MTX dosing schemes on the concentration, distribution and speed of accumulation of the intracellular MTX-PGs we examined the concentration profile of erythrocyte MTX-PGs over 9 months of treatment in 2 study populations of MTX treated patients in ***Chapter 7***.

There have been multiple studies that show a relation between erythrocyte MTX-PGs and clinical response. However, no large prospective studies with a validation cohort have been performed. In ***Chapter 8***, we show the association of erythrocyte MTX-PGs and disease activity using two independent prospective cohorts.

Finally, in ***Chapter 9*** we discuss the results and implications of our studies.

Chapter

1

References

1   Lard LR, Visser H, Speyer I, vander Horst-Bruinsma IE, Zwinderman AH, Breedveld FC, et al. Early versus delayed treatment in patients with recent-onset rheumatoid arthritis: comparison of two cohorts who received different treatment strategies. Am J Med. 2001 Oct 15;111(6):446-51.

2   van der Heide A, Jacobs JW, Bijlsma JW, Heurkens AH, van Booma-Frankfort C, van der Veen MJ, et al. The effectiveness of early treatment with "second-line" antirheumatic drugs. A randomized, controlled trial. Ann Intern Med. 1996 Apr 15;124(8):699-707.

3   van Nies JA, Krabben A, Schoones JW, Huizinga TW, Kloppenburg M, van der Helm-van Mil AH. What is the evidence for the presence of a therapeutic window of opportunity in rheumatoid arthritis? A systematic literature review. Ann Rheum Dis. 2013 Apr 9.

4   Smolen JS, Aletaha D, Bijlsma JW, Breedveld FC, Boumpas D, Burmester G, et al. Treating rheumatoid arthritis to target: recommendations of an international task force. Ann Rheum Dis. 2010 Apr;69(4):631-7.

5   Smolen JS, Landewe R, Breedveld FC, Buch M, Burmester G, Dougados M, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update. Ann Rheum Dis. 2014 Mar 1;73(3):492-509.

6   Smolen JS, Landewe R, Breedveld FC, Dougados M, Emery P, Gaujoux-Viala C, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs. Ann Rheum Dis. 2010 Jun;69(6):964-75.

7   Singh JA, Furst DE, Bharat A, Curtis JR, Kavanaugh AF, Kremer JM, et al. 2012 update of the 2008 American College of Rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis. Arthritis Care Res (Hoboken). 2012 May;64(5):625-39.

8   Stamp LK, Roberts RL. Effect of genetic polymorphisms in the folate pathway on methotrexate therapy in rheumatic diseases. Pharmacogenomics. 2011 Oct;12(10):1449-63.

9   Bulatovic M, Heijstek MW, Van Dijkhuizen EH, Wulffraat NM, Pluijm SM, de Jonge R. Prediction of clinical non-response to methotrexate treatment in juvenile idiopathic arthritis. Ann Rheum Dis. 2012 Sep;71(9):1484-9.

10   Wessels JA, van der Kooij SM, le Cessie S, Kievit W, Barerra P, Allaart CF, et al. A clinical pharmacogenetic model to predict the efficacy of methotrexate monotherapy in recent-onset rheumatoid arthritis. Arthritis Rheum. 2007 Jun;56(6):1765-75.

11   de Jong PH, Quax RA, Huisman M, Gerards AH, Feelders RA, de Sonnaville PB, et al. Response to glucocorticoids at 2 weeks predicts the effectiveness of DMARD induction therapy at 3 months: post hoc analyses from the tREACH study. Ann Rheum Dis. 2013 Oct;72(10):1659-63.

12   Chabner BA, Allegra CJ, Curt GA, Clendeninn NJ, Baram J, Koizumi S, et al. Polyglutamation of methotrexate. Is methotrexate a prodrug? J Clin Invest. 1985 Sep;76(3):907-12.

13   Dalrymple JM, Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Barclay ML. Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. Arthritis Rheum. 2008 Nov;58(11):3299-308.

14   Hornung N, Ellingsen T, Attermann J, Stengaard-Pedersen K, Poulsen JH. Patients with rheumatoid arthritis treated with methotrexate (MTX): concentrations of steady-state erythrocyte MTX correlate to plasma concentrations and clinical efficacy. J Rheumatol. 2008 Sep;35(9):1709-15.

15   Bannwarth B, Pehourcq F, Schaeverbeke T, Dehais J. Clinical pharmacokinetics of low-dose pulse methotrexate in rheumatoid arthritis. Clin Pharmacokinet. 1996 Mar;30(3):194-210.

16   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Caldwell J, et al. Pharmacogenetic and metabolite measurements are associated with clinical status in patients with rheumatoid arthritis treated with methotrexate: results of a multicentred cross sectional observational study. Ann Rheum Dis. 2005 Aug;64(8):1180-5.

17   Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum. 2006 Oct;54(10):3095-103.

18   van der Straaten RJ, Wessels JA, de Vries-Bouwstra JK, Goekoop-Ruiterman YP, Allaart CF, Bogaartz J, et al. Exploratory analysis of four polymorphisms in human GGH and FPGS genes and their effect in methotrexate-treated rheumatoid arthritis patients. Pharmacogenomics. 2007 Feb;8(2):141-50.

19   Kooloos WM, Wessels JA, van der Straaten T, Allaart CF, Huizinga TW, Guchelaar HJ. Functional polymorphisms and methotrexate treatment outcome in recent-onset rheumatoid arthritis. Pharmacogenomics. 2010 Feb;11(2):163-75.

20   de Rotte MC, de Jong PH, Pluijm SM, Calasan MB, Barendregt PJ, van Zeben D, et al. Association of low baseline levels of erythrocyte folate with treatment nonresponse at three months in rheumatoid arthritis patients receiving methotrexate. Arthritis Rheum. 2013 Nov;65(11):2803-13.

21   Angelis-Stoforidis P, Vajda FJ, Christophidis N. Methotrexate polyglutamate levels in circulating erythrocytes and polymorphs correlate with clinical efficacy in rheumatoid arthritis. Clin Exp Rheumatol. 1999 May-Jun;17(3):313-20.

22   Hobl EL, Jilma B, Erlacher L, Duhm B, Mustak M, Broll H, et al. A short-chain methotrexate polyglutamate as outcome parameter in rheumatoid arthritis patients receiving methotrexate. Clin Exp Rheumatol. 2012 Mar-Apr;30(2):156-63.

23   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Walsh M, et al. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum. 2004 Sep;50(9):2766-74.

24   Dervieux T, Kremer J, Lein DO, Capps R, Barham R, Meyer G, et al. Contribution of common polymorphisms in reduced folate carrier and gamma-glutamylhydrolase to methotrexate polyglutamate levels in patients with rheumatoid arthritis. Pharmacogenetics. 2004 Nov;14(11):733-9.

25   Kremer JM, Lee JK. The safety and efficacy of the use of methotrexate in long-term therapy for rheumatoid arthritis. Arthritis Rheum. 1986 Jul;29(7):822-31.

26   Hroch M, Tukova J, Dolezalova P, Chladek J. An improved high-performance liquid chromatography method for quantification of methotrexate polyglutamates in red blood cells of children with juvenile idiopathic arthritis. Biopharm Drug Dispos. 2009 Apr;30(3):138-48.

27   Becker ML, van Haandel L, Gaedigk R, Lasky A, Hoeltzel M, Stobaugh J, et al. Analysis of intracellular methotrexate polyglutamates in patients with juvenile idiopathic arthritis: effect of route of administration on variability in intracellular methotrexate polyglutamate concentrations. Arthritis Rheum. 2010 Jun;62(6):1803-12.

28   Becker ML, Gaedigk R, van Haandel L, Thomas B, Lasky A, Hoeltzel M, et al. The effect of genotype on methotrexate polyglutamate variability in juvenile idiopathic arthritis and association with drug response. Arthritis Rheum. 2011 Jan;63(1):276-85.

29   Stamp LK, Barclay ML, O'Donnell JL, Zhang M, Drake J, Frampton C, et al. Effects of changing from oral to subcutaneous methotrexate on red blood cell methotrexate polyglutamate concentrations and disease activity in patients with rheumatoid arthritis. J Rheumatol. 2011 Dec;38(12):2540-7.

30   Stamp LK, O'Donnell JL, Chapman PT, Zhang M, James J, Frampton C, et al. Methotrexate polyglutamate concentrations are not associated with disease control in rheumatoid arthritis patients receiving long-term methotrexate therapy. Arthritis Rheum. 2010 Feb;62(2):359-68.

31   Bleyer WA. The clinical pharmacology of methotrexate: new applications of an old drug. Cancer. 1978 Jan;41(1):36-51.

32   Lebbe C, Beyeler C, Gerber NJ, Reichen J. Intraindividual variability of the bioavailability of low dose methotrexate after oral administration in rheumatoid arthritis. Ann Rheum Dis. 1994 Jul;53(7):475-7.

33   Kozloski GD, De Vito JM, Kisicki JC, Johnson JB. The effect of food on the absorption of methotrexate sodium tablets in healthy volunteers. Arthritis Rheum. 1992 Jul;35(7):761-4.

34   Kremer JM, Galivan J, Streckfuss A, Kamen B. Methotrexate metabolism analysis in blood and liver of rheumatoid arthritis patients. Association with hepatic folate deficiency and formation of polyglutamates. Arthritis Rheum. 1986 Jul;29(7):832-5.

35   Gangjee A, Dubash NP, Zeng Y, McGuire JJ. Recent advances in the chemistry and biology of folypoly-gamma-glutamate synthetase substrates and inhibitors. Curr Med Chem Anticancer Agents. 2002 May;2(3):331-55.

36   Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Frampton C, James J, et al. Determinants of red blood cell methotrexate polyglutamate concentrations in rheumatoid arthritis patients receiving long-term methotrexate treatment. Arthritis Rheum. 2009 Aug;60(8):2248-56.

37   Dervieux T, Zablocki R, Kremer J. Red blood cell methotrexate polyglutamates emerge as a function of dosage intensity and route of administration during pulse methotrexate therapy in rheumatoid arthritis. Rheumatology (Oxford). 2010 Dec;49(12):2337-45.

38   Jolivet J, Schilsky RL, Bailey BD, Chabner BA. The synthesis and retention of methotrexate polyglutamates in cultured human breast cancer cells. Ann N Y Acad Sci. 1982 Dec 10;397:184-92.

39   Jolivet J, Schilsky RL, Bailey BD, Drake JC, Chabner BA. Synthesis, retention, and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. J Clin Invest. 1982 Aug;70(2):351-60.

40   Assaraf YG. The role of multidrug resistance efflux transporters in antifolate resistance and folate homeostasis. Drug Resist Updat. 2006 Aug-Oct;9(4-5):227-46.

41   Dervieux T, Kremer J. Methotrexate polyglutamate concentrations and association with disease control in rheumatoid arthritis: comment on the article by Stamp et al. Arthritis Rheum. 2010 Aug;62(8):2559-60; author reply 60-1.

42   Dervieux T, Wessels JA, Kremer JM, Padyukov L, Seddighzadeh M, Saevarsdottir S, et al. Patterns of interaction between genetic and nongenetic attributes and methotrexate efficacy in rheumatoid arthritis. Pharmacogenet Genomics. 2012 Jan;22(1):1-9.

43   Dervieux T, Orentas Lein D, Marcelletti J, Pischel K, Smith K, Walsh M, et al. HPLC determination of erythrocyte methotrexate polyglutamates after low-dose methotrexate therapy in patients with rheumatoid arthritis. Clin Chem. 2003 Oct;49(10):1632-41.

44   van Haandel L, Becker ML, Leeder JS, Williams TD, Stobaugh JF. A novel high-performance liquid chromatography/mass spectrometry method for improved selective and sensitive measurement of methotrexate polyglutamation status in human red blood cells. Rapid Commun Mass Spectrom. 2009 Dec;23(23):3693-702.

45   French D, Yang W, Cheng C, Raimondi SC, Mullighan CG, Downing JR, et al. Acquired variation outweighs inherited variation in whole genome analysis of methotrexate polyglutamate accumulation in leukemia. Blood. 2009 May 7;113(19):4512-20.

46   Hayashi H, Fujimaki C, Tsuboi S, Matsuyama T, Daimon T, Itoh K. Application of fluorescence polarization immunoassay for determination of methotrexate-polyglutamates in rheumatoid arthritis patients. Tohoku J Exp Med. 2008 May;215(1):95-101.

47   Inoue S, Hashiguchi M, Kawai S, Mochizuki M. Erythrocyte methotrexate-polyglutamate assay using fluorescence polarization immunoassay technique: application to the monitoring of patients with rheumatoid arthritis. Yakugaku Zasshi. 2009 Aug;129(8):1001-5.

48   Eksborg S, Albertioni F, Rask C, Beck O, Palm C, Schroeder H, et al. Methotrexate plasma pharmacokinetics: importance of assay method. Cancer Lett. 1996 Nov 29;108(2):163-9.

49   Albertioni F, Rask C, Eksborg S, Poulsen JH, Pettersson B, Beck O, et al. Evaluation of clinical assays for measuring high-dose methotrexate in plasma. Clin Chem. 1996 Jan;42(1):39-44.

50   Schroder H, Heinsvig EM. Enzymatic assay for methotrexate in erythrocytes. Scand J Clin Lab Invest. 1985 Nov;45(7):657-9.

51   Li H, Luo W, Zeng Q, Lin Z, Luo H, Zhang Y. Method for the determination of blood methotrexate by high performance liquid chromatography with online post-column electrochemical oxidation and fluorescence detection. J Chromatogr B Analyt Technol Biomed Life Sci. 2007 Jan 1;845(1):164-8.

52   Weigand M, Frei E, Graf N, Wiessler M. Comparative analysis of methotrexate polyglutamates in lymphoblast preparations from bone marrow and blood, and the contribution of residual red blood cells. J Cancer Res Clin Oncol. 2000 Jul;126(7):407-11.

53   Krakower GR, Nylen PA, Kamen BA. Separation and identification of subpicomole amounts of methotrexate polyglutamates in animal and human biopsy material. Anal Biochem. 1982 May 15;122(2):412-6.

54   Cheng HL, Liao YM, Chiou SS, Wu SM. On-line stacking capillary electrophoresis for analysis of methotrexate and its eight metabolites in whole blood. Electrophoresis. 2008 Sep;29(17):3665-73.

55   Chen G, Fawcett JP, Mikov M, Tucker IG. Simultaneous determination of methotrexate and its polyglutamate metabolites in Caco-2 cells by liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal. 2009 Sep 8;50(2):262-6.

56   van Haandel L, Becker ML, Williams TD, Leeder JS, Stobaugh JF. Measurement of methotrexate polyglutamates in human erythrocytes by ion-pair UPLC-MS/MS. Bioanalysis. 2011 Dec;3(24):2783-96.

57   Kumar VS, Law T, Kellogg M. Liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for monitoring methotrexate in the setting of carboxypeptidase-G2 therapy. Methods Mol Biol. 2010;603:359-63.

58   Al-Turkmani MR, Law T, Narla A, Kellogg MD. Difficulty measuring methotrexate in a patient with high-dose methotrexate-induced nephrotoxicity. Clin Chem. 2010 Dec;56(12):1792-4.

59   Zandvliet ML, Jacobs A, Den Boer E, meesters RJW, de Goede AI, Engels FK, et al. Monitoring of methotrexate levels following glucarpidase rescue treatment requires detection by mass spectrometry since immunoassay is not applicable. Eur J Hosp Pharm. 2012;19(2).

Chapter

1

Chapter 2

Assessment of intracellular methotrexate and methotrexate polyglutamate metabolite concentrations in erythrocytes by ultrafast matrix-assisted laser desorption/ionization triple quadrupole tandem mass spectrometry

R.J.W. Meesters
E. den Boer
R. de Jonge
J. Lindemans
T.M. Luider

Abstract

SUMMARY A new ultrafast quantitative and high-throughput mass spectrometric method using Matrix-Assisted Laser Desorption/Ionization-triple quadrupole-tandem mass spectrometry has been developed and validated for determination of intracellular erythrocyte concentrations of the antifolate drug methotrexate (MTX) and its polyglutamate metabolites.

METHODS The method consist a solid phase extraction of MTX and MTX-polyglutamate metabolites from deproteinized erythrocyte lysates spiked with aminopterin as internal standard. The new developed method was validated according to the most recent FDA guidelines on linearity, recovery, within-run and between run accuracy and precision and stability of the analytes. The low limit of quantification (LLOQ) was 10 nmol/L for all analytes while the limit of detection (LOD) determined at a signal-to-noise ratio (S/N=3:1) in drug free erythrocyte lysate was 0.3 nmol/L.

RESULTS After validation, the new method was used in the measurement of intracellular erythrocyte concentrations of MTX and MTX-polyglutamate metabolites (MTX-PG2 to MTX-PG7) in packed human erythrocyte samples collected from patients with rheumatoid arthritis receiving low-dose oral methotrexate therapy. Mean (SD) intracellular erythrocyte concentrations observed in patient samples were 12.8 (12.6), 12.4 (9.4), 44.4 (30.0), 33.6 (35.9) and 9.4 (8.2) nmol/L for MTX to MTX-PG5, respectively in 106 erythrocytes. The highest observed glutamylation degree of MTX was MTX-PG5, the very long chain MTX-polyglutamate metabolites MTX-PG6 and MTX-PG7 were not detected in the packed erythrocyte pellets from rheumatoid arthritis patients.

Introduction

Methotrexate (N-[4[[(2,4-diamino-6-pteridinyl) methyl] methyl-amino] benzoyl]-L-glutamic acid; MTX) is a cytotoxic drug (folate antagonist) which is used to treat diseases such as leukemia, severe psoriasis and rheumatoid arthritis (RA). MTX inhibits competitively and reversibly the enzyme dihydrofolic acid reductase (DHFR) resulting in the inhibition of nucleic acid synthesis and in cell death[1]. MTX is applied in different doses depending on the disease; high dosage (up to 5000 mg/week) for treatment of different cancer types (e.g. leukemia) and in much lower doses for psoriasis [2] and rheumatoid arthritis (RA) [3] (5-25 mg/week). In RA, MTX is used as first-line drug. After admission of MTX, its plasma concentration decrease rapidly. MTX is transported into cells by the reduced folate carrier where it is retained, long after MTX has been eliminated from the plasma. MTX is metabolized intracellular by enzymatic polyglutamylation (folate-γ-glutamyl-tranferase) into MTX-polyglutamate metabolites by the addition of glutamate residues (max. 6 residues are added). Intracellular glutamylation increases the polarity of MTX resulting in intracellular retention prolonging drug action. MTX-polyglutamate metabolites can cause severe adverse events that can be counteracted by supplementation with folic acid (vitamin B9 or B11). Due to the low dose and the relatively short half life (8-15 hrs), it is no use measuring plasma MTX concentrations in low dose MTX therapy [4] and hence, MTX plasma levels do no correlate with disease activity [5]. In contrast, intracellular MTX-polyglutamates predict MTX response in RA patients especially the MTX-polyglutamates with three or more glutamic acid residues (MTX-PG3 to MTX-PG5) are associated with this therapeutic response while MTX and MTX-PG2 are poorly associated with therapeutic efficacy [6].

MTX and MTX-polyglutamate concentrations can be determined by analytical techniques such as high performance liquid chromatography (HPLC) with post column photo oxidation [4,6,7]. Total intracellular MTX can also be assessed after enzymatic hydrolysis of the polyglutamates followed by photometric measurement [8]. MTX-polyglutamate concentrations can also be determined by fluorescence polarization immunoassay (FPIA) [9] and capillary zone electrophoresis [10]. These techniques are laborious and influenced by interference from natural folates or other MTX related compounds. Recently, an ion-pairing liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique was described with increased specificity [11]. We decided to select a different analytical technology to determine intracellular polyglutamate concentrations. We applied a relative new mass spectrometric technology. This technology combines Matrix-Assisted Laser Desorption/Ionization (MALDI) and triple quadrupole (tandem) mass spectrometry (MALDI-QqQ-MS(/MS)) and was launched in 2008. It proved to be a robust and sensitive technology which can be applied for ultrafast and high-throughput analyses of small molecules because it does not necessarily require liquid chromatographic separation of samples prior to mass spectrometric analysis [12-14]. Omitting liquid chromatographic separation in combination with MALDI reduces analysis time considerably to approx. 10 seconds per sample [13] or less. MALDI-QqQ-MS technology has been proven to be a versatile

Chapter

2

quantitative tool in the ultrafast and high-throughput determination of concentrations of drugs (extra-and intracellular) such as antiretroviral drugs (protease inhibitors) [12,14,15], anticancer drugs [13,16] but also other types of drugs [17-19] and as screening tool in enzyme kinetic studies [20]. Moreover, Volmer et al. [21] demonstrated that MALDI-QqQ-MS technology has equal analytical performances in comparison to conventional LC-MS instrumentation using electrospray ionization (ESI)The aim of this study was to develop a new mass spectrometric method for measurement of intracellular MTX and MTX-polyglutamate concentrations in packed erythrocyte pellets from patients on low-dose MTX therapy.

Methods

Materials

4-amino-10-methylpteroylglutamic acid (MTX), 4-amino-10-methylpteroyldiglutamic acid (MTX-PG2), 4-amino-10-methylpteroyltriglutamic acid (MTX-PG3), 4-amino-10-methylpteroyltetraglutamic acid (MTX-PG4), 4-amino-10-methylpteroylpentaglutamic acid (MTX-PG5), 4-amino-10-methylpteroylhexaglutamic acid (MTX-PG6),4-amino-10-methylpteroylheptaglutamic acid (MTX-PG7) and internal standard 4-aminopteroylglutamic acid (Aminopterin; AO) were purchased from Schircks Laboratories (Jona, Switzerland). LC-MS grade methanol and water were obtained from Biosolve (Valkenswaard, the Netherlands) and all MALDI matrices and tricloroacetic acid (TCA) were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands) and were Matrix-Assisted Laser Desorption/ Ionization time-of-flight mass spectrometric (MALDI-TOF) quality. The erythrocyte lysis buffer was purchased from Roche (Almere, the Netherlands. The 96-well SPE plates were purchased from Sigma Aldrich (DSC-C8 and DSC-C18; 25 mg) and from Waters (Oasis HLB-30 mg, Breda, the Netherlands). Primary stock solutions of MTX and MTX-polyglutamate metabolites (MTX-PG2, MTX-PG3, MTX-PG4, MTX-PG5, MTX-PG6 and MTX-PG7) were prepared in potassium hydroxide solution (0.02 mol/L) at 44, 44, 44, 44, 58, 22, 22 µmol/L for MTX, MTX-PG2, MTX-PG3, MTX-PG4, MTX-PG5, MTX-PG6 and MTX-PG7, respectively. Chemical structures of MTX, MTX-polyglutamates and the internal standard aminopterin are illustrated in Figure 1.

MALDI-QqQ-MS/MS conditions

The MALDI-QqQ-MS/MS instrumentation used was a FlashQuant™ workstation containing a high repetition rate solid state UV-laser (FlashLaser; 349 nm, 1000 Hz) combined with a 4000 API mass analyzer (AB Sciex, Concord, Canada) operating in positive ionization mode with selected reaction monitoring (SRM) of the selected analytes at unit resolution. SRM for MTX and MTX-polyglutamate metabolites corresponded to following transitions: $[M+H]+ \rightarrow [M-((C5H9NO4)n-(H2O)m)+H]+$ with n=1 to 7 and m=(n-1) for MTX to MTX-PG7, respectively illustrating the loss of the polyglutamate chain. Optimized MALDI-QqQ-MS instrument parameters used were: laser power 55%, skimmer voltage 0V; CAD gas 8 arbitrary units (3.0-3.33*10^5 torr, N$_2$); source gas 10 arbitrary units (3.33-4.17*10^5 torr, N$_2$), dwell time 10 ms

and laser raster speed of 1 mm/sec. Instrument control and data analyses were performed using Flashquant 1.0 software and Analyst 1.4.2 application software (AB Sciex, Concord, Canada).
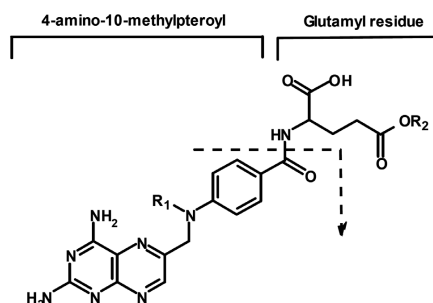
Name	R_1	R_2
MTX	CH_3	H
MTXPG2	CH_3	-[NHCHCOOH(CH$_2$)$_2$COO]-H
MTXPG3	CH_3	-[NHCHCOOH(CH$_2$)$_2$COO]$_2$-H
MTXPG4	CH_3	-[NHCHCOOH(CH$_2$)$_2$COO]$_3$-H
MTXPG5	CH_3	-[NHCHCOOH(CH$_2$)$_2$COO]$_4$-H
MTXPG6	CH_3	-[NHCHCOOH(CH$_2$)$_2$COO]$_5$-H
MTXPG7	CH_3	-[NHCHCOOH(CH$_2$)$_2$COO]$_6$-H
Aminopterin	H	H

Figure 1. Chemical structures of Methotrexate (MTX), MTX-polyglutamate metabolites and internal standard Aminopterin and respective SRM transitions of protonated molecular ions [11].

Determination of MALDI ionization efficacy

The type of MALDI ionization matrix compound used can have a significant impact on sensitivity of the MALDI-QqQ-MS/MS measurements since the ionization efficacy of the analyte by selected MALDI matrix compound can be significant different. Therefore, we determined the influence of the MALDI matrix compounds on the ionization efficacy of MTX and all MTX-polyglutamate metabolites. We applied different MALDI matrix compounds which are frequently applied in MALDI-TOF. Tested were: 2,5-dihydroxy benzoic acid (2,5-DHB),7-hydroxy-4-(trifluoromethyl)-coumarin (HFMC) [22], super-DHB (SDHB; mixture of 2,5-DHB and 5-methoxysalicylic acid), 9-amino acridine (9-AA) and α-cyano-hydroxy-cinnamic acid (α-CHCA). MALDI matrices such as 2,5-DHB and SDHB were used at a concentration of 30 mg/mL, HFMC and 9-AA at 10 mg/mL and α-CHCA at 6.2 mg/mL, respectively.

The ionization efficacy of each individual MALDI matrix compound was determined by the measurement of the total counts per second (CPS) signal for all analytes at one concentration using each MALDI ionization compound, respectively. The CPS signal was

measured using following protocol: 20 µL of individual MTX and MTX-polyglutamate metabolite stock solutions (100 nmol/L in 50% (v/v) methanol/water) were mixed at a ratio of (1:1) with the different MALDI matrix solutions and subsequently five spots of 0.5 µL were spotted onto the MALDI target plate. Detection of the positive charged (protonated) ions were done in full scan mode (m/z= 450 to 1250) using a scan time of 1s. After the experiments, the MALDI matrix compound with the highest ionization efficacy was used for further fine-tuning of MALDI-QqQ-MS/MS instrument settings and the optimal sensitivity by determination the best mixing ratio between analyte solution and MALDI matrix solution and the maximum sample amount (µL) to be spotted onto the stainless steel MALDI target plate.

Development of solid phase extraction procedure

A solid phase extraction (SPE) procedure was developed because MTX and MTX-polyglutamate metabolite concentrations are very low (fmol/106 erythrocytes) [4, 6, 9, 23]. At first, we determined the best SPE adsorbent material by determination of recovery rates for MTX and all MTX-polyglutamate metabolites applying 96-well SPE plates containing following adsorbents: octyl (C8), octadecyl (C18) and hydrophilic modified styrene polymer (HLB). The C8 and C18 well plates contained 25 mg adsorbent while the 96-well SPE-HLB plate contained 30 mg of adsorbent. The recovery rates of MTX and all other individual MTX-polyglutamate metabolites on the C8, C18 and HLB adsorbents were determined by spiking four different amounts of erythrocyte pellets (from healthy controls) with MTX and MTX-polyglutamate metabolites at one concentration (MTX, MTX-PG2, MTX-PG4; 44 nmol/L, MTX-PG3; 58 nmol/L and MTX-PG5, MTX-PG6, MTX-PG7 at 22 nmol/L,respectively). Erythrocyte pellet volumes of 25, 50, 75 and 100 µL (in triplicate) were homogenized and lyzed with 65 µL water, 10 µL of internal standard (500 nmol/L) and 150 µL of the erythrocyte lysis buffer solution followed by deproteinized of the lysate by 50 µL TCA (50% w/v). Collected deproteinized supernatants were diluted with 1000 µL of water and further processed by SPE using the three different types of adsorbents and recovery rates for MTX and all MTX-polyglutamate metabolites were determined.

Calibration curve of MTX and MTX-polyglutamate metabolites

The linear concentration ranges of the method for MTX and all MTX-polyglutamate metabolites were determined by applying calibrators prepared in whole blood by spiking MTX and MTX-polyglutamate metabolites at different concentrations. The whole blood applied for the preparation of calibrators was obtained from a healthy control (Sanquin Blood Supply Foundation, Rotterdam, the Netherlands) and calibrators were made by dilution of primarily stock solution containing MTX and MTX-polyglutamate metabolites (1000 nmol/L per analyte) with drug free whole blood yielding following calibrator concentrations: 1000, 500, 250, 100, 50, 25, 10 and 0 nmol/L (blank). The calibration curves were prepared by spiking each calibrator (25µL in triplicate) with 10 µL of the internal standard aminopterin

(AO; 500 nmol/L) followed by lysis of the erythrocytes with 65 µL of water, 150 µL of erythrocyte lysis buffer (Roche, Almere, the Netherlands) and deproteinization of the erythrocyte lysates by 50 µL of TCA. Precipitated proteins were removed by centrifugation for 5 min at 400 x g at ambient temperature.After collection of the supernatants, 1000 µL of water were added and analytes were extracted from the supernatants by solid phase extraction (SPE) using an Oasis HLB 96-SPE well plate (Waters, Etten-Leur, the Netherlands) containing 30 mg adsorbent. The SPE adsorbent was conditioned by washing the adsorbent with 1 mL of methanol followed by 2 x1 mL of water. After adsorption of the analytes and washing of the SPE adsorbent by 1 mL of water, elution of the analytes from the SPE adsorbent was achieved by 200 µL of methanol.Aliquots of 20 µL from collected SPE extracts were mixed with 40 µL of α-HCA-MALDI matrix solution and 0.5 µL were spotted in fivefold onto a 96-well stainless steel MALDI target plate (123 x 81 mm).Pipetted spots were let to dry and crystallize for 5 min at ambient temperature prior to MALDI-QqQ-MS/MS analysis. Calibration curves from all analytes were fit by linear regression of the ratio between the SRM peak areas of analyte and internal standard versus analyte concentrations by using GraphPad Prism software version 5.00 for Windows (GraphPad software, San Diego, USA).

Accuracy and precision

Within-run and between run accuracy and precision were determined by analyzing erythrocyte lysates prepared from 25 µL of whole blood in threefold spiked at three different concentration levels with all analytes at low, middle and high concentrations [24]. Within-run accuracy and precision were assessed with 3 replicate erythrocyte lysates spiked at 500, 100 and 20 nmol/L for each analyte, whereas between run accuracy and precision were assessed with 3 replicates of each concentration level analyzed on 3 subsequently different days. Accuracy was determined from the difference between measured concentrations and spiked nominal concentrations and was expressed as %error. Precision was determined by calculation of the coefficient of variation (%CV) of the replicate measurements (%CV).

Application of method to RA patient samples

During MTX administration, 8 mL CPT cell preparation tubes (Becton Dickinson, Breda, the Netherlands) were collected on t=3 months (patients 1-7) and 9 months (patients 8-10) during therapy by venapuncture from RA patients receiving low dose MTX therapy (15-25 mg MTX/week). The included patients had inflammatory joint complaints for less than 1 year. Patient blood samples were collected in compliance with the Helsinki regulations and patients gave written consent (MEC-2006-252).

Hematocrit, erythrocyte cell count, (differential) white blood cell count, hemoglobin concentration and platelet counts were determined from EDTA whole-blood using a Sysmex XE-5000 hemocytometer (Sysmex, Etten-Leur, The Netherlands). CPT tubes were centrifuged at room temperature for 20 minutes at 1500-1800 x g to separate blood cells (erythrocytes and monocytes) and obtained plasma was immediately stored at -80°C.

From all RA patients hematological parameters such as hematocrit, erythrocyte count (10^{12}/L), hemoglobin concentration and platelet count were known, erythrocyte count and hematocrit values were used to determine the number of erythrocytes/μL in the packed erythrocyte pellet. Mean hematocrit (SD) was 0.43 (0.03) and and mean erythrocyte count (SD) was 4.56 x 10^{12}/L (0.37). Collected erythrocyte pellets contained approx. 10 million erythrocytes/μL and for the measurement of the intracellular erythrocyte MTX and MTX-polyglutamate metabolite concentrations in average 250 million erythrocytes were used.

Results and discussion

Method development

MALDI ionization efficacy and sensitivity

The highest ionization efficacy for all MTX-polyglutamate metabolites was obtained using α-CHCA as MALDI matrix in combination with a sample/MALDI matrix solution ratio of 1:2 and sample spots of 0.5 μL. Application of α-CHCA as MALDI matrix compound resulted in significant higher total ion counts (CPS) for all protonated molecular ions of MTX polyglutamates (Figure 2).
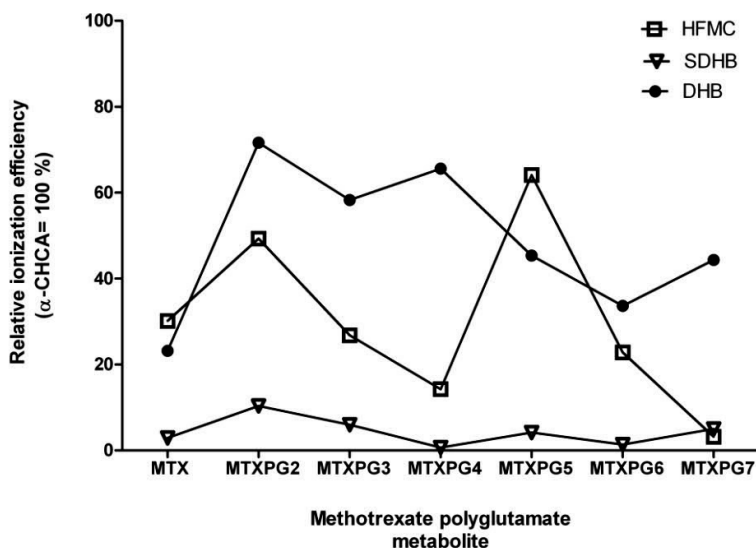
Figure 2. Ionization efficiencies of studied MALDI ionization compounds on methotrexate and metothrexate polyglutamate metabolites. Applied MALDI-QqQ-MS instrument parameters (full scan m/z 450 to 1250) laser power 55%, skimmer voltage 0V; source gas 10 arbitrary units (3.33-4.17*10^5 torr, N_2) and laser raster speed of 1 mm/sec

Protonated molecular ions of MTX and MTX-polyglutamate metabolites using α-CHCA were used for further optimization of MALDI and MS instrument parameters such as plate voltage (V), collision energy (CE), collision cell exit potential (CXP), collision gas

(CAD), source gas, and skimmer voltage setting. Optimized MALDI-QqQ-MS/MS instrument settings for MTX and MTX-polyglutamate metabolites and the internal surrogate standard aminopterin are presented in Table 1.

Mass spectrometric analysis of MTX and MTX-polyglutamate metabolites
Collision induced fragmentation (CID) of the protonated molecular ions from MTX and MTX-polyglutamate metabolites resulted in MS/MS spectra where one main high abundant fragment ion at a mass-to-charge ratio of m/z 308.2 was observed, although also few smaller fragment ions with lower abundance were observed; for the internal standard aminopterin, identical fragmentation behavior was observed with one difference that a high abundant fragment ion was observed at m/z 294.2. The high abundant fragment ions observed at m/z 308.2 for all analytes were selected for SRM analyses. The formation of this high abundant fragment ion originate from an intermolecular cleavage of the amide bond in MTX and MTX-polyglutamate metabolites situated between the first glutamyl-moiety and the 4-amino-10-methylpteroyl-backbone of MTX-polyglutamate molecules (Figure 1). The cleavage of the amide bond resulted after hydrogen rearrangement in the loss of a neutral glutamate molecule (M-$C_5H_9NO_4$; M-147) for MTX as well as the loss of neutral charged polyglutamyl-peptide containing between two and seven glutamate moieties for MTX-PG2 until MTX-PG7, respectively as also reported by van Haandel et al. [11] using LC-ESI-MS/MS.

Validation
Significant different recovery rates for MTX and MTX-polyglutamate metabolites were observed for the three tested different SPE adsorbent materials. The lowest recoveries for all analytes were observed using the SPE-C8 adsorbent material, recovery ranges obtained applying this SPE adsorbent material ranged between 62.4% for MTX and 17.6% for MTX-PG6. Higher recovery rates were obtained for the SPE-C18 material, recovery ranged here between 85.6% and 57.9 % for MTX and MTX-PG7, respectively. The highest recoveries were observed for the HLB-SPE material, recoveries observed were 71.3, 75.1, 96.7, 86.3, 88.5, 90.3 and 97.7% for MTX to MTX-PG7, respectively.

The recovery of the internal standard aminopterin was in the range of MTX and the MTX-polyglutamate metabolites, the recovery was 81.2%. After determination of the SPE adsorbent material with the highest recovery rates for all analytes, the maximum sample loading of this adsorbent was determined by applying different amounts of packed erythrocyte pellet, tested were 25, 50, 75 and 100 µL of packed erythrocyte pellet and recoveries of MTX and MTX-polyglutamate metabolites were determined. Highest recovery rates for all analytes were observed when 25 µL of lyzed erythrocyte pellets were applied. Higher loading amounts resulted in significant lower recovery rates for all analytes (Figure 3). Obviously, higher amounts of erythrocyte cell pellets overloaded the SPE adsorbent and caused desorption (breakthrough) of the analytes.

Table 1. MALDI-QqQ-MS/MS instrument settings for the quantitative measurement of MTX-PGs[a,b]

Analyte	Abbr.	SRM (m/z)	Plate Voltage (V)	CE (V)	CXP (V)
4-amino-10-methylpteroylglutamic acid	MTX	455.2 → 308.2	45	25	10
4-amino-10-methylpteroyldiglutamic acid	MTX-PG2	584.4 → 308.2	30	43	10
4-amino-10-methylpteroyltriglutamic acid	MTX-PG3	713.4 → 308.2	35	43	10
4-amino-10-methylpteroyltetraglutamic acid	MTX-PG4	842.4 → 308.2	45	60	10
4-amino-10-methylpteroylpentaglutamic acid	MTX-PG5	971.6 → 308.2	60	70	15
4-amino-10-methylpteroylhexaglutamic acid	MTX-PG6	1100.3 → 308.2	80	80	10
4-amino-10-methylpteroylheptaglutamic acid	MTX-PG7	1229.4 → 308.2	75	90	25
4-amino-pteroyldiglutamic acid	AO	441.2 → 284.2	50	28	15

[a]Skimmer voltage (0 Volts), [b]As collision gas (CAD) N_2 was applied
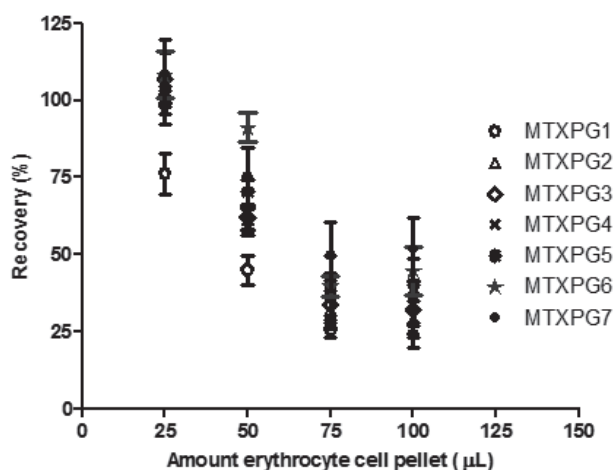
Figure 3. Recovery rates for individual MTX-polyglutamates in spkied erythrocyte pellets in relation to the erythrocyte pellet volume used for solid-phase extraction using Oasis HLB 96-well plates containing 30 mg adsorbent.

The selected concentration range using lysates produced from erythrocyte pellets based calibration standards indicated a linear relationship between the analyte peak area/ IS ratio of each individual MTX-polyglutamate metabolites and their concentration (MTX: y=1.3224x+0.3546, r^2=0.9981; MTX-PG2: y=0.4042x-0.0443, r^2=0.9936; MTX-PG3: y=0.1649x-0.0317, r^2=0.9905; MTX-PG4: y=0.0709x-0.0078, r^2=0.9945; MTX-PG5: y=0.1137x-0.0189, r^2=0.9912; MTX-PG6: y=0.2063-0.004, r^2=0.9928 and MTX-PG7: y=0.01113x-0.0011, r^2=0.9961). The LLOQ for each individual MTX-polyglutamate metabolite was defined as the calibrator with the lowest concentration used to calculate the calibration

curve which could be measured with an accuracy and precision of <20 %CV [24]; the LLOQ for the MTX-polyglutamate metabolites was 10 nmol/L for MTX to MTX-PG7, respectively. The upper limit of quantification (ULOQ) was defined as the highest concentration calibrator which could by measured with acceptable accuracy and precision of <15 %CV; the ULOQ was 1000 nmol/L for all MTX-polyglutamates.

The limit of detection (LOD) defined as three times the signal-to-noise ratio (3:1) of drug free control whole blood was 1 nmol/L for MTX and MTX-PG2 and 3 nmol/L for MTX-PG3 to MTX-PG7, respectively. Validation of the method's accuracy and precision by determination of with-run and between run accuracy and precision using quality control samples prepared at three different concentration levels (500, 100 and 20 nmol/l for each analyte) were in compliance with FDA criteria (CV and %error < 20%) (Table 2). Precision of the new method was expressed as %CV from within-run validation and ranged between 5.5 and 14.4%, while within-run precision ranged between 4.9 and 14.6%, respectively.
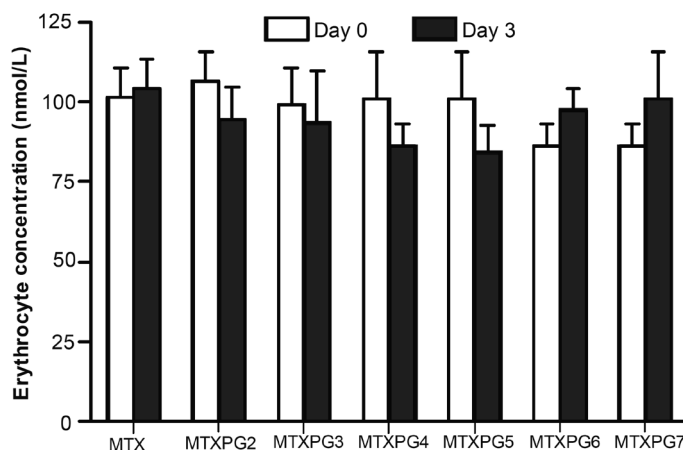
Figure 4. Stability of MTX and MTX-polyglutamate metabolites in a packed erythrocyte pellet spiked at a concentration of 100 nmol/L per analyte, illustrated are measured concentration of all analytes at day 0 and day 3 (mean ± SD).

Accuracy of the new method expressed as %error ranged for within-run validation between -8.5% and 9.8%, while the %error for the between run measurements were between -7.5% and 4.5%, respectively. The stability of MTX and its MTX-polyglutamate metabolites in erythrocyte matrix were tested by an erythrocyte pellet (25 μL) spiked with MTX and MTX-polyglutamate metabolites at a concentration of 100 nmol/L by measuring at day 0 and day 3. The stability of all analytes in the erythrocyte matrix was stable (expressed as %error) and were found to be <20 %error (Figure 4).Dervieux et al. [6] reported a mean sum of individual MTX- polyglutamate metabolite in seventy RA patients of 120 nmol/L (MTX to MTX-PG5). The intracellular concentrations were determined applying HPLC in combination with fluorescence detection of the MTX-polyglutamate metabolites. Van Haandel et al. [11]

used a LC-MS/MS assay for the determination of erythrocyte intracellular MTX and MTX-polyglutamate metabolite concentrations and reported a concentration of 153 nmol/L (sum of MTX to MTX-PG6) for a JIA patient (Juvenile Idiopatic Arthritis) on MTX therapy.
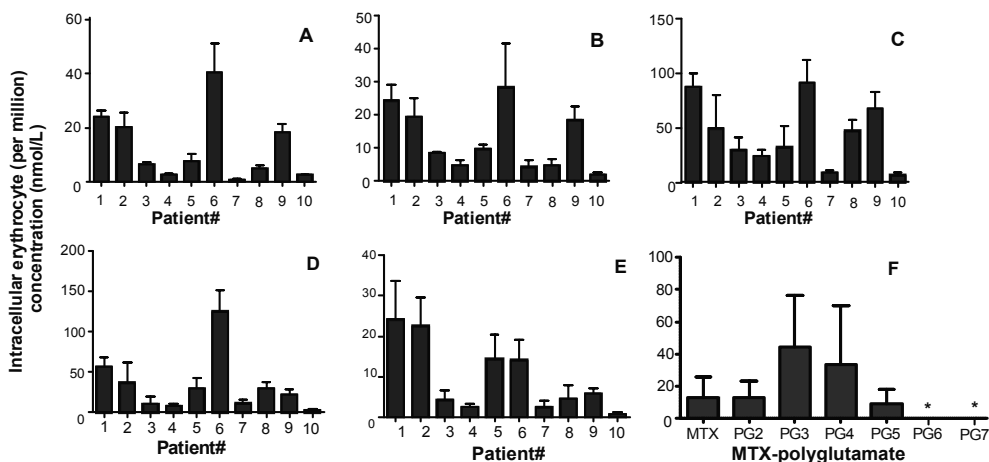
Figure 5. Individual intracellular erythrocyte MTX and MTX-polyglutamate metabolite concentrations in ten RA patients; (a) MTX, (b) MTX-PG2, (c) MTX-PG3, (d) MTX-PG4 and (e) MTX-PG5, (f) observed concentrations of individual MTX-polyglutamates. The concentrations of the very long chain MTX-PG6 and MTX-PG7 were < LOD. Illustrated are mean concentrations and SD obtained from an analysis of 25 µL of a packed erythrocytes pellet in triplicate by MALDI-QqQ-MS/MS. (*) depicts concentration of this MTX-polyglutamate metabolite is <LOD.

In comparison to measured sum of individual MTX and MTX-polyglutamate metabolite concentrations (139 nmol/L; MTX to MTX-PG5) in our study of RA patients both publications are consistent with both publications. In contrast to reported concentrations we observed a concentration (SD) of the MTX-PG4 metabolite of 33.6 nmol/L (35.9) was almost equal to the MTX-PG3 metabolite concentration level which was 43.6 nmol/L (30.0). An unambiguous explanation is difficult to give since many parameters could cause this significant difference. These include differences in pharmacokinetics between patients, time point of sample collection, and storage of samples [7]. The time reaching steady state concentrations for MTX and MTX-polyglutamate metabolites can be significant different, especially for MTX-PG4 time ranges between 16 and 832 weeks (mean 146 weeks) were observed with patients while for MTX to MTX-PG3 metabolite 6, 11 and 41 weeks and for MTX-PG5 140 weeks were reported [7]. Depending on the point of the sample collection
higher or lower concentrations can be expected [7]. Higher MTX-PG4 concentrations were

Table 2. Precision and accuracy of the MALDI-QqQ-MS/MS assay at three different MTX polyglutamate concentration levels

Within-run validation[a]

	MTX			MTX-PG2			MTX-PG2			MTX-PG4			MTX-PG5			MTX-PG6			MTX-PG7		
Nominal concentration (nmol/L)	500	100	20	500	100	20	500	100	20	500	100	20	500	100	20	500	100	20	500	100	20
Observed conc. (nmol/L)	507	105	18	486	99	21	486	94	20	497	97	22	494	92	20	495	92	20	502	98	21
Accuracy (% error[c])	1.4	4.9	-7.9	-2.7	-1.3	4.4	-2.8	-6.1	0.26	-0.7	-2.7	9.8	-1.1	-8.3	0.9	1.0	-8.5	1.1	0.3	-2.3	5.5
Precision (% CV)	5.5	8.0	14.1	7.5	11.2	13.6	9.2	13.4	10.7	12.6	14.4	12.7	6.0	12.7	5.5	12.1	11.3	13.3	1.8	13.6	5.7

Between-run validation[b]

	MTX			MTX-PG2			MTX-PG2			MTX-PG4			MTX-PG5			MTX-PG6			MTX-PG7		
Observed conc. (nmol/L)	502	103	19	494	100	20	490	96	20	498	102	20	484	93	20	503	99	20	491	102	21
Accuracy (% error[c])	0.3	3.2	-5.2	-1.1	0.3	-1.3	-2.1	-3.8	0.8	-0.5	1.9	-2.3	-3.1	-7.5	0.1	0.6	-1.3	-2.5	-1.7	1.8	4.5
Precision (% CV)	5.00	9.2	13.2	9.8	11.0	7.9	9.1	14.5	6.8	11.4	13.0	11.9	7.9	14.6	13.4	7.4	8.1	7.6	5.9	4.9	6.8

[a]within-run results summarize 3 spots per QC sample at each concentration level in one experiment, [b]between-run results summarize three different experiments from 3 consecutive days with 3 spots per QC sample at each concentration level, [c]% error = (mean observed concentration–nominal concentration)/(nominal concentration)* 100%

Chapter

2

reported as being associated with improved MTX efficacy in RA patients [23]. In general, our findings are consistent with previously reported intracellular concentrations [6,26,27] although also a large variation in individual MTX-polyglutamate metabolite concentrations between our ten included RA patients per MTX-polyglutamate metabolite was observed (Figure 5). This large variation in individual MTX-polyglutamate metabolite concentrations was also observed by Dalrymple et al. [7] who reported a large interpatient variability of erythrocyte MTX and MTX-polyglutamate metabolite accumulation and elimination in adults with RA.

Conclusion

The newly developed MALDI-QqQ-MS/MS method allows a sensitive and accurate measurement of therapeutically relevant concentrations of MTX and MTX-polyglutamate metabolites in erythrocytes from patients receiving low dose MTX therapy. This new technology is comparable to other analytical methods such as fluorescence polarization immunoassay (FPIA) and HPLC in combination with fluorescence detection after photo-oxidation or LC-MS/(MS) is that it is ultrafast and therefore can be applied for high-throughput measurements of large number of samples of large patient population studies. A major advantage of the MALDI-QqQ-MS instrumentation is that the instrumentation is directly available for analysis of other substances without downtime of the instrumentation compared with common LC-MS instrumentation due to exchange of separation columns and equilibration of the applied instrumentation.

Chapter

2

References

1   M. Krajinovic, A. Moghrabi (2004) Pharmacogenetics of methotrexate. Pharmacogenomics 5: 819.

2   R.B. Warren, R.J. Chalmers, C.E. Griffiths, A. Menter (2008) Methotrexate for psoriasis in the era of biological therapy. Clin Exp Dermatol 33:551.

3   B.N. Cronstein (2005) Low-dose methotrexate: a mainstay in the treatment of rheumatoid arthritis. Pharmacol Rev 57: 163.

4   A.J. Brooks, E.J. Begg, M. Zhang, C.M. Frampton, M.L. Barclay (2007) Red blood cell methotrexate polyglutamate concentrations in inflammatory bowel disease. Ther Drug Monit 29:619.

5   P. Lafforgue, S. Monjanel-Mouterde, A. Durand, J. Catalin, P.C. Acquaviva (1995) Lack of correlation between pharmacokinetics and efficacy of low dose methotrexate in patients with rheumatoid arthritis. J Rheumatol 22:844.

6   T. Dervieux, D. Orentas Lein, J. Marcelletti, K. Pischel, K. Smith, M. Walsh, R. Richerson (2003) HPLC determination of erythrocyte methotrexate polyglutamates after low-dose methotrexate therapy in patients with rheumatoid arthritis. Clin Chem 49:1632.

7   J.M. Dalrymple, L.K. Stamp, J.L. O'Donnell, P.T. Chapman, M. Zhang, M.L. Barclay (2008) Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. Arthritis Rheum 58:3299.

8   P. Angelis-Stoforidis, F.J. Vajda, N. Christophidis (1999) Methotrexate polyglutamate levels in circulating erythrocytes and polymorphs correlate with clinical efficacy in rheumatoid arthritis. Clin Exp Rheumatol 17:313.

9   H. Hayashi, C. Fujimaki, S. Tsuboi, T. Matsuyama, T. Daimon, K. Itoh (2008) Application of fluorescence polarization immunoassay for determination of methotrexate-polyglutamates in rheumatoid arthritis patients. Tohoku J Exp Med 215:95.

10   C.Y. Kuo, H.L. Wu, H.S. Kou, S.S. Chiou, D.C. Wu, S.M. Wu (2003) Simultaneous determination of methotrexate and its eight metabolites in human whole blood by capillary zone electrophoresis. J Chromatogr A 1014:93.

11   L. van Haandel, M.L. Becker, J.S. Leeder, T.D. Williams, J.F. Stobaugh (2009) A novel high-performance liquid chromatography/mass spectrometry method for improved selective and sensitive measurement of methotrexate polyglutamation status in human red blood cells. Rapid Commun Mass Spectrom 23:3693.

12   R.J. Meesters, J.J. Van Kampen, R.D. Scheuer, M.E. Van der Ende, R.A. Gruters, T.M. Luider (2011) Determination of the antiretroviral drug tenofovir in plasma from HIV-infected adults by ultrafast isotope dilution MALDI-triple quadrupole tandem mass spectrometry. J Mass Spectrom 46:282.

13   R.J. Meesters, R. Cornelissen, R.J. van Klaveren, R. de Jonge, E. den Boer, J. Lindemans, T.M. Luider (2010) A new ultrafast and high-throughput mass spectrometric approach for the therapeutic drug monitoring of the multi-targeted anti-folate pemetrexed in plasma from lung cancer patients. Anal Bioanal Chem. 398:2943.

14   R.J. Meesters, J.J. van Kampen, M.L. Reedijk, R.D. Scheuer, L.J. Dekker, D.M. Burger, N.G. Hartwig, A.D. Osterhaus, T.M. Luider, R.A. Gruters (2010) Ultrafast and high-throughput mass spectrometric assay for therapeutic drug monitoring of antiretroviral drugs in pediatric HIV-1 infection applying dried blood spots. Anal Bioanal Chem 398:319.

15   J.J. van Kampen, M.L. Reedijk, P.C. Burgers, L.J. Dekker, N.G. Hartwig, I.E. van der Ende, R. de Groot, A.D. Osterhaus, D.M. Burger, T.M. Luider, R.A. Gruters (2010) Ultra-fast analysis of plasma and intracellular levels of HIV protease inhibitors in children: a clinical application of MALDI mass spectrometry. PLoS One 5:e11409.

16   R.J. Meesters, E. den Boer, R.A. Mathot, R. de Jonge, R.J. van Klaveren, J. Lindemans, T.M. Luider (2011) Ultrafast selective quantification of methotrexate in human plasma by high-throughput MALDI-isotope dilution mass spectrometry. Bioanalysis 3:1369.

17   M. Wagner, E. Varesio, G. Hopfgartner (2008) Ultra-fast quantitation of saquinavir in human plasma by matrix-assisted laser desorption/ionization and selected reaction monitoring mode detection. J Chromatogr B Analyt Technol Biomed Life Sci 872:68.

18   P. Hatsis, S. Brombacher, J. Corr, P. Kovarik, D.A. Volmer (2003) Quantitative analysis of small pharmaceutical drugs using a high repetition rate laser matrix-assisted laser/desorption ionization source. Rapid Commun Mass Spectrom 17: 2303.

19   L. Sleno, D.A. Volmer (2005) Some fundamental and technical aspects of the quantitative analysis of pharmaceutical drugs by matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 19: 1928.

20   R. Rathore, J. Corr, G. Scott, P. Vollmerhaus, K.D. Greis (2008) Development of an inhibitor screening platform via mass spectrometry. J Biomol Screen 13: 1007.

21   D.A. Volmer, L. Sleno, K. Bateman, C. Sturino, R. Oballa, T. Mauriala, J. Corr (2007) Comparison of MALDI to ESI on a triple quadrupole platform for pharmacokinetic analyses. Anal Chem 79:9000.

22   J.J. van Kampen, P.C. Burgers, R.A. Gruters, A.D. Osterhaus, R. de Groot, T.M. Luider, D.A. Volmer (2008) Quantitative analysis of antiretroviral drugs in lysates of peripheral blood mononuclear cells using MALDI-triple quadrupole mass spectrometry. Anal Chem 80:4969.

23   T. Dervieux, R. Zablocki, J. Kremer. Red blood cell methotrexate polyglutamates emerge as a function of dosage intensity and route of administration during pulse methotrexate therapy in rheumatoid arthritis. Rheumatology (Oxford). 49, 2337.

24   CDER Center for Drug Evaluation and Research (CDER) Guidance for Industry: Bioanalytical Method Validation (2001) Volume,

25   M.L. Becker, L. van Haandel, R. Gaedigk, A. Lasky, M. Hoeltzel, J. Stobaugh, J.S. Leeder (2010) Analysis of intracellular methotrexate polyglutamates in patients with juvenile idiopathic arthritis: effect of route of administration on variability in intracellular methotrexate polyglutamate concentrations. Arthritis Rheum 62: 1803.

26   H. Schroder, K. Fogh (1988) Methotrexate and its polyglutamate derivatives in erythrocytes during and after weekly low-dose oral methotrexate therapy of children with acute lymphoblastic leukemia. Cancer Chemother Pharmacol 21: 145.

27   J.M. Kremer, J. Galivan, A. Streckfuss, B. Kamen (1986) Methotrexate metabolism analysis in blood and liver of rheumatoid arthritis patients. Association with hepatic folate deficiency and formation of polyglutamates. Arthritis Rheum 29:832.

Chapter

2

Chapter 3

Measuring methotrexate polyglutamates in red blood cells; a new LC-MS/MS based method

E. den Boer
R.J.W. Meesters
B.D. van Zelst
T.M. Luider
J.M.W. Hazes
S.G. Heil
R. de Jonge

Abstract

BACKGROUND: The folate antagonist methotrexate (MTX) is the anchor drug in the treatment of rheumatoid arthritis. The therapeutic effects of MTX are attributed to the intracellular levels of MTX, present in the cell as polyglutamates (MTX-PGs). We developed a new LC-ESI-MS/MS based assay to separately quantitate MTX-PGs in red blood cells using stable isotope internal standards.

METHODS: Samples were analysed by LC-ESI-MS/MS using a Waters Acquity UPLC BEH C18 column with a 5-100% organic gradient of 10 mM ammonium bicarbonate (pH10) and methanol. The analysis consisted of simple sample preparation and 6 minute run time. Detection was done using a Waters Acquity UPLC coupled to a Waters Quattro Premier XE with electrospray ionization operating in the positive ionization mode. Assay validation was performed following recent FDA guidelines.

RESULTS: The method was linear from 1-1000 nmol/L for all MTX-PGs (r^2 >0.99). The coefficient of variation ranged from 1-4% for intraday precision and 6-15% for interday precision. Samples were stable for at least 1 month at −80°C. Recovery ranged from 98-100% and the relative matrix-effect varied from 95-99%. The LLOQ was 1 nmol/L for each MTX-PG. 50 patients samples from the tREACH study were analyzed. The MTX-PG concentration and distribution of these samples were comparable with values reported in literature.

CONCLUSIONS: The developed LC-ESI-MS/MS method for the quantitative measurement of MTX-PGs in erythrocytes is both sensitive and precise within the clinically relevant range. The method can be easily applied in clinical laboratories due to the combination of simple pre-treatment with robust LC-ESI-MS/MS.

Introduction

Although low-dose methotrexate (MTX) is the most widely used treatment for rheumatoid arthritis (RA), approximately 30% of patients do not reach sufficient response or suffer from adverse events [1]. There is no therapeutic drug-monitoring based model for predicting response or adverse events during low-dose MTX treatment.

Although MTX levels can be measured easily in plasma, low-dose MTX is rapidly cleared from plasma and is therefore not routinely measured. The therapeutic effects of MTX are thought to be mediated by its intracellular levels [2], which in contrast to plasma levels is difficult to measure and is not performed clinically.

MTX is transported into the cell primarily by the reduced folate carrier. In the cell it is rapidly converted by folylpolyglutamate synthase to MTX polyglutames (MTX-PGs) by γ-linked sequential addition of glutamic acid residues. In a competing reaction, the MTX-PGs are deconjugated by γ-glutamyl hydrolase, leading to a variety of chain-lengths (MTX-PG2-7) [2, 3]. In low-dose MTX treatment, the pentaglutamate (MTX-PG5) is the highest order of glutamylation detected, while the triglutamate form (MTX-PG3) of MTX predominates [4, 5]. Polyglutamylation retains MTX in the cell because MTX-PGs are a poor substrate for the MTX efflux proteins. Assessment of intracellular MTX-PG levels might predict how patients respond to treatment [6-10] and can also be used to assess adherence to MTX therapy.

Intracellular MTX-PG concentrations can be determined by analytical techniques such as capillary-zone electrophoresis [11] and high performance liquid chromatography (HPLC) with post-column photo-oxidation [4, 8]. Total intracellular MTX can also be assessed by fluorescence polarization immunoassay [12, 13] or after enzymatic hydrolysis of the polyglutamates, followed by photometric measurement [8]. However, these techniques are laborious, influenced by interference from endogenous folates [5] or other MTX-related compounds [14, 15] and therefore not suitable for routine measurement in patients.

Recently, an ion-pairing LC-MS/MS technique with increased specificity was described [16]. However, it did not use stable-isotope-labelled internal standards for each MTX-PG, and is therefore unable to compensate for recovery and matrix-effects for the individual MTX-PG. Also, the use of ion-pairing on a multi-assay MS can lead to interference with the other assays, which is not ideal for routine clinical application. We recently developed an ultrafast MALDI-MS/MS method to measure MTX-PGs [17]. Although this method is ideal for large cohort studies, stable isotopes were not used, it lacks the precision needed for routine clinical monitoring, and the equipment is not routine for hospital laboratories. Because of these issues, we developed a new LC-ESI-MS/MS based assay to separately determine individual MTX-PGs in erythrocytes using stable-isotope internal standards for the quantitation of intracellular MTX-PGs to ensure the method is specific, robust and precise in order to be used in clinical laboratories.

Chapter

3

Methods

Materials

MTX-PG1-5 standards were purchased from Schircks Laboratories (Jona, Switzerland). Stable isotope labeled internal standards ($^{13}C_5$, ^{15}N), MTX-PG1-5(M+6), were purchased from Pepscan (Lelystad, the Netherlands). The chemical structures of MTX and the stable isotope labeled standards are illustrated in Figure 1. LC-MS grade methanol and water were obtained from Biosolve (Valkenswaard, the Netherlands). Ammonium bicarbonate (NH_4HCO_3) and perchloric acid ($HClO_4$ 70%) were purchased from Sigma (Zwijndrecht, Netherlands). Ammonium hydroxide (NH_4OH, 25% v/v) was purchased from Merck (Schiphol-Rijk, Netherlands).

Blinded drug-free K2–ethylenediaminetetraacetic acid (EDTA) erythrocyte pellets were used for development and validation. The drug-free erythrocyte pellets were obtained by centrifugation of EDTA whole-blood tubes at 2700 × g and removing the supernatant. Pellets were pooled per 10 ml and stored at −80°C until further use. As these patient samples were leftover from routine analysis at the department of clinical chemistry, no medical-ethical approval was necessary for this study.

Equipment

Analyses were performed on an LC-MS/MS system consisting of a Waters Acquity UPLC with a 20 µl sample loop and a Quattro Premier XE (Waters Corporation, Etten-Leur, Netherlands) triple quadrupole mass spectrometer with an electrospray ionization source (ESI) operated in the positive mode.

Chromatographic conditions

Chromatography was done using partial-loop injection of 10 µl sample on a Waters Acquity BEH C18 column (2.1 × 100 mm, 1.7 µmol/L) at 35°C. The mobile phase consisted of (A) 10 mM ammonium bicarbonate adjusted to pH 10 with 25% ammonium hydroxide and (B) methanol. A flow rate of 0.3 ml/min was maintained and the analytes were eluted using the following program: 0-0.5 min isocratic hold 5% B, 0.5-4 min linear gradient 5–40% B; 4.0–4.25 min linear gradient 40–100% B; 4.25–4.75 min isocratic 95% B; 4.75–5 min linear gradient 100–5% B; 5–6 min, isocratic 5% B.

MS detection

The ESI was operated in the positive mode with the following fixed settings: capillary voltage 1.00 kV, desolvation temperature 350°C at a gas flow of 1000 l/hr nitrogen, and cone gas flow of 50 l/hr nitrogen. Argon was used as collision gas at a flow rate of 0.20 ml/min. MTX-PG specific settings were as follows; dwell time was 0.1s for all MTX-PGs, cone voltage was 30, 30, 50, 60 and 55V for MTX-PG1-5, respectively. Collision energy was set at 20, 20, 40, 40 and 50eV for MTX-PG1-5, respectively. Mass transitions are shown in Figure 1.

Preparation of standards

Stock solutions were prepared in 0.1 mol/L ammonium hydroxide at 88.75 µmol/L, 64.48 µmol/L, 85.7 µmol/L, 40.71 µmol/L and 51.28 µmol/L for MTX-PG1-5 respectively, and 10 µmol/L for each IS. Stock solutions were stored at -80°C. For Each analysis the IS stock solutions were diluted with LC-MS grade water to a working solution containing 100 nmol/L of each MTX-PG(M+6).

Mass transitions	SRM MTXPGn (IS) (m/z)[a]
MTX-PG1	455.2 (461.2) → 308.2
MTX-PG2	584.4 (590.4) → 308.2
MTX-PG3	713.4 (719.4) → 308.2
MTX-PG4	842.4 (848.4) → 308.2
MTX-PG5	971.6 (977.6) → 308.2
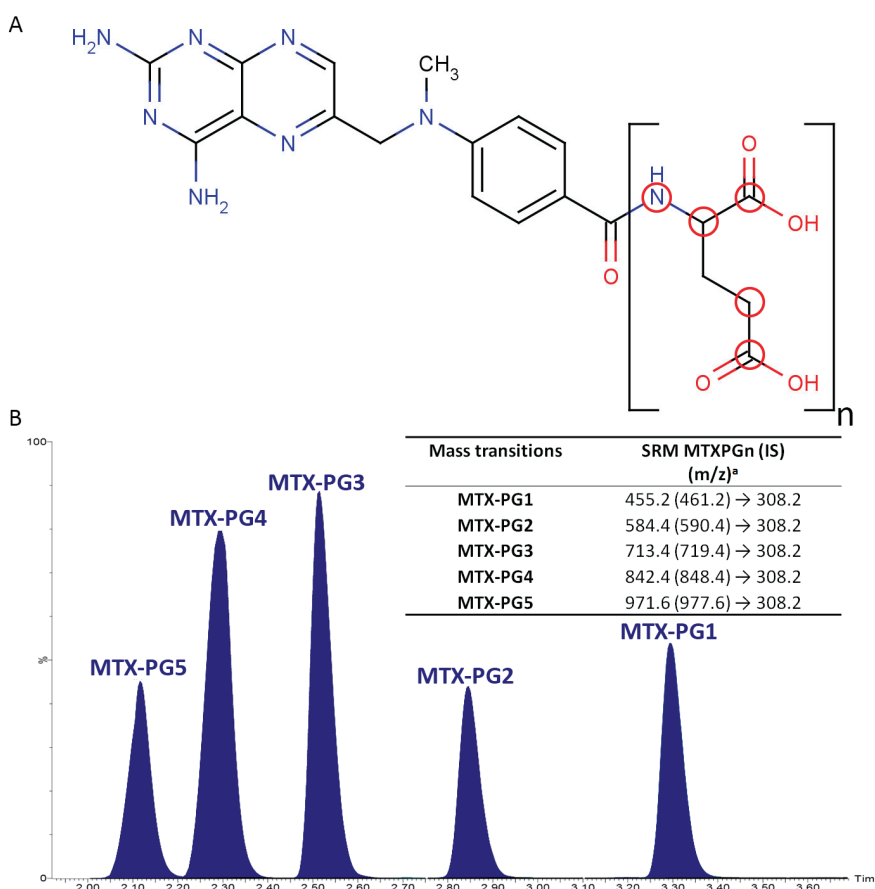
Figure 1. (A) MTX-PG structure, the glutamate group is indicated in brackets. Cirkels denotate stable isotope labels. **(B)** A typical total ion count chromatogram obtained from spiked erythrocyte samples (50 nmol/L). Dwell time was 0.1s for all MTX-PGs, cone voltage was 30, 30, 50, 60 and 55V for MTX-PG1-5 respectively. Collision energy was set at 20, 20, 40, 40 and 50eV for MTX-PG1-5, respectively. [a]internal standard (MTX-PG mass+6 $^{13}C_5$, ^{15}N) denoted between brackets

Preparation of calibrators and quality control samples

A batch of frozen pooled drug-free erythrocyte pellet was thawed at room temperature while rotating and spiked with 500 nmol/L of each MTX-PG. A ten-point calibration curve was obtained by a 1:1 serial dilution with drug-free erythrocyte pellet resulting in 0.97, 1.95,

3.91, 7.81, 15.63, 31.25, 62.5, 125 and 250 nmol/L of each MTX-PG and a blank (0 nmol/L) calibrator. A fresh calibration curve was prepared for each run.

Quality-control (QC) samples were prepared in drug-free erythrocyte pellet at four different concentrations: 5, 25, 50 and 100 nmol/L of each individual MTX-PG. Aliquots were stored at -80 °C and thawed on ice. QC samples were prepared with every 50 patient samples. A run was considered successful if the QC samples did not deviate more than 15% from the nominal value, as indicated by the FDA guidelines for industry [18].

Sample preparation

Patient samples were obtained by centrifuging EDTA whole-blood tubes at 2700 × g. Patient and validation samples were all prepared as follows; 200 μL erythrocyte pellet was supplemented with 200 μL cold IS solution (100 nmol/L MTX-PG1-5). Protein was precipated by adding 320 μl cold 16% perchloric acid to the diluted sample while vortexing and incubating on ice for 30 min, after which they were centrifuged for 15 min at 2700 × g. The clear supernatant was transferred to a sample vial and placed in the autosampler at 4°C until analysis.

Validation procedures

Method validation was adapted from the 2001 FDA guidelines [18] and Matuszewski et al. [19]. It consisted of selectivity, IS integrity, carry-over, linearity, stability (freeze/thaw, short-term storage), recovery, matrix-effects, lower limit of quantitation (LLOQ), and precision (interday/intraday) and accuracy. All samples were measured in duplicate unless otherwise specified

Selectivity and IS integrity

Selectivity was determined by measuring 20 different drug-free erythrocyte pellets without addition of MTX-PGs or IS. The average background signal was calculated for each MTX-PG and IS, and used as noise for the signal/noise ratio. The IS was also tested for the presence of MTX-PG, MTX-PG mass+1 and MTX-PG mass +2 in drug-free erythrocyte pellet spiked with 50 nmol/L IS to determine isotope interference.

Carry-over

Carry-Over was assessed by triplicate measurement of a spiked sample with high concentration (250 nmol/L of each MTX-PG) directly followed by triplicate measurement of a sample with low (5 nmol/L) concentration and calculated as *((Low1 − Low3)/(High3 − Low3)) × 100%* [20]. Carry-over was considered acceptable if it was less than 0.5%.

Linearity

Linearity was determined by spiking a batch of pooled drug-free erythrocyte pellet with 12 different concentrations of MTX-PGs in a range of 0.97-1000 nmol/L and containing a blank (0

nmol/L) sample. Each concentration was measured five times. This was repeated in a second drug-free erythrocyte pellet. Linearity was expressed as the expected concentration against the measured concentration and was considered acceptable if the squared correlation coefficient (R^2) was >0.99 for each calibration curve.

Recovery

Recovery of the sample preparation was determined by spiking ten different batches of pooled drug-free erythrocyte pellets with 50 nmol/L of each MTX-PG and 50 nmol/L IS before, and after sample preparation. The recovery was calculated as: *recovery (%) = (peak area of an analyte spiked into erythrocyte pellet before sample preparation / peak area of an analyte spiked into the supernatant obtained from erythrocytes subjected to sample preparation) × 100%.* After correction with internal standard, the relative recovery should be approximately 100% and reproducible; the CV% of the ten batches should be <10%.

Matrix-effects

Matrix-effects can have a detrimental effect on MS-based analysis [21], and both absolute and relative matrix-effects can occur [19]. Absolute matrix-effect is defined as the difference in response between analyte in sample matrix and analyte in pure solvent. Relative matrix-effect is defined as the variation in response between different samples in a similar matrix, and can be expressed as the CV% of the slopes of reference curves in different samples of the same matrix. The evaluation of matrix-effects was performed according to the recommendations of Matuszewski et al. [19]. Ten different batches of pooled drug-free erythrocyte pellet were spiked with five concentrations (6.25 nmol/L, 12.5 nmol/L, 25 nmol/L, 50 nmol/L and 100 nmol/L) of each MTX-PG after sample preparation. The slopes of these reference curves were compared with the slope of a reference curve prepared in pure solvent as a measure of absolute matrix-effect: *Absolute matrix-effect = (slope pooled erythrocyte pellet/slope distilled water) × 100%.* The relative matrix-effect was calculated as: *100% – CV%* of the slopes of the ten drug-free pellets calibration lines. We also calculated the maximum slope difference between the lowest and highest slope of the 10 standard lines, which was expressed as %difference. Matrix-effects were calculated with and without correction by the internal standard. Slopes were calculated by linear regression analysis. A relative matrix-effect of <5% was considered acceptable.

Stability

The stability of MTX-PGs in stored erythrocytes was studied by measuring the stored QC samples after 1 week at -80 °C, 4 weeks at -80 °C, and 3 months at -80 °C. Stability after sample preparation was studied by preparing a concentration range of MTX-PGs (1-250 nmol/L of each MTX-PG and a blank sample) and measuring this directly and after storage in the autosampler at 4 °C for up to 2 weeks.

Freeze/thaw stability was investigated by measuring QC samples after three successive cycles of freezing at -80 °C and unassisted thawing on ice. All results from these experiments were compared with results from freshly prepared and measured samples.

Lower limit of quantitation (LLOQ)
LLOQ was determined by spiking drug-free erythrocyte pellet with different levels of MTX-PGs covering a range of 0.97-1000 nmol/L of each MTX-PG and containing a blank (0 nmol/L) sample. Each sample was measured ten times. This experiment was repeated in separate drug-free erythrocyte pellet. The LLOQ was defined as the lowest concentration with a CV<20% and having a signal-to-noise ratio of >10:1. To calculate the signal-to-noise ratio, the result of the selectivity experiment was used.

Precision and Accuracy
Interday precision was determined by measuring QC samples five times consecutively per day for twenty successive working days. For each day a fresh batch of QC samples was prepared. The intraday precision was assessed by measuring one set of QC samples 20 times on the same day. This was repeated on a different day in a different batch of QC samples. The samples were prepared as described in 2.7. Precision was expressed as CV%.

Due to the lack of reference material and reference methods, accuracy could not be determined with samples of known concentration. However, accuracy was determined according to FDA guidelines for industry and expressed as the bias (%error) from a known concentration which should not deviate more than 15% from the known concentration.

Patient samples
To compare our measurements with previously reported erythrocyte MTX-PG levels in RA patients, 50 samples were obtained from RA patients enrolled in the treatment in Rotterdam Early Arthritis CoHort (tREACH) study [22, 23]. These patients were treated with a similar amount (median 25 mg/wk, range 10-25 mg/wk) of MTX for the same period (median 97 days, range 50-121 days). Patient consent was obtained for the tREACH study and the study was approved by the local ethics committee. Samples were prepared as described in paragraph 2.7.

Statistics
Quantitation was performed using peak-area ratio of analyte to internal standard. Microsoft Excel and Analyse-it for Microsoft Excel (version 2.20) were used to assess linearity according to CLSI EP-6 (matrix-effects studies and linearity studies)[24]. Precision was expressed as coefficient of variation (CV): *CV% = (SD/mean) × 100%*. Accuracy was expressed as %bias and calculated as the %error.

Results

MS optimization

To establish the appropriate MS conditions for each individual MTX-PG, a 1 µmol/L standard solution was directly infused into the MS system. Collision-induced dissociation of the protonated molecules was performed and the product ion with the highest abundance was chosen for selected reaction monitoring (SRM) analysis. All MTX-PGs and MTX-PGs(M+6) had an identical fragment ion of m/z 308.2 (Figure 1). Other mass transitions were detected but their S/N ratio was too low to be used for quantitation.

Selectivity and IS integrity

When 20 drug-free erythrocyte pellets were measured, the signal of the MTX-PG and MTX-PG(M+6) mass transition did not rise above the noise, showing good selectivity of the assay. Individual MTX-PG(M+6) IS were tested for the presence of MTX-PG and MTX-PG(n+1) and were not found to contain significant traces of MTX-PG (<0.044% MTX-PG; <0.026% MTX-PG(M+1); <0.025% MTX-PG(M+2)). Injecting 1 µM clean standard solution of each MTX-PG(M+6) yielded a background signal <1.1% for each of the MTX-PGs. When drug-free pellets were spiked with 50 nmol/L of each of the MTX-PGs, there was a small amount of background for MTX-PG1-4 (~0.5 nmol/L for each MTX-PG). The blank standard in the calibration line was used to correct for this background.

Carry-over and Linearity

No significant carry-over was detected for any of the MTX-PGs (mean carry-over 0.04% range 0.0-0.1%). The method was linear for all MTX-PGs from 0.975-1000 nmol/L (R^2>0.99 for all MTX-PGs, Table 1).

Table 1. Linearity[a]

	$R^2 \pm SD$[b]	Slope $\pm$ SD[b]
MTX-PG1	0.9995 ± 0.0001	0.9996 ± 0.0003
MTX-PG2	0.9972 ± 0.0037	0.9973 ± 0.0038
MTX-PG3	0.9969 ± 0.0045	0.9991 ±0.0007
MTX-PG4	0.9991 ± 0.0003	0.9994 ± 0.0006
MTX-PG5	0.9996 ± 0.0001	0.9997 ± 0.0002

[a]Linearity based on a 1-1000 nmol/L calibration curve including a blank sample.

[b]R^2 and slope represent the average of 3 linearity experiments in 3 different batches of pooled erythrocytes.

Recovery

Recovery was measured in 10 batches of pooled pellet and ranged from 54-98% (mean recovery 80% ± 11%) with a CV% of 14%. After correction with the internal standard, recovery ranged from 89-108% (mean recovery 100 ± 3%) with a CV% of 5% for all MTX-PGs at all concentrations tested.

Matrix-effects

When not corrected for the IS, a noticeable absolute matrix-effect was present for each of the MTX-PGs (63-105%) with a prominent relative matrix-effect (87-95%, maximum slope difference of 15-47%) for all MTX-PGs. After correction with the IS, the absolute matrix-effect was compensated for all MTX-PGs but to a lesser extent for MTX-PG4. However, the relative matrix-effect was low for each of the MTX-PGs, with an acceptable maximum slope difference (3-17%, Table 2). All slopes that were used to determine matrix-effects were linear ($R^2 > 0.995$).

Table 2. Matrix-effects

	IS corrected (response)			Not IS corrected (Area)		
	Absolute Matrix-effect[a] (CV)	Min/Max[b]	Relative Matrix-effect	Absolute Matrix-effect (CV)	Min/Max	Relative Matrix-effect
MTX-PG1	102% (2%)	4%	98%	65% (13%)	47%	87%
MTX-PG2	100% (1%)	4%	99%	63% (12%)	42%	88%
MTX-PG3	101% (3%)	11%	97%	82% (5%)	16%	95%
MTX-PG4	84% (5%)	17%	95%	74% (5%)	15%	95%
MTX-PG5	100% (1%)	3%	99%	105% (10%)	34%	90%

All slopes were $r^2 > 0.999$ when corrected for IS.
[a]Matrix-effect is expressed as % recovery and calculated by comparing the slopes of reference curves made in 10 different plasma matrices with the slope of a reference curves made in water.
[b]The maximum slope difference shows the % difference between the largest and smallest slope, indicating the variance between slopes in different matrices.

Stability

When storing untreated QC samples at −80°C, a 10% loss in recovery was observed (mean recovery: 90% range 89-98%). However, no further loss was observed for at least 3 months at −80°C (Table 3). Pre-treated QC samples were stable when kept in the autosampler at 4°C for up to 2 weeks (mean recovery: 101% range 96-113%) (Table 3). Three subsequent freeze-thaw cycles did not influence the recovery of MTX-PGs in erythrocytes (mean recovery 100%, range 93%-107%).

Lower limit of quantitation (LLOQ)

LLOQs were determined as the lowest concentration that resulted in a CV% <20% and S/N >10:1. However, as all concentrations tested had a CV% <20% and an S/N ratio >10:1, the LLOQ was set at 1 nmol/L for all MTX-PGs. At this concentration there was always a visual and distinguishable peak in every calibration curve, and a response at least five times higher than the response of the lowest sample of the calibration curve (0 nmol/L).

Table 3. Stability

	Before sample preparation stored at −80°C[a]			After sample preparation stored at 4 °C[b]	
	1 week (Recovery % ± SD)	**1 month** (Recovery % ± SD)	**3 months** (Recovery % ± SD)	**1 week** (Recovery % ± SD)	**1 month** (Recovery % ± SD)
MTX-PG1	90 ± 3%	91 ± 3%	92 ± 4%	99 ± 3%	99 ± 3%
MTX-PG2	98 ± 5%	86 ± 5%	87 ± 3%	101 ± 3%	99 ± 6%
MTX-PG3	89 ± 7%	89 ± 8%	95 ± 6%	98 ± 3%	103 ± 8%
MTX-PG4	89 ± 2%	96 ± 10%	92 ± 11%	96 ± 4%	98 ± 2%
MTX-PG5	95 ± 2%	87 ± 1%	86 ± 1%	113 ± 4%	106 ± 6%

[a]The mean recovery represents the recovery of the different QC sample concentrations, measured in fivefold and compared to the results of the freshly prepared QC samples.
[b]The mean recovery represents the recovery of a prepared calibration curve, measured in twofold and compared to the results of the freshly prepared calibration curve

Precision and Accuracy

The median CV for intraday precision was 2.1% (range 1.0-4.3%), whereas for interday precision the median CV was 8.4% (range 5.9-14.7%) for all MTX-PGs at all concentrations. The bias was <15% for all MTX-PGs at all concentrations (Table 4).

Table 4. Precision

		Intraday precision[a]			Interday precision[b]		
	Concentration (nmol/L)	**Mean ± SD** (nmol/L)	**CV%**	**Bias** (%)	**Mean ± SD** (nmol/L)	**CV%**	**Bias** (%)
MTX-PG1	5	5.9 ± 0.19	3.0	-5.5	5.2 ± 0.43	8.3	-3.6
	50	52.3 ± 0.83	1.6	-1.3	49.8 ± 3.12	6.3	0.5
	100	98.6 ± 1.91	1.9	5.6	93.5 ± 6.03	6.4	6.5
MTX-PG2	5	5.68 ± 0.12	1.9	4.1	5.1 ± 0.47	9.2	-1.5
	50	51.4 ± 1.23	2.3	-2.4	50.1 ± 3.54	7.1	-0.2
	100	99.6 ± 1.46	1.4	5.1	94.9 ± 7.94	8.4	5.1
MTX-PG3	5	5.4 ± 0.17	2.8	-9.7	4.8 ± 0.51	10.6	3.4
	50	48.7 ± 0.88	1.8	-1.6	47.5 ± 4.14	8.7	5.1
	100	92.2 ± 1.62	1.7	-5.3	90.8 ± 6.66	7.3	9.2
MTX-PG4	5	5.1 ± 0.2	3.4	-10.0	4.5 ± 0.67	14.7	9.1
	50	46.0 ± 0.77	1.7	-1.6	47.3 ± 4.66	9.8	5.4
	100	89.1 ± 0.91	1.0	5.5	92.3 ± 10.61	11.5	7.7
MTX-PG5	5	4.8 ± 0.21	4.3	7.0	4.6 ± 0.56	12.1	7.1
	50	44.7 ± 0.62	1.4	8.0	46.7 ± 2.75	5.9	6.6
	100	84.9 ± 1.46	1.7	15.0	87.2 ± 6.76	7.7	12.8

[a]The intraday precision summarizes 20 replicate measurements at each concentration of MTX during one day.
[b]The interday precision summarizes 20 different experiments from 20 consecutive days with measurement done in fivefold each day.

Patient samples

The 50 patient samples obtained from the tREACH study contained a mean concentration of 139.0 ± 54.3 nmol/L of total MTX and an average of 35.3 ± 28.1 nmol/L MTX-PG1, 21.3 ± 9.2 nmol/L MTX-PG2, 51.4 ± 19.6 nmol/L MTX-PG3, 23.4 ± 17.1 nmol/L MTX-PG4 and 7.7 ± 10.4 nmol/L MTX-PG5 respectively (Figure 2). The pre-dominant glutamate form of MTX in erythrocytes was MTX-PG3, which constituted 37% of the total erythrocyte MTX content. MTX-PG5 was <LLOQ in 5 out of 50 samples.
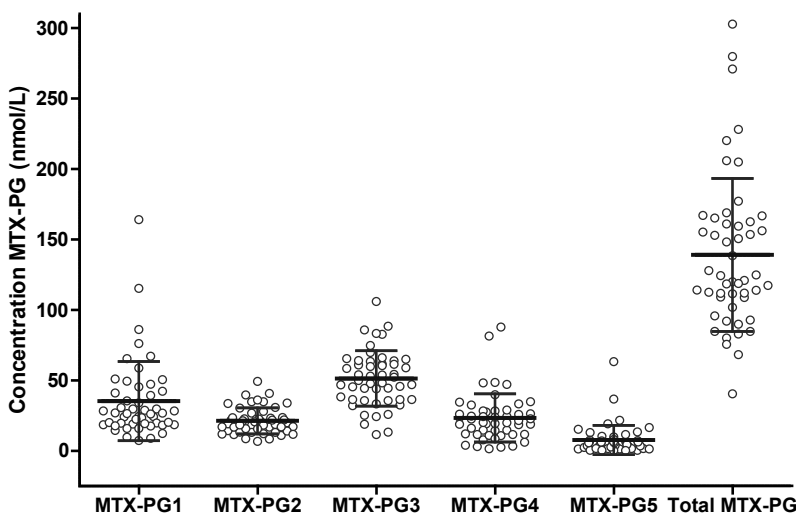
Figure 2. MTX-PG content of 50 RA patients treated with MTX for 3 months (mean treatment duration 93.0 ± 13.1 days). Mean MTX dose was 24.0 ± 3.4 mg/week. MTX was administered orally in all patients

Discussion

We developed a simple LC-MS/MS-based method for the separate quantitation of individual MTX-PGs in human erythrocytes. To our knowledge this is the first method using stable isotope internal standards for the measurement of MTX-PG1-5.

While other methods have been reported for measuring MTX-PGs in erythrocytes, most have longer sample-preparation and analysis times, and most do not use mass spectrometry for detection [4, 11, 25]. Therefore, these methods might be less specific and prone to interference by compounds similar to MTX, such as folates [5]; this translates to lower sensitivity and higher limits of quantitation.

The described method has a linear range of 1-1000 nmol/L, which contains the known clinical range of the different MTX-PGs [6, 10]. At 1 nmol/L the LLOQ is comparable to [16], or lower than previously reported methods [4, 5, 17]. This is especially important for the longer-chain MTX-PGs as these are present in lower concentrations than the short-chain MTX-PGs [6, 10]. Improvements in LLOQ can still be achieved by better sample clean-up such as SPE, but this would increase the cost and preparation time of the method.

As shown in our validation experiments, recovery and matrix-effects differ strongly for each MTX-PG and between different erythrocyte pellets. The use of a stable-isotope labeled IS is important for a reliable quantitation in a complex matrix such as erythrocytes.

The stable-isotope IS compensates for absolute matrix-effect in all MTX-PGs except MTX-PG4, where the internal standard did not fully compensate for absolute matrix-effect even after correction with the IS. However, this was not considered a problem, as the stable isotope IS compensates sufficiently for the relative matrix-effects.

Recovery based on peak area was moderate with high variance, which was compensated by the stable-isotope-labeled IS. As this resulted in more reproducible measurements and a recovery of approximately 100%, it emphasizes the need for a proper internal standard.

QC samples were stable for at least three months at −80°C with approximately 90% recovery. Although no freeze-thaw effect was found, the stability study suggests there is indeed a small freeze-thaw effect as QC samples stored at −80°C had a stable 90% recovery at 4 weeks, 8 week and 12 weeks. QC samples stored after sample preparation were stable, with approximately 100% recovery.

The intraday CV of <2.5% and interday CV of <12.0% are improved over our previous method (intraday CV<13.2%, interday CV<12.1% [17]) and are well within acceptable limits [18]. Moreover, our precision is better than the ion-pairing-based MS method reported recently (intraday <20.0%, interday <23.7% [16]).

We used our method to analyze the erythrocyte MTX-PG content of 50 patient samples obtained from the tREACH study [22]. The concentrations and distribution of MTX-PG1-5 as measured by our method were in line with those previously reported [4, 5, 7, 10, 26].

Theoretically, MTX-PG6 and MTX-PG7 are also present in the erythrocyte. However, these have not been proven conclusively. As analysis of multiple patients in literature and in our patient samples did not reveal detectable amounts of MTX-PG6 and MTX-PG7 [4, 10], and because of the prohibitive cost of the MTX-PG6 and MTX-PG7 stable-isotopes, we decided not to focus on MTX-PG6 and MTX-PG7.

In conclusion, we describe an LC-MS/MS-based method to measure MTX in erythrocytes with minimal sample pretreatment, relatively short analysis time, and good diagnostic performance that can be applied in routine clinical TDM. The method is validated for the clinical lab and is currently the only method that measures MTX-PG1-5 separately using stable isotope internal standards.

Chapter

3

References

1   Strand V, Cohen S, Schiff M, Weaver A, Fleischmann R, Cannon G, et al. Treatment of active rheumatoid arthritis with leflunomide compared with placebo and methotrexate. Leflunomide Rheumatoid Arthritis Investigators Group. Arch Intern Med. 1999 Nov 22;159(21):2542-50.

2   Chabner BA, Allegra CJ, Curt GA, Clendeninn NJ, Baram J, Koizumi S, et al. Polyglutamation of methotrexate. Is methotrexate a prodrug? J Clin Invest. 1985 Sep;76(3):907-12.

3   Jolivet J, Schilsky RL, Bailey BD, Drake JC, Chabner BA. Synthesis, retention, and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. J Clin Invest. 1982 Aug;70(2):351-60.

4   Dervieux T, Orentas Lein D, Marcelletti J, Pischel K, Smith K, Walsh M, et al. HPLC determination of erythrocyte methotrexate polyglutamates after low-dose methotrexate therapy in patients with rheumatoid arthritis. Clin Chem. 2003 Oct;49(10):1632-41.

5   van Haandel L, Becker ML, Leeder JS, Williams TD, Stobaugh JF. A novel high-performance liquid chromatography/mass spectrometry method for improved selective and sensitive measurement of methotrexate polyglutamation status in human red blood cells. Rapid Commun Mass Spectrom. 2009 Dec;23(23):3693-702.

6   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Caldwell J, et al. Pharmacogenetic and metabolite measurements are associated with clinical status in patients with rheumatoid arthritis treated with methotrexate: results of a multicentred cross sectional observational study. Ann Rheum Dis. 2005 Aug;64(8):1180-5.

7   Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum. 2006 Oct;54(10):3095-103.

8   Hroch M, Tukova J, Dolezalova P, Chladek J. An improved high-performance liquid chromatography method for quantification of methotrexate polyglutamates in red blood cells of children with juvenile idiopathic arthritis. Biopharm Drug Dispos. 2009 Apr;30(3):138-48.

9   Becker ML, Gaedigk R, van Haandel L, Thomas B, Lasky A, Hoeltzel M, et al. The effect of genotype on methotrexate polyglutamate variability in juvenile idiopathic arthritis and association with drug response. Arthritis Rheum. 2011 Jan;63(1):276-85.

10   Becker ML, van Haandel L, Gaedigk R, Lasky A, Hoeltzel M, Stobaugh J, et al. Analysis of intracellular methotrexate polyglutamates in patients with juvenile idiopathic arthritis: effect of route of administration on variability in intracellular methotrexate polyglutamate concentrations. Arthritis Rheum. 2010 Jun;62(6):1803-12.

11   Cheng HL, Liao YM, Chiou SS, Wu SM. On-line stacking capillary electrophoresis for analysis of methotrexate and its eight metabolites in whole blood. Electrophoresis. 2008 Sep;29(17):3665-73.

12   Hayashi H, Fujimaki C, Tsuboi S, Matsuyama T, Daimon T, Itoh K. Application of fluorescence polarization immunoassay for determination of methotrexate-polyglutamates in rheumatoid arthritis patients. Tohoku J Exp Med. 2008 May;215(1):95-101.

13   Inoue S, Hashiguchi M, Kawai S, Mochizuki M. Erythrocyte methotrexate-polyglutamate assay using fluorescence polarization immunoassay technique: application to the monitoring of patients with rheumatoid arthritis. Yakugaku Zasshi. 2009 Aug;129(8):1001-5.

14   Al-Turkmani MR, Law T, Narla A, Kellogg MD. Difficulty measuring methotrexate in a patient with high-dose methotrexate-induced nephrotoxicity. Clin Chem. 2010 Dec;56(12):1792-4.

15   Albertioni F, Rask C, Eksborg S, Poulsen JH, Pettersson B, Beck O, et al. Evaluation of clinical assays for measuring high-dose methotrexate in plasma. Clin Chem. 1996 Jan;42(1):39-44.

16   van Haandel L, Becker ML, Williams T, Leeder JS, Stobaugh JF. Measurement of methotrexate polyglutamates in human erythrocytes by ion-pair UPLC-MS/MS. Bioanalysis. 2011 Dec;3(24):2783-96.

17   Meesters RJ, den Boer E, de Jonge R, Lindemans J, Luider TM. Assessment of intracellular methotrexate and methotrexate-polyglutamate metabolite concentrations in erythrocytes by ultrafast matrix-assisted laser desorption/ionization triple quadrupole tandem mass spectrometry. Rapid Commun Mass Spectrom. 2011 Oct 30;25(20):3063-70.

18   CDER. Center for Drug Evaluation and Research (CDER) Guidance for Industry: Bioanalytical Method Validation. 2001.

19   Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 2003 Jul 1;75(13):3019-30.

20   Broughton PM. Carry-over in automatic analysers. J Automat Chem. 1984;6(2):94-5.

21   Vogeser M, Seger C. Pitfalls associated with the use of liquid chromatography-tandem mass spectrometry in the clinical laboratory. Clin Chem. 2010 Aug;56(8):1234-44.

22   Claessen SJ, Hazes JM, Huisman MA, van Zeben D, Luime JJ, Weel AE. Use of risk stratification to target therapies in patients with recent onset arthritis; design of a prospective randomized multicenter controlled trial. BMC Musculoskelet Disord. 2009;10:71.

23   de Jong PH, Hazes JM, Barendregt PJ, Huisman M, van Zeben D, van der Lubbe PA, et al. Induction therapy with a combination of DMARDs is better than methotrexate monotherapy: first results of the tREACH trial. Ann Rheum Dis. 2012 Jun 7.

24   Evaluation of the Linearity of Quantitative Analytical Methods, NCCLS Document EP6-P,. 1986;6(18).

25   Uchiyama M, Matsumoto T, Jimi S, Takamatsu Y, Tamura K, Hara S. Simple and sensitive HPLC method for the fluorometric determination of methotrexate and its major metabolites in human plasma by post-column photochemical reaction. Biomed Chromatogr. 2011 Mar 25.

26   Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Frampton C, James J, et al. Determinants of red blood cell methotrexate polyglutamate concentrations in rheumatoid arthritis patients receiving long-term methotrexate treatment. Arthritis Rheum. 2009 Aug;60(8):2248-56.

Chapter

3

Chapter 4

A new U-HPLC-ESI-MS/MS based stable isotope dilution method for the detection and quantitation of methotrexate in plasma

E. den Boer

S.G.Heil

B.D. van Zelst

R.J.W. Meesters

B.C.P. Koch

M.L. te Winkel

M.M. van den Heuvel-Eibrink

T.M. Luider

R. de Jonge

Abstract:

BACKGROUND: High dose methotrexate (MTX) is used in the treatment of proliferative diseases such as acute lymphoblastic leukemia. Therapeutic drug monitoring of plasma MTX is important to monitor efficacy and adverse events. The authors aimed to develop a new mass spectrometry based method for determining MTX in plasma for patient diagnosis and pharmacokinetic studies.

METHODS: Samples were analysed using a Waters Acquity U-HPLC and Quattro Premier XE. A Waters Acquity U-HPLC BEH C18 column (2.1 mm x 100 mm, 1.7 μm) was used running an isocratic mobile phase of 21% methanol and 10 mM ammonium bicarbonate. The electrospray was operated in the positive ionization mode monitoring the following mass transitions: m/z 455.2>308.2 for MTX and m/z 458.2>311.2 for MTX-d3. The analysis combines straightforward sample preparation, consisting of dilution and protein precipitation, with a 3 minute run time.

RESULTS: The method was linear up to 50 μmol/L (r^2>0.99) and a coefficient of variation (CV) of <6% for intraday and <10% for interday precision was found. Average recovery was 99%. There were no significant matrix effects. The lower limit of quantitation, defined as the lowest concentration where CV<20% and S/N>1:10, was 5 nmol/L. Method comparison with the Abbott TDx FPIA immunoassay showed excellent agreement and a small but significant negative constant bias was detected (LC-MS/MS=0.98 × FPIA-7.3).

CONCLUSIONS: The authors developed a specific and sensitive stable isotope dilution LC-ESI-MS/MS method to monitor MTX levels in plasma within the clinically relevant range. The method can be easily applied in clinical laboratories because it combines straightforward sample pre-treatment with LC-MS/MS.

Introduction

Methotrexate (MTX) is a folate antagonist that has been used in clinical practice for five decades. It is used in the treatment of proliferative diseases such as acute lymphoblastic leukemia (ALL) [1, 2], which is the most frequent cancer in children aged less than 15 years, [3] and other proliferative diseases [4]. Treatment is often limited due to severe toxicity [5] and monitoring of plasma MTX is important to assess efficacy and adverse events [3, 6, 7].

Plasma MTX is commonly measured using immunoassays like the Fluorescent Polarization Immuno Assay (FPIA) and Enzyme Multiplied Immunoassay Technique (EMIT) [8], from Abbott and Siemens, respectively. Immunoassays need limited sample preparation and relatively fast measuring times can be achieved, which make them suitable for routine clinical application.

However, immunoassays are expensive and are hampered by interference such as cross reaction of folates and MTX metabolites leading to low specificity [9-12]. While immunoassays are easy to implement and run, the large concentration range of MTX plasma in high-dose (HD) MTX treated patients (20 nmol/L to 100 µmol/L) imposes the need for testing samples in serial dilution [12, 13]. This compromises the assay's throughput unless an extensive infrastructure is present. Furthermore, immunoassays generally display little sensitivity [9]; the lower limit of quantitation (LLOQ) of most immunoassays for MTX lies in the 20 nmol/L range. While this range is below the clinically relevant levels (therapeutic drug monitoring (TDM) of MTX plasma levels generally stops below 100 nmol/L) pharmacokinetic studies might benefit from a lower LLOQ.

Several high performance liquid chromatography (HPLC) based methods have been developed to improve specificity and sensitivity of plasma MTX detection. Commonly used detection methods include fluorescence detection [6, 14-16], UV detection [17] and mass spectrometry [18-21]. While HPLC based methods have many advantages, most HPLC methods rely on UV detection of fluorescence which makes these methods subject to interference by other compounds, most notoriously folates and the deconjugated MTX form DAMPA [22], leading to poor specificity and lower sensitivity (high LLOQ).

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is becoming a more routine method in the clinical laboratory [23]. LC-MS/MS is generally more specific than either conventional HPLC or immuno-assay methods. In addition, these LC-MS/MS based methods will in most cases result in shorter analysis times than HPLC methods, leading to higher throughput [24]. Therefore, we aimed to develop and validate a new LC-MS/MS method for the measurement of MTX in plasma.

Methods

Chemicals and blood specimens

MTX was purchased from Schircks Laboratories (Jona, Switzerland). Deuterium labeled MTX (MTXd3) was used as internal standard (IS) and was purchased from Toronto Research Chemicals Inc. (North York, Canada). Chemical structures of MTX and the deuterated

analogue are illustrated in Figure 1. LC-MS grade methanol and water were obtained from Biosolve (Valkenswaard, the Netherlands). Ammonium bicarbonate and perchloric acid (70% v/v) were purchased from Sigma (Zwijndrecht, the Netherlands). Ammonia (25% v/v) was purchased from Merck (Schiphol-Rijk, the Netherlands).

Blinded drug-free and patient blood samples were used for development and validation of the method. Blinded samples were left-over from routine analysis at the department of Clinical Chemistry and the Hospital Pharmacy and hence, no medical-ethical approval was necessary for this study.

Instruments

Analyses were performed on a LC-MS/MS system consisting of a Waters Acquity UPLC (Waters Corporation, Etten-Leur, the Netherlands) equipped with a 20 µl sample loop and a Quattro Premier XE (Waters Corporation, Etten-Leur, the Netherlands) triple quadrupole mass spectrometer with an electrospray ionization source (ESI) operated in the positive ionization mode.

Chromatographic conditions

Chromatography was done using partial loop injection of 10 µl sample on a Waters Acquity UPLC BEH C18 column (2.1 mm x 100 mm, 1.7 µm) maintained at 35°C. The mobile phase consisted of (A) 10 mM ammonium bicarbonate adjusted to pH 10 with 25% ammonia and (B) methanol. The system was maintained at a flow rate of 0.3 ml/min. keeping an isocratic concentration of 21% B. After every 80 samples, the column was flushed 10 min with 100% methanol at a flow rate of 0.3 ml/min followed by re-equilibration with 21% B at a flow rate of 0.3 ml/min.

MS detection

The ESI was operated in the positive mode with the following selected reaction monitoring mass transitions: m/z 455.2>308.2 for MTX and 458.2>311.2 for MTXd3 (Figure 1). Other mass spectrometer settings were: capillary voltage 1.00 kV, cone voltage 30 V, collision energy 20 eV, source temperature 120°C desolvation temperature 350°C at a gas flow of 700 l/hr and cone gas flow 50 l/hr. Argon was used as collision gas at a flow rate of 0.20 ml/min.

Preparation of standards

Stock solutions were prepared in 0.1 mol/L ammonia at concentrations of 1836.6 µmol/L for MTX and 10 µmol/L for MTXd3. Both stock solutions were stored at −80°C. The stock solution of MTXd3 was diluted with LC-MS grade water to a working solution of 100 nmol/L to serve as internal standard (IS). The working solution was prepared freshly for each batch of prepared samples.

Preparation of calibrators and quality control samples

Frozen drug-free plasma was thawed at room temperature while rotating and spiked with MTX to a final concentration of 50 µmol/L. A sixteen point calibration range was obtained by 1:1 serial dilution, containing 3.05, 6.10, 12.21, 24.41, 48.83, 97.66, 195.31, 391.63, 781.25, 1562.5, 3125, 6250, 12500, 25000 and 50000 nmol/L MTX as well as a blank sample (0 nmol/L). A fresh calibration curve was prepared for each run.

Quality control (QC) samples were prepared in drug-free plasma at five different concentrations: 10, 50, 500, 5000 and 50000 nmol/L MTX. Aliquots were stored at −80 °C and thawed at 4°C while rotating.

Sample preparation

Plasma was obtained by centrifuging K_2-EDTA whole-blood tubes at 2700 × g. A 50 µl plasma aliquot was added to 450 µl drug-free plasma and then supplemented with 500 µl cold IS solution (100 nmol/L MTXd3 in H2O). Protein precipitation was done by the addition of 800 µl cold 16% perchloric acid to the diluted sample while vortexing. The samples were placed on ice for 30 min to enhance precipitation, after which they were centrifuged for ten minutes at 2700 × g. The clear supernatant was transferred to a sample vial and placed in the autosampler of the LC-MS/MS system maintained at 4°C. The injection volume was 10 µl. Calibrators and QC samples were also prepared as described above. Concentrations were multiplied by 10 to correct for dilution.

Validation procedures

Method validation was adapted from the 2001 FDA guidelines [25] and Matuszewski et al. [26]. It consisted of selectivity, IS integrity, carry-over, stability (short/long term storage, freeze/thaw), recovery, matrix effects, linearity, LLOQ, precision (interday/intraday) and accuracy in comparison to FPIA (gold standard). All samples were measured in duplicate unless otherwise specified.

Selectivity of the detection was determined by measuring 20 different drug-free samples, which were prepared as described in the "materials and methods" section but replacing MTX and IS solution with LC-MS grade water. The average background signal was calculated for both MTX and MTXd3 and was used as noise when calculating the signal/noise ratio.

IS integrity was tested by measuring the presence of MTX, MTX mass+1, MTX mass+2 and MTX mass+3 in LC-MS/MS grade water spiked with 100 nmol/L IS.

Carry-Over was assessed by triplicate measurement of a spiked plasma sample with high concentration (50 µM) directly followed by triplicate measurement of a sample with low (50 nmol/L) concentration and calculated as ((Low1 − Low3)/(High3 − Low3)) × 100% [27]. This was repeated in 3 different plasma batches.

Stability of MTX in plasma was studied by measuring the stored QC samples after 1 week, 6 weeks and 6 months at −80 °C. The stability of MTX after sample preparation was

studied by storing a prepared concentration range (3-50,000 nmol/L) in the autosampler at 4 °C for 16 days. Freeze/thaw stability was investigated by measuring QC samples after three successive cycles of freezing at −80 °C and unassisted thawing on ice. All samples were measured five times from the same vial and the results from these stability experiments were compared with freshly prepared and directly measured samples.

Recovery of the sample preparation was determined by spiking ten different blank plasma samples with three different MTX concentrations (50, 500, 5000 nmol/L) and IS (100 nmol/L) before and after sample preparation. The recovery was calculated with the following formula: Recovery (%) = (MTX spiked before sample prep/MTX spiked after sample prep) × 100%.

Matrix effects can have a detrimental effect on MS based analysis [28] and both absolute and relative matrix effects can occur [26]. Absolute matrix effect was defined as the difference in response between analyte spiked in sample matrix versus analyte spiked in pure solvent. A relative matrix effect is described as the variation in response between different samples in a similar matrix and can be expressed as the coefficient of variation (CV%) between the slopes of reference curves made in different samples of the same matrix. The evaluation of matrix effects was performed according to the recommendations of Matuszewski et al. [26]. Ten reference curves were prepared by spiking ten different drug-free plasma batches with five concentrations (10 nmol/L, 50 nmol/L, 500 nmol/L, 5 µmol/L and 50 µmol/L) of MTX after sample preparation. The slopes of these reference curves in the different drug-free plasma batches were compared with the slope of a reference curve prepared in purified water to determine the absolute matrix-effect. The absolute matrix-effect was defined as: *Absolute matrix effect = (mean slope plasma sample/slope distilled water) × 100%*. By comparing the reference curves of the ten standard lines from the ten different plasma samples the relative matrix-effect was calculated, which was expressed as: *Relative matrix-effect = 100% - CV% of the slopes of the ten reference curves*. Furthermore, the maximum slope difference was calculated as the difference in percentage between the lowest slope and highest slope of the ten reference curves. All matrix effects were calculated with and without correction by the internal standard. Slopes were calculated by linear regression analysis.

Linearity of the method was determined by spiking drug-free plasma with 22 different concentrations of MTX in a range of 3-250 µmol/L. This range reflects the full clinical range of MTX concentrations reported the past year at the Hospital Pharmacy of the Erasmus MC. Samples were measured five times from the same vial and the experiment and the experiment was repeated in a second drug-free plasma batch. Linearity was considered acceptable if the coefficient of determination (R^2) was >0.99 for each calibration curve and if the lack of fit was <3.75.

LLOQ was determined by spiking drug-free plasma samples with different levels of MTX covering a range of 3-50000 nmol/L. These were measured ten times from the same vial for each concentration level and the experiment was repeated in a second drug-free

plasma batch. The LLOQ was defined as the lowest concentration of a serial dilution with a CV<20%, while having an area of at least ten times the average area of the drug-free plasma samples used for the selectivity experiment and five times the area of the blank sample (0 nmol/L).

Precision and accuracy were determined by measuring QC samples five times on twenty consecutive working days for interday precision. The QC samples were thawed and prepared as described in the "materials and methods" section. Everyday a new batch of drug-free plasma was used for the dilution of the QC samples. The intraday precision was assessed by measuring QC samples twenty times in one run. This was repeated on a different day with another drug-free plasma batch for the dilution of the QC samples. Precision was expressed as CV% and accuracy as bias (%error).

Method comparison

Blood samples from ALL patients were collected in K_2-EDTA tubes from November, 2009 to March 2011 by the Hospital Pharmacy of the Erasmus MC. These were used for routine measurement of plasma MTX using the fluorescence polarization immunoassay (FPIA) on an Abbott TDx FLx Immunology Analyzer (Abbott Diagnostics, Hoofddorp, the Netherlands).

In total, 194 plasma samples from 34 ALL patients receiving 2-5 g/m² MTX for 24 h were measured both with FPIA and with LC-MS/MS. After measurement by the Hospital Pharmacy, left over plasma was kept at -20 °C before measurement on the LC-MS/MS. All samples were measured twice from the same vial on the LC-MS/MS to be able to detect carry-over.

Samples were measured in random order to reduce sample selection bias. Samples were re-measured by LC-MS/MS when: i) duplicate measurements differed more than 15%, ii) samples were below the LLOQ or iii) above the upper limit of quantitation (ULOQ). All samples that were re-measured were both obtained from the original prepared sample as well as from freshly prepared sample. Samples that were below LLOQ were repeated undiluted, whereas samples that were above ULOQ were diluted 1:100 in drug-free plasma before preparation.

Statistics

Quantitation was performed using peak area ratio of analyte to internal standard. Microsoft Excel and Analyse-it for Microsoft Excel (version 2.20) were used to perform linear regression (matrix effects studies, calibration lines, linearity). Validation studies were done according to CLSI EP-6 and Passing & Bablok regression analysis with 95% confidence intervals (method comparison). Precision was expressed as coefficients of variation (CV): CV% = (mean/stdev) × 100%. MassLynx software (Waters) was used for the analysis of chromatograms. The processing of MS data was done using QuanLynx software (Waters).

Chapter

4

Results

Method performance

To establish the appropriate MS conditions, a standard solution was directly infused into the MS for optimization. Collision induced dissociation of the protonated molecules was performed and the product ions with the highest abundance were selected for selected reaction monitoring (SRM) analysis. Mass transitions obtained in positive mode were: 455.2>308.2 for MTX and 458.2>311.2 for MTXd3 (Figure 1). Other mass transitions were detected but their S/N ratio was too low to be used for quantitation.

When measuring 20 drug-free samples the signal did not rise above the noise, showing good selectivity of the assay and that no interferences were detected. 100 nmol/L MTXd3 in water yielded 0%, 0%, 3% and 97% for MTX, MTX mass+1, MTX mass+2 and MTX mass+3 respectively, showing good integrity and purity. Mean carry-over was 0.021%.
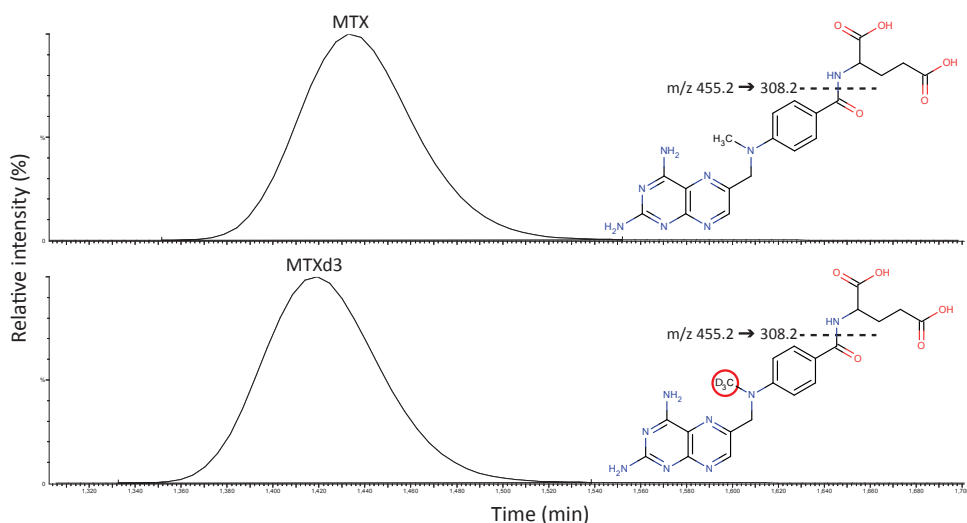
Figure 1. Chromatograms and chemical structures of methotrexate and internal standard (MTXd3). The location of the deuterium enriched methyl-group is highlighted. The SRM for both MTX and MTXd3 are depicted above the molecule.

The results for the stability experiments are presented in Table 1. Untreated plasma samples were stable when stored for 1 week at −80 °C (mean recovery: 106%), 6 weeks at −80°C (mean recovery: 106%), or 6 months at −80 °C (mean recovery: 107%). After preparation samples were stable when kept in the autosampler at 4 °C for up to 16 days (mean recovery: 102%). Furthermore, this was confirmed in 11 patient samples that were re-measured after one week at 4 °C (mean recovery: 110%). Three subsequent freeze-thaw cycles did not significantly influence MTX concentration. The observed decrease in response after freeze-thaw was 3%.

Table 1. Recovery MTX in plasma (n=10)

Sample concentration (nmol/L)	Measured concentration (nmol/L ± SD)	Average recovery (± SD)	CV%
50 nM	50.4 ± 2.5	101% ± 5%	5%
500 nM	503.2 ± 14.6	99% ± 5%	5%
5000 nM	5001.6 ± 175.7	98% ± 6%	6%

Average recovery ranged from 96-102% for all concentrations (50 nmol/L, 500 nmol/L, 5000 nmol/L) of MTX with a CV% of <15% for each concentration when based on the area of the MTX peaks. After correction with the IS, average recovery ranged from 98-101% for all concentrations with a CV% of <6% for each concentration (Table 2).

The absolute matrix effect calculated without correction for the IS was 71% with a relative matrix effect of 81% and a maximum slope difference of 67%, showing large variation between the different samples. After correction with the IS, the absolute matrix effect improved to 94% with a relative matrix effect of 98% and a maximum slope difference of 7%. All slopes showed an r^2 >0.999. Matrix effects tested in sample without dilution in drug-free plasma yielded similar results (Table 3).

Table 2. Stability of stored spiked plasma

Conditions		Average recovery (range)
Before sample preparation[a]	1 week -80 °C	106% (95-115%)
	6 weeks -80 °C	106% (100-115%)
	6 months -80 °C	107% (103-114%)
Freeze/Thaw[a]	(3 cycles -80 °C)	97% (92-99%)
After sample preparation[b]	1 day 4 °C	102% (93-115%)
	1 week 4 °C	102% (91-110%)
	2 weeks 4 °C	102% (93-113%)

[a]The average recovery represents the recovery of the different QC sample concentrations, measured in fivefold and compared to the results of the freshly prepared QC samples.
[b]The average recovery represents the recovery of a prepared calibration curve, measured in twofold and compared to the results of the freshly prepared calibration curve

The method was linear up to 50 μmol/L (y=-0.326 + 0.997x) and subsequent validation samples and QC samples were done using calibration curves of 3 nmol/L – 50 μmol/L (supplementary data 1). These were linear (r^2>0.998, lack-of-fit <0.85) throughout the validation.

When determining the LLOQ the CV% did not become larger than 20% at any concentration other than the blank (0nmol/L) sample (supplementary data 2). In all samples the signal to noise ratio was better than 10:1. The LLOQ was set at 5 nmol/L because at this concentration there was always; 1) a visual and distinguishable peak present in every

calibration curve and 2) a response five times higher than the response of the lowest sample of the calibration curve (0 nmol/L). The precision profile of the method is shown in Table 4. Precision ranged between 2.1 and 6.5% for intraday variation and between 5.8 and 11.0% for interday variation for the total range of tested MTX concentrations. The mean bias (%error) ranged from −11.3 to 14.5% for intraday precision and −9.7% to 14.4 for the total range of tested MTX concentrations.

Table 3. Matrix effects of 10 different plasma samples[a]

Sample concentration		Average Matrix effect	CV%	Relative matrix effect	Maximum slope difference[b]
Diluted plasma (1:10 in drug-free plasma)	With IS	94%	2%	98%	7%
	Without IS	71%	19%	81%	67%
Undiluted plasma	With IS	96%	2%	98%	6%
	Without IS	72%	17%	93%	67%

All slopes were $r^2 > 0.999$ when corrected for IS.
[a]Matrix effect is expressed as % recovery and calculated by comparing the slopes of reference curves made in 10 different plasma matrices with the slope of a reference curves made in water.
[b]The maximum slope difference shows the % difference between the largest and smallest slope, indicating the variance between slopes in different matrices.

Table 4. Intraday and interday precision

Expected concentration (nmol/L)	Measured concentration intraday precision (nmol/L; n=20)[a]			Measured concentration interday precision (nmol/L; n=20)[b]		
	Mean ± SD	CV%	Bias (%error)	Mean ± SD	CV%	Bias (%error)
10	11.4 ± 0.76	6.5	14.5	11.6 ± 1.27	11.0	14.4
50	54.9 ± 1.95	3.5	9.7	53.5 ± 3.13	5.8	7.5
500	543.9 ± 17.84	3.3	8.8	536.3 ± 40.43	7.5	7.1
5000	4947.4 ± 168.85	3.4	−1.1	5073.1 ± 465.15	9.2	1.3
50000	44335.1 ± 918.77	2.1	−11.3	45413.0 ± 3322.88	7.3	−9.7

[a]The intraday precision summarizes 20 replicate measurements at each concentration of MTX during one day.
[b]The interday precision summarizes 20 different experiments from 20 consecutive days with measurement done in fivefold each day.

Method comparison

194 HD-MTX patient plasma samples were compared between methods. Duplicate measurements on the LC-MS/MS always showed a CV <10% with the exception of 14 samples, of which 10 samples were below the LLOQ of either the FPIA or the LC-MS/MS and were therefore excluded from the comparison. The other 4 samples contained low concentrations of MTX (20-36 nmol/L) and were preceded by high samples (35088-146851 nmol/L). The observed difference between duplos (6-45 nmol/L) was similar to the expected carry-over (7-31 nmol/L) assuming the 0.02% found previously, suggesting carry-over as the

cause. Another 9 samples were excluded because MTX plasma concentrations were below the LLOQ of the FPIA and therefore not reported as numerical data but as "<20 nmol/L" by the FPIA. All samples where the CV was >10% were re-measured both from the original original prepared sample and from freshly prepared samples after which all samples had duplicates with a CV<10%.

The comparison of the remaining 175 samples showed good agreement between methods: (LC-MS/MS=0.98 [95% CI: 0.95 to 1.02]×FPIA - 7.3 nmol/L [95% CI: -10.96 to -5.34]) (Figure 2, insert). A slight, non-significant proportional bias was present and a small but significant constant bias of -7.3 nmol/L was found for the complete sample set. Changing the method comparison to the low (0-500 nmol/L), high (500-50000 nmol/L) or complete sample concentration range did not give a significant difference in slope or bias. For the lower concentration range (0-500 nmol/L), which contained 75% of all patient samples, good agreement was obtained (LC-MS/MS=1.00 [95% CI: 0.94 to 1.06] × FPIA -8.51 nmol/L [95% CI: -11.06 to -5.88]) (Figure 2).

Discussion

We developed a straightforward and robust LC-MS/MS based method for the measurement of MTX in human EDTA plasma. While other mass spectrometry based methods have been reported for the detection of MTX in plasma, most methods do not use stable isotope labeled IS. Furthermore, these methods generally have higher LLOQs [29-31], or use more expensive and work-intensive sample preparation and machinery [18, 32]. Compared with traditional methods of MTX TDM (such as FPIA), our method has improved LLOQ and a large dynamic range.

The presented method does not require expensive and time-consuming sample preparation, such as solid phase extraction, and because of the use of a stable isotope labeled internal standard, it is a very useful method for implementation within a clinical laboratory. The short analysis time of 3 minutes makes the method attractive for high-throughput settings and clinical studies. Improvements in LLOQ can be made with the addition of better sample clean-up like SPE, but this would also drive up the cost and preparation time of the method. Alternatively, LLOQ may be improved by reducing the dilution factor. However, due to the dilution of patient sample in drug-free plasma, less volume is needed making the method suitable for use in very small sample volumes such as in pediatric samples.

Although carry-over is only 0.02%, the difference in maximum plasma levels in HD-MTX treatment can be as high as 100 fold. When measuring very low samples (<100 nmol/L) after very high samples (>50 µmol/L) this could lead to falsely increased MTX levels in the low concentration range as was seen when patient samples were measured in random order. Samples with a low concentration of MTX measured directly after samples with a high concentration of MTX had a discrepancy between duplicates that fitted roughly with the 0.02% carry-over that was determined. The use of duplicate measurement is important to detect and counter this. For most TDM purposes, this will not pose any problems as the

Chapter

4

clinical cut-off for TDM is usually ~200 nmol/L. However, when dealing with samples in the setting of carboxylase-G2 treatment, carry-over might present a problem due to the large difference in MTX concentration between patient samples as a result of MTX breakdown by the enzyme. [12, 13, 33]
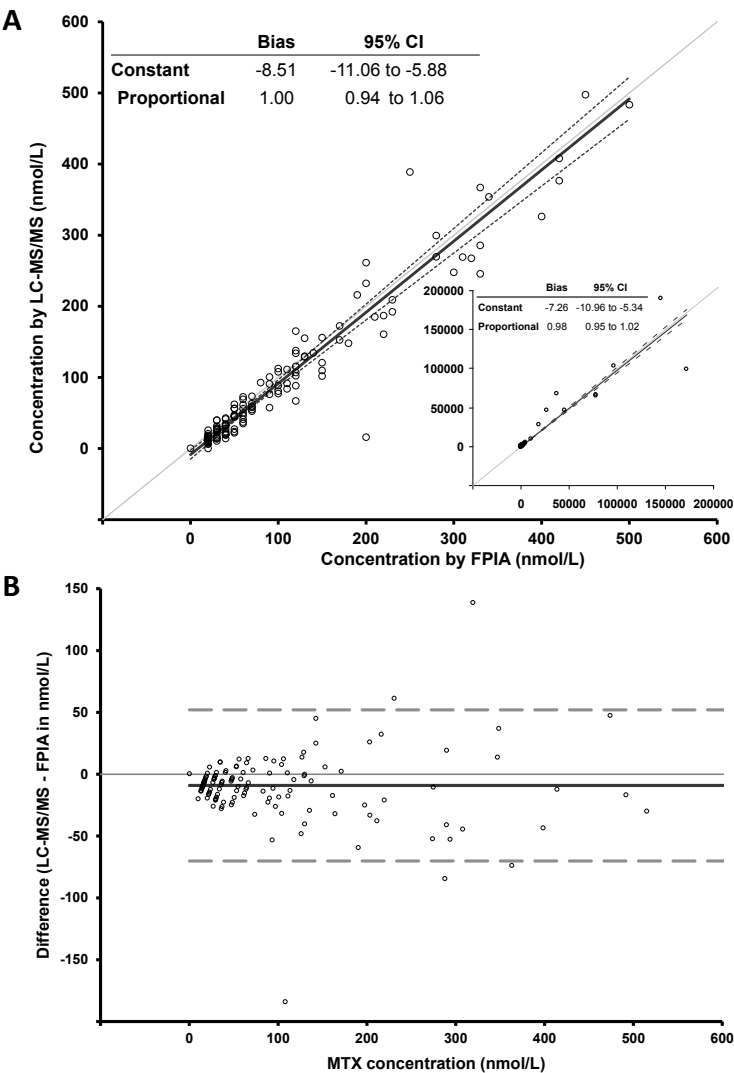
Figure 2. Comparison of plasma MTX between the Abbott FPIA on the TDx analyzer (x-axis) compared to the LC-MS/MS method (y-axis) (n=175). Samples shown are in the range of 0-500 nmol/L MTX which contains 75% of all patient samples (n=131). **A)** Passing & Bablok regression was used to compare both methods. The insert shows the comparison of the whole concentration range (0-170 μmol/L MTX). **B)** Bland-Altman plot of differences between observed MTX concentrations by FPIA and LC-MS/MS methods. Solid line represents bias (-9.02 nmol/L), and dotted lines represent 95% limits of agreement (-70.13 to 52.08)

Recovery based on peak area was moderate with high variance, but this was corrected for by the use of the stable isotope labeled IS, leading to more reproducible measurements and a recovery of approximately 100%. The use of a deuterium labeled IS also compensated for matrix effects. When using the peak areas to calculate matrix effects, a prominent absolute matrix effect was detected, which was almost completely corrected for by the use of the IS. The relative matrix effect caused by using different plasma batches is also corrected for by use of the internal standard. Both the recovery and matrix effect experiments emphasize the need for a proper IS when working with complex matrices such as biological samples in an LC-MS/MS setting.

Matrix effects in undiluted samples were also very low after correction for the IS which indicates that the dilution step in drug-free plasma is optional and leads to the possibility of measuring very low samples concentration (<LLOQ) without dilution.

As shown by the method comparison, good agreement was found with the FPIA analysis. This correlation was somewhat unexpected due to reports about the a-specificity of the FPIA method [9, 11, 33]. The few outliers that were found and the observed small constant bias might be caused by several reasons. First, the FPIA might measure higher concentrations because of its a-specific measurement of MTX metabolites like DAMPA. Second, differences might exist in the calibration between methods (traceability chain). Third, calibration errors within the laboratory might also have caused the constant bias between FPIA and LC-MS/MS. Because there is neither reference method nor standard reference material for MTX in plasma it is difficult to speculate about the accuracy of methods. However, in line with FDA guidelines our LC-MS/MS method showed small positive bias (%error <7.1 for interday and <8.8 for intraday) in the clinically relevant concentration range based on the spiked samples and the intraday recovery (see Table 4). In the higher concentration range the bias was slightly negative (%error −9.7 for interday and −11.3 for intraday). In certain situations, immunoassays like the FPIA method may be hampered by interference of MTX metabolites. For example, the presented method was able to measure the rapid disappearance of plasma MTX in patients treated with carboxylase-G2 for adverse events whereas the FPIA method was hampered by falsely high MTX levels due to interference from the methotrexate metabolite DAMPA (data not shown). This observation was confirmed by literature [12, 13].

In conclusion, we describe a LC-MS/MS based method to measure MTX in plasma with minimal sample pretreatment, relative short analysis time, and good diagnostic performance that can be applied in routine clinical TDM.

Chapter

4

References

1   Panetta J, Yanishevski Y, Pui C-H, Sandlund J, Rubnitz J, Rivera G, et al. A mathematical model of in vivo methotrexate accumulation in acute lymphoblastic leukemia. Cancer Chemotherapy and Pharmacology. 2002;50(5):419-28.

2   Rubino FM. Separation methods for methotrexate, its structural analogues and metabolites. J Chromatogr B Biomed Sci Appl. 2001 Nov 25;764(1-2):217-54.

3   Martelli N, Mathieu O, Marguerite G, Bozonnat MC, Daurès JP, Bressolle F, et al. Methotrexate pharmacokinetics in childhood acute lymphoblastic leukaemia: a prognostic value ? Journal of Clinical Pharmacy and Therapeutics. 2011;36(2):237-45.

4   Krajinovic M, Moghrabi A. Pharmacogenetics of methotrexate. Pharmacogenomics. 2004 Oct;5(7):819-34.

5   Faganel Kotnik B, Grabnar I, Bohanec Grabar P, Dolžan V, Jazbec J. Association of genetic polymorphism in the folate metabolic pathway with methotrexate pharmacokinetics and toxicity in childhood acute lymphoblastic leukaemia and malignant lymphoma. European Journal of Clinical Pharmacology. 2011;67(10):993-1006.

6   Uchiyama M, Matsumoto T, Jimi S, Takamatsu Y, Tamura K, Hara S. Simple and sensitive HPLC method for the fluorometric determination of methotrexate and its major metabolites in human plasma by post-column photochemical reaction. Biomed Chromatogr. 2011 Mar 25.

7   Alnaim L. Therapeutic drug monitoring of cancer chemotherapy. J Oncol Pharm Pract. 2007 Dec;13(4):207-21.

8   Mendu DR, Chou PP, Soldin SJ. An improved application for the enzyme multipled immunoassay technique for caffeine, amikacin, and methotrexate assays on the Dade-Behring Dimension RxL Max clinical chemistry system. Ther Drug Monit. 2007 Oct;29(5):632-7.

9   Eksborg S, Albertioni F, Rask C, Beck O, Palm C, Schroeder H, et al. Methotrexate plasma pharmacokinetics: importance of assay method. Cancer Lett. 1996 Nov 29;108(2):163-9.

10   Hayashi H, Fujimaki C, Tsuboi S, Matsuyama T, Daimon T, Itoh K. Application of fluorescence polarization immunoassay for determination of methotrexate-polyglutamates in rheumatoid arthritis patients. Tohoku J Exp Med. 2008 May;215(1):95-101.

11   Albertioni F, Rask C, Eksborg S, Poulsen JH, Pettersson B, Beck O, et al. Evaluation of clinical assays for measuring high-dose methotrexate in plasma. Clin Chem. 1996 Jan;42(1):39-44.

12   Al-Turkmani MR, Law T, Narla A, Kellogg MD. Difficulty measuring methotrexate in a patient with high-dose methotrexate-induced nephrotoxicity. Clin Chem. 2010 Dec;56(12):1792-4.

13   Esteve MA, Devictor-Pierre B, Galy G, Andre N, Coze C, Lacarelle B, et al. Severe acute toxicity associated with high-dose methotrexate (MTX) therapy: use of therapeutic drug monitoring and test-dose to guide carboxypeptidase G2 rescue and MTX continuation. Eur J Clin Pharmacol. 2007 Jan;63(1):39-42.

14   Dervieux T, Orentas Lein D, Marcelletti J, Pischel K, Smith K, Walsh M, et al. HPLC determination of erythrocyte methotrexate polyglutamates after low-dose methotrexate therapy in patients with rheumatoid arthritis. Clin Chem. 2003 Oct;49(10):1632-41.

15   Hroch M, Tukova J, Dolezalova P, Chladek J. An improved high-performance liquid chromatography method for quantification of methotrexate polyglutamates in red blood cells of children with juvenile idiopathic arthritis. Biopharm Drug Dispos. 2009 Apr;30(3):138-48.

16   McCrudden EA, Tett SE. Improved high-performance liquid chromatography determination of methotrexate and its major metabolite in plasma using a poly(styrene-divinylbenzene) column. J Chromatogr B Biomed Sci Appl. 1999 Jan 8;721(1):87-92.

17   Belz S, Frickel C, Wolfrom C, Nau H, Henze G. High-performance liquid chromatographic determination of methotrexate, 7-hydroxymethotrexate, 5-methyltetrahydrofolic acid and folinic acid in serum and cerebrospinal fluid. J Chromatogr B Biomed Appl. 1994 Nov 4;661(1):109-18.

18   Meesters RJ, den Boer E, Mathot RA, de Jonge R, van Klaveren RJ, Lindemans J, et al. Ultrafast selective quantification of methotrexate in human plasma by high-throughput MALDI-isotope dilution mass spectrometry. Bioanalysis. 2011 Jun;3(12):1369-78.

19   Barbieri A, Sabatini L, Indiveri P, Bonfiglioli R, Lodi V, Violante FS. Simultaneous determination of low levels of methotrexate and cyclophosphamide in human urine by micro liquid chromatography/electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom. 2006;20(12):1889-93.

20   Przybylski M, Preiss J, Dennebaum R, Fischer J. Identification and quantitation of methotrexate and methotrexate metabolites in clinical high-dose therapy by high pressure liquid chromatography and field desorption mass spectrometry. Biomed Mass Spectrom. 1982 Jan;9(1):22-32.

21   Turci R, Fiorentino ML, Sottani C, Minoia C. Determination of methotrexate in human urine at trace levels by solid phase extraction and high-performance liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom. 2000;14(3):173-9.

22   van Haandel L, Becker ML, Leeder JS, Williams TD, Stobaugh JF. A novel high-performance liquid chromatography/mass spectrometry method for improved selective and sensitive measurement of methotrexate polyglutamation status in human red blood cells. Rapid Commun Mass Spectrom. 2009 Dec;23(23):3693-702.

23   Honour JW. Development and validation of a quantitative assay based on tandem mass spectrometry. Ann Clin Biochem. 2011 Mar;48(Pt 2):97-111.

24   Shushan B. A review of clinical diagnostic applications of liquid chromatography-tandem mass spectrometry. Mass Spectrom Rev. 2010 Nov-Dec;29(6):930-44.

25   CDER. Center for Drug Evaluation and Research (CDER) Guidance for Industry: Bioanalytical Method Validation. 2001.

26   Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 2003 Jul 1;75(13):3019-30.

27   Broughton PM. Carry-over in automatic analysers. J Automat Chem. 1984;6(2):94-5.

28   Vogeser M, Seger C. Pitfalls associated with the use of liquid chromatography-tandem mass spectrometry in the clinical laboratory. Clin Chem. 2010 Aug;56(8):1234-44.

29   Rule G, Chapple M, Henion J. A 384-well solid-phase extraction for LC/MS/MS determination of methotrexate and its 7-hydroxy metabolite in human urine and plasma. Anal Chem. 2001 Feb 1;73(3):439-43.

30   Guo P, Wang X, Liu L, Belinsky MG, Kruh GD, Gallo JM. Determination of methotrexate and its major metabolite 7-hydroxymethotrexate in mouse plasma and brain tissue by liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal. 2007 Apr 11;43(5):1789-95.

31   Koufopantelis P, Georgakakou S, Kazanis M, Giaginis C, Margeli A, Papargiri S, et al. Direct injection liquid chromatography/positive ion electrospray ionization mass spectrometric quantification of methotrexate, folinic acid, folic acid and ondansetron in human serum. J Chromatogr B Analyt Technol Biomed Life Sci. 2009 Nov 15;877(30):3850-6.

32   Steinborner S, Henion J. Liquid-liquid extraction in the 96-well plate format with SRM LC/MS quantitative determination of methotrexate and its major metabolite in human plasma. Anal Chem. 1999 Jul 1;71(13):2340-5.

33   Kumar VS, Law T, Kellogg M. Liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for monitoring methotrexate in the setting of carboxypeptidase-G2 therapy. Methods Mol Biol. 2010;603:359-63.

Chapter

4
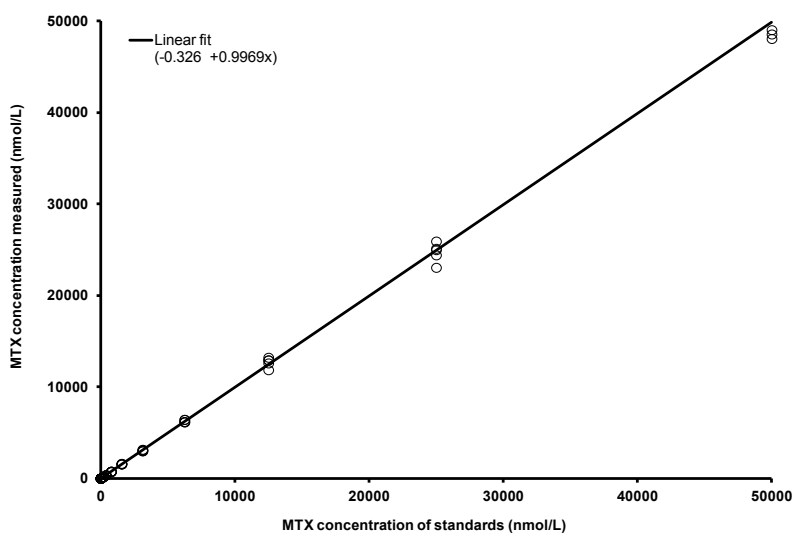
Supplementary data 1. Linearity of plasma MTX
MTX concentration as measured by LC-MS/MS (y-axis) is plotted against the spiked concentrations of the samples (x-axis). Linearity was determined by five-fold measurement of a 0-50 µmol/Lconcentration range, R^2 was always >0.99.
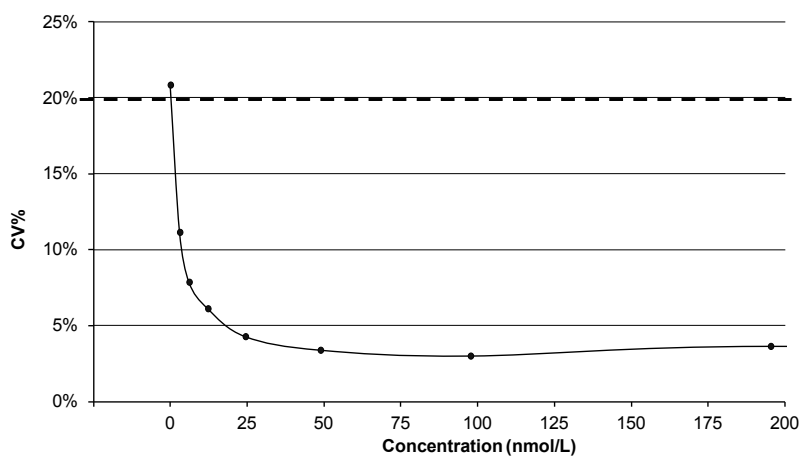
Supplementary data 2. LLOQ determination of MTX in plasma as measured by LC-MS/MS.
Each point represents the CV% of a ten-fold measurement of drug-free plasma samples spiked with MTX. The concentration of the standards are represented on the x-axis, the CV% on the y-axis.

Chapter

4

Chapter 5

Using fluorescence polarization immunoassay for determination of erythrocyte methotrexate-polyglutamates, a quick and easy test?

E. den Boer
B.C.P. Koch
R. Huisman
R. de jonge

Abstract

BACKGROUND: The folate antagonist methotrexate (MTX) is the anchor drug in the treatment of rheumatoid arthritis. The therapeutic effects of MTX are attributed to the intracellular levels of MTX, present in the cell as polyglutamates (MTX-PG). We aimed to validate an immunoassay for the measurement of MTX-PG in erythrocytes.

METHODS: Samples were analysed by an adapted fluorescent polarisation immune assay (FPIA) method on the FLx analyser (Abbott). Cross-reactivity was determined in both plasma and erythrocyte pellet. In erythrocyte pellet, the imprecision, linearity, and lower limit of quantitation (LLOQ) was determined. The method was compared to our in-house LC-MS/MS method for total MTX-PG.

RESULTS: For the adapted FPIA method, a linear range of 25-1000 nmol/L (R^2 = 0.993) was obtained for total MTX-PG in erythrocytes. A coefficient of variation (CV) of <17% for interday and <8% for intraday imprecision was found and average recovery was 91%. LLOQ was determined at 50 nmol/L total MTX-PG with a CV of 15%. There was no significant proportional bias of the FPIA assay compared with our in-house LC-MS/MS method but a (non-significant) constant positive bias was present [FPIA = 1.00 (95% CI: 0.60 - 1.95) × LC-MS/MS + 31.00 nmol/L (95% CI: -11.83 - 61.00)]. Results could be very different for individual patients as reflected in the poor R^2 of 0.419.

CONCLUSIONs: The FPIA method can be used to measure total MTX-PG in erythrocytes.. Although there was no significant bias detected compared with the LC-MS/MS method, the FPIA method showed constant positive bias, probably due to interference from folates and MTX metabolites DAMPA and 7-OH-MTX. The correlation between both methods was average and resulted in large differences in individual patients, most likely due to problems during sample preparation.

Introduction

Methotrexate (MTX) is the anchor drug in the treatment of arthritic diseases due to its safety and efficacy. In low-dose MTX treatment, plasma-MTX is eliminated from plasma within 24 hours[1] and is unrelated to response.[2] Therefore, plasma MTX is not a reliable tool for therapeutic drug monitoring in low-dose MTX treatment.[3] However, methotrexate polyglutamates (MTX-PGs) accumulate intracellularly, which are related to clinical response and have been proposed to be of use for TDM purposes.[4-7] In low-dose MTX treatment, the pentaglutamate (MTX-PG5) is the highest order of glutamylation detected, while the triglutamate form (MTX-PG3) of MTX predominates.[5, 8] Polyglutamylation retains MTX in the cell because MTX-PGs are a poor substrate for the MTX efflux proteins.

While methods for the measurement of plasma MTX are commercially available, methods for measuring intracellular MTX-PG are not and require more complex procedures and equipment such as HPLC[5, 9] or mass spectrometry.[8, 10, 11] However, measurement of intracellular total MTX-PG concentration is also possible by adaptation of commercial plasma methods.[12, 13]

Although the individual MTX-PG species are good predictors of response, we have recently shown that total MTX-PG concentration at three months of treatment can also be used to predict clinical response in rheumatoid arthritis (RA)[14] and juvenile idiopathic arthritis (JIA).[15] In RA, a threshold of 74 nmol/L of total erythrocyte MTX-PG was able to differentiate between responders and non-responders over 9 months of treatment.

Cross-reactivity of the antibody against all MTX-PG species in the MTX fluorescent polarisation immune assay (FPIA) can be used for the measurement of erythrocyte total MTX-PG from RA patients.[12] Cross reactivity was shown by comparing erythrocytes spiked with MTX-PG7 to those spiked with equal amounts of MTX-PG1.[12] However, the authors did not assess bias of the method by comparing with more specific methods to measure intracellular MTX-PG species such as mass spectrometry.

Therefore, the aim of our study was to compare the adapted Abbott FLx MTX FPIA assay to measure erythrocyte total MTX-PG with the more specific measurement of erythrocyte MTX-PG species measurement as described by us earlier.[11]

Material and methods

Materials and samples

MTX-PG1-5 were purchased from Schircks Laboratories (Jona, Switzerland). Dichloromethane, 10 mol/L ammonium hydroxide, sodium hydroxide, 70% perchloric acid were bought at Merck (Schiphol-Rijk, the Netherlands). Protease K was bought at Qiagen (Venlo, the Netherlands). Methotrexate II Reagent Pack for FPIA, calibrators and controls were purchased from Abbott Laboratories (Hoofdorp, The Netherlands) and the assay was performed on an Abbott FLx analyzer.

For both methods erythrocyte pellets were obtained by centrifuging K_2-ethylenediaminetetraacetic acid (EDTA) whole-blood tubes for 10 minutes at 2700 × g.

Chapter

5

Pellets were not washed. Blinded, drug-free erythrocyte pellets were used for development and validation. Drug-free pellets were pooled per 10 ml and stored at −80 °C until further use. After thawing, samples were vortexed vigorously before handling. Positive displacement pipettes (Gilson, den Haag) were used for pipetting the erythrocyte pellets. 20 samples of patients treated with MTX were used to compare the adapted FPIA method with LC-MS/MS. All samples were blinded after selection. Patient samples were pelleted by centrifugation for 10 minutes at 2700 × g and stored at −80 °C until analysis. As these patient samples were leftover from routine analysis at the department of clinical chemistry, no medical-ethical approval was necessary for this study.

Sample preparation
For the FPIA assay, sample preparation was based on Hayashi et al.[12] and was adapted where the article did not specify or optimization was needed in our hands. Briefly, samples were prepared by adding 40 µl of proteinase K to 275 µl of erythrocyte pellet, incubated for 15 minutes at 50 °C and cooled on ice. 60 µl 1 mol/L NaOH and 500 µl methylene chloride were added to the sample and vortex mixed for 30 seconds at room temperature. Samples were then incubated at −30 °C for one hour followed by centrifugation at 20000 × g for 15 minutes; 200 µl clear supernatant was transferred and mixed with 4 µl of 10% perchloric acid. Samples were kept on ice during the sample preparation unless mentioned otherwise. Samples for LC/MS-MS analyses were prepared as described earlier.[11]

Preparation of calibrators and control samples
Stock solutions of MTX-PG were prepared in 0.1 M ammonium hydroxide at 88.75 µmol/L (MTX-PG1), 64.48 µmol/L (MTX-PG2), 85.7 µmol/L (MTX-PG3), 40.71 µmol/L (MTX-PG4) and 51.28 µmol/L (MTX-PG5). A batch of frozen pooled drug-free erythrocyte pellet was thawed at room temperature while rotating and spiked with 0, 40, 80, 120, 160 or 200 nmol/L of each MTX-PG standard, yielding a total MTX-PG calibration curve of 0, 200, 400, 600, 800 and 1000 nmol/L. A fresh calibration curve was prepared for each run.

Quality-control (QC) samples were prepared in drug-free erythrocyte pellet at three different concentrations: 200, 500, and 800 nmol/L of each individual MTX-PGn. Aliquots were stored at −80 °C and thawed on ice. QC samples were prepared for every run. LC-MS/MS calibrators and QC samples were prepared as published earlier.[11]

LC-MS/MS analysis
Sample preparation and LC-MS/MS analysis were performed as published previously [11].

Validation of the adapted FPIA assay
Cross reactivity of the FPIA assay was determined by comparing drug-free plasma samples spiked with 200, 400, 600, 800 and 1000 nmol/L of the separate MTX-PGs with samples spiked with the same concentrations of MTX-PG1. To test cross reactivity in erythrocytes,

drug-free erythrocyte pellets were spiked with 200, 400, 600, 800 and 1000 nmol/L of either MTX-PG1 or MTX-PG5. Cross reactivity was expressed as *(concentration MTX-PG/ concentration MTX-PG1) × 100%.*

Linearity of the method was determined by spiking a batch of drug-free erythrocyte pellet with 9 different concentrations of MTX-PG1-5 in a range of 12.5–1000 nmol/L. The samples were measured 5 times from the same vial. Linearity was considered acceptable if the squared coefficient of correlation (R^2) was >0.99 for each calibration curve.

Lower limit of quantitation (LLOQ) was determined by spiking a batch of drug-free erythrocyte pellets with 9 different concentrations of MTX-PG1-5 in a range of 12.5–1000 nmol/L. These were measured 5 times from the same vial for each concentration level. The LLOQ was defined as the lowest concentration with a coefficient of correlation (CV) <20%. MTX-PG interday imprecision was determined by measuring quality control samples 10 consecutive working days. Intraday imprecision was determined by measuring a set of quality control samples 5 times on a single day. Imprecision was expressed as CV% (mean/ standard deviation × 100%).

Recovery of the sample preparation was determined by spiking 3 different batches of pooled drug-free erythrocyte pellets with 40, 100, and 160 nmol/L of each MTX-PGn before and after sample preparation. The recovery was calculated as *recovery (%) = (sample spiked before preparation/ sample spiked after sample preparation)×100 %.*

Method Comparison.

The methods were compared using two sets of samples, one set of calibration samples (n=10), and one set of 20 blood samples of RA patients treated with MTX which were left over from routine analysis. These samples were measured on the same day with both the adapted FPIA method and our in-house LC-MS/MS method which is capable of measuring the separate MTX-PGs. In the LC-MS/MS method, total erythrocyte MTX-PG was calculated by summing up all individual MTX-PG species. Passing and Bablok regression analysis was used to compare both methods. The patients received 15-25 mg/week oral MTX.

Results

When the MTX-PGs were separately spiked into drug-free plasma samples, we found 103 ± %, 102 ± 4%, 100 ± 2%, and 106 ± 6% cross reactivity for MTX-PG2 to MTX-PG5 respectively when compared to MTX-PG1 in plasma, demonstrating the high cross reactivity of this assay (Table 1). In erythrocyte samples, a cross reactivity of 98 ± 8% for MTX-PG5 was found. When the 5 MTX-PGs were added in equimolar amounts, the measured amount of total MTX-PG did not differ from equal amounts of MTX-PG1 (Table 1). Using a dilution of equimolar amounts of MTX-PG1-5 spiked into erythrocyte pellet, the method was linear from 25 nmol/L to 1000 nmol/L (R^2 = 0.993; y= x + 9 nmol/L), which reflects most of the clinically relevant range. LLOQs were determined as the lowest concentration that resulted in a CV% <20%, and was set at 50 nmol/L total MTX-PG.

Chapter

5

Table 1. Cross-reactivity of MTX-PGs in the Abbott FPIA assay for spiked plasma and erythrocyte samples.

MTX-PG measured in plasma (nmol/L)					
Concentration (nmol/L)	**200**	**400**	**600**	**800**	**1000**
MTX-PG1 (nmol/L)	195	390	584	793	1002
MTX-PG2 (nmol/L)	208	403	611	806	1000
MTX-PG3 (nmol/L)	209	386	611	805	998
MTX-PG4 (nmol/L)	188	393	598	802	990
MTX-PG5 (nmol/L)	226	403	613	823	1000
Crossreactivity (%)					
MTX-PG2	107%	103%	105%	102%	100%
MTX-PG3	107%	99%	105%	101%	100%
MTX-PG4	96%	101%	102%	101%	99%
MTX-PG5	116%	103%	105%	104%	100%
MTX-PG measured in spiked erythrocyte samples (nmol/L)[a]					
MTX-PG1 (nmol/L)	233	481	652	776	1040
MTX-PG5 (nmol/L)	231	432	601	863	987
Crossreactivity (%)					
MTX-PG5	99%	90%	92%	111%	95%
MTX-PG1 (nmol/L)	198	350	517	700	1065
MTX-PG1-5 (nmol/L)	228	404	596	772	1035
Crossreactivity (%)					
MTX-PG1-5	115%	115%	115%	110%	97%

[a]Erythrocyte samples were measured in two batches of pooled drug-free erythrocytes, one batch to asses cross-reactivity of MTX-PG5, and the other batch to assess cross-reactivity of the combined MTX-PGs at equimolar concentration.

The CV% for interday imprecision was 17%, 6% and 5% for the 200, 500 and 800 nmol/L samples respectively, For intraday imprecision the CV% was 8%, 2% and 1% for for the 200, 500 and 800 nmol/L samples, respectively for the combined MTX-PGs in erythrocyte pellets. Recovery was measured in 3 batches of spiked pooled pellet and ranged from 80-100% (mean recovery 91±7%) with a CV% of 8%. Figure 1 and 2 show the results for the method comparison of the 10 calibration samples and the 20 patient samples, respectively. The FPIA method showed no proportional bias but a (non-significant) constant positive bias compare to LC-MS/MS, both in the calibrator samples [FPIA = 0.99 (95% CI: 0.96-1.09) × LC-MS/MS + 22.23 nmol/L (95% CI: −23.21-34.18)] and the patient samples [FPIA = 1.00 (95% CI: 0.60 - 1.59) x LC-MS/MS + 31.00 nmol/L (95% CI: -11.83 - 61.00)].

Of the 20 patient samples, 1 sample was excluded because it did not mix properly during the sample preparation for the FPIA assay. The squared correlation coefficient was excellent in the calibration samples (R^2 = 0.998) whereas it was average in the 19 patient

samples (R^2 = 0.419). The results in individual patients samples could be very different, which was reflected by the average correlation (Figure 2).

	Bias	95% CI
Constant	22.23	-23.21 to 34.18
Proportional	0.99	0.96 to 1.09

R^2 = 0.998

FPIA (nmol/L)	LC-MS/MS (nmol/L)
34	12
240	216
443	429
584	574
834	788
1053	1040
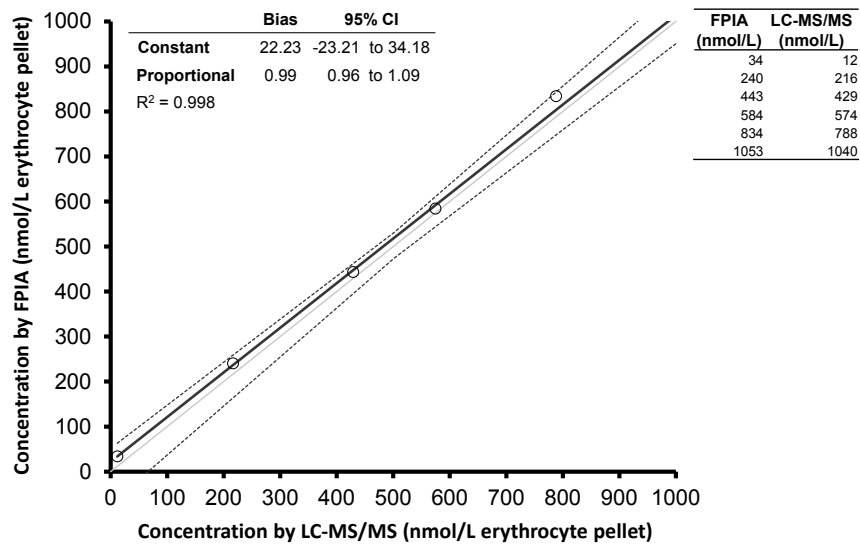
Figure 1. Passing-Bablok comparison of MTX-PGs in spiked erythrocyte pellets between the adapted Abbott FPIA on the FPIA analyzer (y-axis) and LC-MS/MS method (x-axis) (n=19). Solid line (grey) represents an x=y comparison, Solid line (Blue) represents Passing and Bablok comparison, dotted lines represent the 95% CI. Right panel shows the measured values in nmol/L per liter erythrocyte pellet.

	Bias	95% CI
Constant	31.00	-11,83 to 61,00
Proportional	1.00	0.60 to 1.59

R^2 = 0.419

FPIA (nmol/L)	LC-MS/MS (nmol/L)
31	0
31	88
61	20
61	60
77	56
77	53
92	1
92	51
107	65
107	94
107	77
107	148
123	87
123	146
153	8
169	162
184	116
184	114
230	193
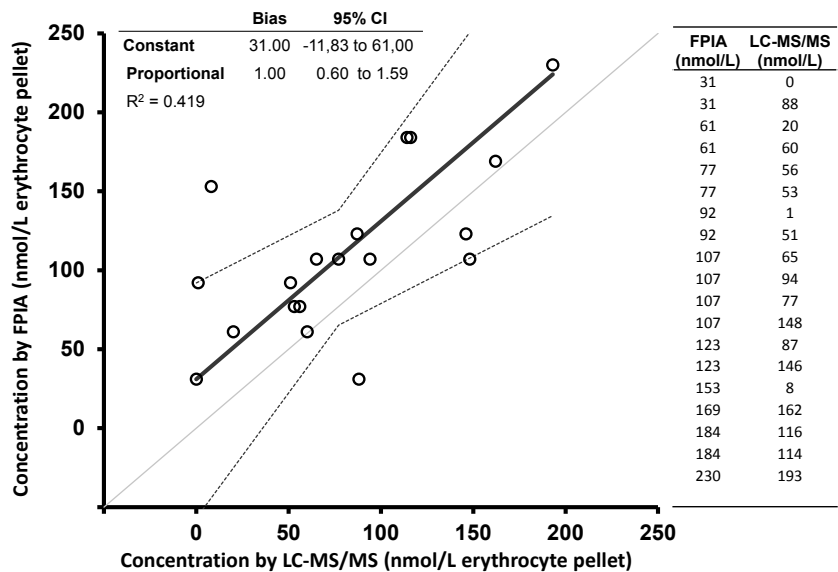
Figure 2. Passing-Bablok comparison of MTX-PGs determined by the adapted Abbott FPIA assay on the FLx analyzer (y-axis) and the LC-MS/MS method (x-axis) using erythrocyte samples from patients treated with MTX (n=19). Solid line (grey) represents an x=y comparison, Solid line (Blue) represents Passing and Bablok comparison, dotted lines represent the 95% CI. Right panel shows the measured values in nmol/L per liter erythrocyte pellet.

Chapter

5

Discussion

Due to the increasing evidence of the relation between methotrexate polyglutamates and response in RA and JIA it would be beneficial to have a quick, easy-to-use assay for the measurement of the total MTX-PG content in erythrocytes that utilizes an existing, robust platform like the FLx in order to enhance the clinical availability of this measurement. We tried to utilize the adapted FLx based FPIA MTX-PG assay published previously,[12] which makes use of the assay's high cross-reactivity between MTX-PG species in order to measure total erythrocyte MTX-PG. We were able to replicate this high cross-reactivity and we did not find a notable difference in specificity for any of the MTX-PGs compared to MTX-PG1 which is the analyte for which the FPIA method has originally been developed.

In our hands, the method performed well during validation using spiked erythrocyte samples. When comparing patient samples measured with both the FPIA method and our in-house validated LC-MS/MS assay,[11] Passing and Bablok analysis showed no significant proportional bias. However, while no proportional bias was found, the plot shows a substantial constant bias (31 nmol/L) between both methods; FPIA measured samples generally yielded higher concentrations of total MTX-PG than the same samples measured on LC-MS/MS. The constant bias found with Passing and Bablock analysis will likely be significant when more samples are included.

Overestimation by the FPIA assay has been reported before [16, 17] and is likely to be partially due to cross-reactivity of the FPIA assay to folate or the MTX metabolites 7-hydroxy-MTX (7-OH MTX) and 2,4-diamino-N10-methylpteroic acid (DAMPA)[18, 19] reflecting the lower specificity. In theory part of the overestimation might be due to the very long chain MTX-PGs (MTX-PG6 and MTX-PG7) as our total MTX-PG measurement on the LC-MS/MS does not take these into account. However, these have not been conclusively shown in low-dose MTX treatment and we have never encountered these MTX-PG species before.

In a previous publication, we reported a significant bias of 7.3 nmol/L MTX in plasma when samples were measured with the FPIA assay. The cross-reactivity for MTX metabolites might be the reason for the observed constant positive bias for the FPIA assay. Furthermore, we showed that the correlation between FPIA and LC-MS/MS in patients samples was much poorer than for calibration samples (R^2 = 0.419 vs 0.998), which sometimes generated large differences in total erythrocyte MTX-PG levels in individual patients for both methods (Figure 2). The difference in MTX levels measured by both methods might be partially due to the colour and turbidity of the samples measured using the FPIA assay because the concentration of the patient samples was calculated using calibration samples from a separate batch of erythrocyte pellet. The colour and turbidity of these batches was as variable as the colour and turbidity of the patient samples. Although FPIA is less influenced by colour and turbidity differences than other immune assays, the preparation of the erythrocyte pellets for the FPIA assay did not yield colourless samples or samples of consistent colour and turbidity and these sample to sample differences may contribute to the discrepancy between methods.

These sample to sample differences might also reflect a difference in extraction efficiency, yielding varying amounts of extracted total MTX-PG from the erythrocytes.

During the initial method comparison there was good agreement between methods when using spiked samples which did not contain intracellular MTX and were obtained from a single batch of pooled erythrocytes (Figure 1). Although a clear, though non-significant bias was present, these samples exhibited similar behaviour. However, patient samples containing intracellular MTX did not compare favourably between methods (Figure 2). The sample preparation might therefore not be able to extract all intracellular MTX-PG, combined with differences between patients in cross-reacting metabolites such as 7-OH MTX and DAMPA might have led to the discrepancy between methods. These differences between methods might in fact reflect variations in folate metabolism between individual patients and it might be useful to compare these differences with clinical response or adverse events.

Compared with the in-house LC-MS/MS method for the measurement of MTX-PG in erythrocytes, the FPIA assay has higher CV% for interday and intraday imprecision, as well as a higher LLOQ and a smaller linear range,[11] making the FPIA assay less suitable for patients with low levels of erythrocyte MTX-PG.

One other method for the measurement of total MTX-PG in erythrocytes with the FPIA assay has been published, using a different sample preparation. However, that method uses solid phase extraction (SPE) to obtain a clean supernatant,[13] although this should prevent inter-sample variation in colour, it is very difficult to compensate for the recovery rate of the SPE, which is known to be very variable. Both FPIA based assays that have been published were not compared with a reference method to test if the newly developed method does indeed measure consistently and correctly.

While the FPIA assay seems promising, sample preparation needs to be further optimized before it can be utilised in a clinical setting and it cannot be translated directly from the original articles. We strongly advice laboratories to do a cross-validation with a (reference) method on a different platform using real patient material when adapting similar methods.

In conclusion, we demonstrate that an adapted FPIA assay can be used to measure erythrocyte total MTX-PG levels but that the method in general shows a positive bias and low specificity, probably due to interference MTX metabolites DAMPA and 7-OH-MTX. Furthermore, due to problems in the sample preparation FPIA results may substantially deviate between individual patients.

Chapter

5

References

1   Hornung N, Ellingsen T, Attermann J, Stengaard-Pedersen K, Poulsen JH. Patients with rheumatoid arthritis treated with methotrexate (MTX): concentrations of steady-state erythrocyte MTX correlate to plasma concentrations and clinical efficacy. J Rheumatol. 2008 Sep;35(9):1709-15.

2   Dalrymple JM, Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Barclay ML. Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. Arthritis Rheum. 2008 Nov;58(11):3299-308.

3   Bannwarth B, Pehourcq F, Schaeverbeke T, Dehais J. Clinical pharmacokinetics of low-dose pulse methotrexate in rheumatoid arthritis. Clin Pharmacokinet. 1996 Mar;30(3):194-210.

4   Chabner BA, Allegra CJ, Curt GA, Clendeninn NJ, Baram J, Koizumi S, et al. Polyglutamation of methotrexate. Is methotrexate a prodrug? J Clin Invest. 1985 Sep;76(3):907-12.

5   Dervieux T, Orentas Lein D, Marcelletti J, Pischel K, Smith K, Walsh M, et al. HPLC determination of erythrocyte methotrexate polyglutamates after low-dose methotrexate therapy in patients with rheumatoid arthritis. Clin Chem. 2003 Oct;49(10):1632-41.

6   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Walsh M, et al. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum. 2004 Sep;50(9):2766-74.

7   Becker ML, Gaedigk R, van Haandel L, Thomas B, Lasky A, Hoeltzel M, et al. The effect of genotype on methotrexate polyglutamate variability in juvenile idiopathic arthritis and association with drug response. Arthritis Rheum. 2011 Jan;63(1):276-85.

8   van Haandel L, Becker ML, Leeder JS, Williams TD, Stobaugh JF. A novel high-performance liquid chromatography/mass spectrometry method for improved selective and sensitive measurement of methotrexate polyglutamation status in human red blood cells. Rapid Commun Mass Spectrom. 2009 Dec;23(23):3693-702.

9   Hroch M, Tukova J, Dolezalova P, Chladek J. An improved high-performance liquid chromatography method for quantification of methotrexate polyglutamates in red blood cells of children with juvenile idiopathic arthritis. Biopharm Drug Dispos. 2009 Apr;30(3):138-48.

10   Meesters RJ, den Boer E, de Jonge R, Lindemans J, Luider TM. Assessment of intracellular methotrexate and methotrexate-polyglutamate metabolite concentrations in erythrocytes by ultrafast matrix-assisted laser desorption/ionization triple quadrupole tandem mass spectrometry. Rapid Commun Mass Spectrom. 2011 Oct 30;25(20):3063-70.

11   den Boer E, Meesters RJ, van Zelst BD, Luider TM, Hazes JM, Heil SG, et al. Measuring methotrexate polyglutamates in red blood cells: a new LC-MS/MS-based method. Anal Bioanal Chem. 2013 Feb;405(5):1673-81.

12   Hayashi H, Fujimaki C, Tsuboi S, Matsuyama T, Daimon T, Itoh K. Application of fluorescence polarization immunoassay for determination of methotrexate-polyglutamates in rheumatoid arthritis patients. Tohoku J Exp Med. 2008 May;215(1):95-101.

13   Inoue S, Hashiguchi M, Kawai S, Mochizuki M. Erythrocyte methotrexate-polyglutamate assay using fluorescence polarization immunoassay technique: application to the monitoring of patients with rheumatoid arthritis. Yakugaku Zasshi. 2009 Aug;129(8):1001-5.

14   de Rotte MC, den Boer E, de Jong PH, Pluijm SM, Bulatovic Calasan M, Weel AE, et al. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in patients with rheumatoid arthritis. Ann Rheum Dis. 2013 Dec 5.

15   Bulatovic Calasan M, den Boer E, de Rotte MC, Vastert SJ, Kamphuis S, de Jonge R, et al. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in juvenile idiopathic arthritis patients. Ann Rheum Dis. 2013 Nov 28.

16   Najjar TA, Matar KM, Alfawaz IM. Comparison of a new high-performance liquid chromatography method with fluorescence polarization immunoassay for analysis of methotrexate. Ther Drug Monit. 1992 Apr;14(2):142-6.

17   den Boer E, Heil SG, van Zelst BD, Meesters RJ, Koch BC, Te Winkel ML, et al. A U-HPLC-ESI-MS/MS-based stable isotope dilution method for the detection and quantitation of methotrexate in plasma. Ther Drug Monit. 2012 Aug;34(4):432-9.

18   Al-Turkmani MR, Law T, Narla A, Kellogg MD. Difficulty measuring methotrexate in a patient with high-dose methotrexate-induced nephrotoxicity. Clin Chem. 2010 Dec;56(12):1792-4.

19   Pesce MA, Bodourian SH. Evaluation of a fluorescence polarization immunoassay procedure for quantitation of methotrexate. Ther Drug Monit. 1986;8(1):115-21.

Chapter

5

Chapter 6

Clinical, metabolic and genetic determinants of erythrocyte methotrexate-polyglutamate concentrations at 3 months of treatment in rheumatoid arthritis

E. den Boer
M.C.F.J. de Rotte
S.M.F. Pluijm
S.G. Heil
J.M.W. Hazes
R. de Jonge

Abstract

BACKGROUND: Low-dose methotrexate (MTX) is the anchor drug in the treatment for rheumatoid arthritis. Response to MTX is related to the intracellular MTX-polyglutamate (MTX-PG) levels and little is known about its determinants. We aimed to define the determinants of erythrocyte MTX-PG concentrations in two prospective cohorts of RA patients.

METHODS: MTX treated RA patients from two longitudinal cohorts were included: 93 from the MTX-R study (derivation cohort), and 247 from the tREACH study (validation cohort). MTX-PG concentrations were measured at 3 months of treatment using LC-MS/MS. The MTX-PGs were used as outcome measure. Various socio-demographic, clinical, biochemical, and genetic factors were assessed at baseline. Associations with MTX-PG levels were analyzed using multivariate regression analysis.

RESULTS: Age was positively associated with MTX-PG1 (stβ 0.23; p=0.033) and total MTX-PGs (stβ 0.23; p=0.018) in the derivation cohort, and with all MTX-PGs in the validation cohort (PG1: stβ 0.13, p=0.04; PG2: stβ 0.21, p=0.001; PG3: stβ 0.22, p<0.001; PG4+5: stβ 0.25, p<0.001; and total: stβ 0.32, p<0.001). Erythrocyte folate levels were positively associated with MTX-PG3 (stβ 0.32; p=0.021) and total MTX-PG levels (stβ 0.32; p=0.022) in the derivation cohort, which was replicated for MTX-PG3 (stβ 0.15, p=0.04) in the validation cohort. Patients with the FPGS rs4451422 wildtype-genotype had higher concentrations of MTX-PG3 (p<0.05), MTX-PG4+5 (p<0.05) and total MTX-PG (p<0.05) in both cohorts. In the combined cohort, MTX dose was positively associated with levels of MTX-PG3 (stβ 0.23; p<0.001), MTX-PG4+5 (stβ 0.30; p<0.001), and total Total MTX-PG (stβ 0.20; p=0.002), but negatively associated with MTX-PG2 levels (stβ -0,22; p<0.001).

CONCLUSIONS: This prospective study shows that higher age, higher MTX dose, higher erythrocyte folate status and the FPGS rs4451422 wildtype genotype are associated with higher MTX-PG concentrations. While only up to 21% of inter-patient variability can be explained by these determinants, this knowledge may aid in the development of personalized treatment in RA.

Introduction

Low dose methotrexate (MTX) is the most widely used treatment for rheumatoid arthritis (RA) and other arthritic diseases. Although MTX is effective and save, approximately 30% of RA patients do not reach sufficient response or suffer from adverse events [1]. A pharmacogenetic model for the prediction of MTX efficacy has been proposed previously [2]. However, at the moment there is no therapeutic drug-monitoring (TDM) based model for predicting compliance, response or adverse events during low-dose MTX treatment.

While MTX plasma levels can be measured easily, low-dose MTX is rapidly cleared from plasma and is not routinely measured. Hence, plasma MTX levels do not correlate with response in RA patients [3]. The therapeutic effects of MTX are thought to be mediated by its intracellular levels [4], which are difficult to measure. Intracellular levels of methotrexate can predict treatment response, making intracellular MTX an interesting target for TDM [5-13]. We recently developed a stable isotope dilution LC-MS/MS assay to measure erythrocyte MTX-PGs [14].

MTX is transported into the cell primarily by the reduced folate carrier. Once in the cell, MTX is converted by folylpolyglutamate synthase (FPGS) to MTX polyglutamates (MTX-PGs) by γ-linked sequential addition of glutamic acid residues. In a competing reaction, the MTX-PGs are deconjugated by γ-glutamyl hydrolase (GGH), leading to a variety of chain-lengths (MTX-PG2-7). In low-dose MTX treatment, the pentaglutamate (MTX-PG5) is the highest order of glutamylation detected, while the triglutamate form (MTX-PG3) of MTX predominates [15, 16]. Polyglutamylation retains MTX in the cell because the MTX-PGs are a poor substrate for the MTX efflux proteins.

In low dose MTX, the median time to reach steady-state erythrocyte MTX levels is highly variable between patients and increases with the number of PGs attached to MTX [17]. For example, MTX-PG3 has a median time to reach steady-state of 41.2 weeks (range 19.8-66.7 weeks) compared to 139.8 weeks (range 15.5-264.0 weeks) for MTX-PG5 [17]. Steady-state levels also are highly variable between patients: total erythrocyte MTX-PG concentration varied between 90.9-351.5 nmol/8*10^{12} erythrocytes [17]. The mechanisms behind the highly variable intracellular MTX-PG levels are still not known.

Previous research has shown that increased age, higher dose, route of administration and decreased renal function [18, 19] are associated with higher MTX-PG levels, as well as multiple single nucleotide polymorphisms (SNP) in MTX pathway genes [8, 13]. However, these studies used cross-sectional cohorts with a wide range of treatment duration between patients. Therefore, the aim of this study was to examine clinical, genetic, socio-demographic, and biochemical determinants of erythrocyte MTX-PG concentrations in patients treated with low-dose oral MTX using two different prospective cohorts.

Methods

Patients

This study includes data of RA patients treated with MTX from two prospective cohorts: For

Chapter

6

the derivation cohort, patients from the methotrexate in Rotterdam, Netherlands cohort (MTX-R) were used. The MTX-R is a longitudinal prospective cohort of patients diagnosed with RA who started MTX between January 2006 and March 2011 in the department of Rheumatology, University Medical Center Rotterdam (Erasmus MC), Netherlands. The validation cohort consisted of patients from the treatment in Rotterdam Early Arthritis Cohort (tREACH). The tREACH is a clinical multicentre, stratified single-blinded trial (ISRCTN26791028) and was described earlier.[20] Patients were included in the validation cohort if they met the 2010 ACR/EULAR criteria for RA. The medical ethics committee from the Erasmus MC approved both studies and patients gave written informed consent before inclusion. Patients from the derivation and validation cohorts were included in our study if they were prescribed MTX at baseline and three months of treatment and had at least one MTX-PG measurement at three months of treatment. All patients were MTX naïve at inclusion.

In the derivation cohort, dosage and co-medication was chosen by the physician. MTX was generally given orally. Patients from the validation cohort started with 25 mg/week MTX per os (dosage reached after 3 weeks) and were randomized to treatment with or without sulfasalazine, hydroxychloroquine and glucocorticosteroids. Patients in both cohorts received folic acid (10 mg/week) during MTX treatment as recommended by the Dutch Rheumatology Society [21]. In both cohorts, patients were assessed at baseline and after 3 months.

Patient material

During the first and second study visits, an extra EDTA tube was drawn from the patient. The sample from the first visit was used for DNA isolation, whereas the sample from the second visit was immediately put on ice after collection and centrifuged for 10 min at 1700 x g and 4 °C. Plasma and erythrocyte cell-pellet aliquots were stored at -80 °C.

MTX-PG quantification

MTX-PGs were measured in the cell-pellet aliquots sampled at 3 months of treatment using a recently developed LC-MS/MS method [14]. MTX-PG1 and MTX-PG2 are considered as short chain, MTX-PG3 as medium chain and MTX-PG4 and MTX-PG5 as long chain. The sumscore of MTX-PG2 to MTX-PG5 was used as the total MTX-PG content. Considering the finding that MTX-PG1 can diffuse over the erythrocyte membrane, [22] we decided to remove MTX-PG1 out of the model for total MTX-PGs.

SNP selection

SNP in genes involved in MTX transport and polyglutamylation were selected based on the following criteria: minor allele frequency (MAF) > 0.10 in the Hapmap and National Center for Biotechnology Information (NCBI) SNP database [23, 24] or a proven functionality in relation to MTX, JIA, RA, or folate metabolism[25-33]. If no information was known for a

particular gene, we selected tagging SNP by Hapmap database and Haploview (version 4.2, 29 April 2008) . Preferably, 2 SNP were selected per gene, which were located in different haplotype blocks.

The following 28 SNP in 19 genes were selected: ABCB1 rs1128503, rs2032582, rs1045642; ABCC1 rs35592, rs3784862; ABCC2 rs4148396, rs717620; ABCC3 rs4793665, rs3785911; ABCC4 rs868853, rs2274407; ABCC5 rs2139560; ABCG2 rs13120400, rs2231142; ADA rs7359874; ADORA2A rs5751876; AMPD1 rs17602729; ATIC rs2372536; FPGS rs4451422; FOLR2 rs514933; GGH rs10106587, rs3758149; ITPA rs1127354; MTHFR rs1801131, rs1801133; MTRR rs1801394; SLC46A1 rs2239907; and SLC19A1 rs1051266. The major allele was analysed as wild-type allele.

Genotyping
SNP genotyping has been described earlier [34].

Clinical, biochemical and socio-demographic parameters
Various clinical, biochemical and socio-demographic parameters were assessed at baseline. In the derivation cohort, the use of other DMARDs, hydroxychloroquine, sulfasalazine, corticosteroids, biological, route of administration of corticosteroids, dose of methotrexate, and route of administration of methotrexate were reported by patients using question forms. In the validation cohort, these items were scored by research nurses. The eGFR-MDRD was calculated using the 4-variable MDRD formula and body surface area (BSA) was calculated using the Mosteller formula. During the study visit, blood was obtained from patients to determine rheumatoid factor, anti-cyclic citrullinated peptide antibody (Anti-CCP), C-reactive protein (CRP), one-hour erythrocyte sedimentation rate (ESR), albumin, enzymatic creatinine, erythrocyte folate, serum folate, vitamin B12, vitamin B6, and homocysteine. Questionnaires were used to determine smoking habit and the consumption of alcohol, cola, coffee, tea and cigarettes (amount per day).

Statistics
Comparison of patient characteristics between cohorts was made by Student t-test, X2 test or the Mann-Withney u-test where appropriate. Multivariate multiple linear regression analysis, stratified by cohort, was used to examine the associations between these potential determinants and the different MTX-PG concentrations. First, univariate linear regression was performed for all potential determinants with the MTX-PG concentrations as outcome measure. The strength of the associations was expressed as standardized beta's. Univariate relations between variables and any MTX-PG with a p-value less than 0.2 were entered in subsequent multivariate multiple regression analyses with adjustment for age, gender and other potential determinants that had a p-value of less than 0.2 in the univariate analysis. Continuous determinants were analyzed as continuous variable and transformed into quintiles to examine possible non-linear associations. In order to establish non-linear

Chapter

6

associations the quintiles were plotted against the total MTX-PG levels were used. Variables with a non-linear association were transformed into categorical variables and categories were combined where appropriate. This was done for ESR, GFR, creatinine, alcohol consumption, tea consumption and days of treatment. Dummy variables were used to analyze categorical variables with more than two categories in linear regression using the first category as reference. Non-normal distributed variables were transformed using the natural logarithm (ln) for linear regression; this was done for homocysteine, erythrocyte folate and C-reactive protein.

SNPs were divided into dominant, recessive or additive models depending on the distribution of the total MTX-PG levels per genotype to ensure pre-analysis selection of an analysis model. ANCOVA was used to determine significant associations between SNPs and MTX-PG levels. For SNPs, estimated marginal means + standard error are reported. SNPs in dominant model were ITPA rs1127354, AMPD1 rs17602729, ABCC4 rs2274407, ABCC2 rs717620. SNPs in recessive model were ABCC1 rs35592, ABCC4 rs868853, FPGS rs4451422, SLC19A1 rs1051266. Other SNPs were analyzed as an additive model. All SNPs were corrected for age and gender

It was not possible to test the influence of MTX dose in the separate cohorts because MTX dose was protocolled at 25 mg/wk in the validation cohort and there was low variation in the derivation cohort. To evaluate dose as potential determinant, both cohorts were combined. MTX dose was entered in an ANCOVA together with age, gender, erythrocyte folate, and FPGS rs4451422.

Multiple testing was not corrected for as the included variables in the study were carefully chosen for an expected relation to MTX-PG based on literature and physiology. Statistical analyses were done with SPSS PASW 20.0.0.1 for Windows (SPSS Inc., Chicago, IL, USA) unless stated otherwise. P values less than 0.05 were considered significant.

Results

Patient characteristics

93 out of 102 patients from the derivation cohort and 247 out of 285 patients from the validation cohort could be included into our study, the remaining patients were excluded because there was no erythrocyte pellet sample for MTX-PG measurement at three months.

The derivation and validation cohorts were very similar for most baseline characteristics (table 1). In the derivation cohort, a smaller percentage of patients used hydroxychloroquine (44.7% vs 58.4%; p=0.32), sulfasalazine (35.3% vs 58.4%; p<0.001) and corticosteroids (12.9% vs 89.1%; p<0.001). DAS28 was lower in the derivation cohort (4.1 vs 4.7) and the derivation cohort had slightly higher eGFR-MDRD (88.1 ml/min/1.73m^2 vs 80.7 ml/min/1.73m^2; p<0.05) and erythrocyte folate content (1075.7 nmol/L vs 925.5 nmol/L; p<0.001) than the validation cohort. Treatment dose of MTX was significantly different between both cohorts (p<0.001). Patients in the derivation were treated with 15 mg/wk and patients in the validation were treated with 25 mg/wk as per study protocol.

MTX-PG levels

After 3 months of MTX treatment, median [IQR] MTX-PG concentrations in the derivation cohort were: 33.8 [22.7-61.6], 23.1 [17.2-31.6], 39.8 [24.8-53.6], 8.4 [4.2-17.3], 1.0 [0.0-2.8] nmol/l for MTX-PG1 to MTX-PG5 respectively, and 79.0 [49.3-106.0] nmol/l for total MTX-PG (Figure 1a). In the validation cohort, median [IQR] MTX-PG concentrations were: 30.0 [19.8-47.4], 21.2 [15.9-27.4], 49.0 [36.5-61.4], 20.0 [11.4-30.2], 4.7 [2.0-9.3] nmol/l for MTX-PG1 to MTX-PG5, respectively (Figure 1a), and 97.9 [71.6-125.3] for total MTX-PG. MTX-PG1 did not differ between the derivation cohort and the validation cohort despite the difference in MTX dose between cohorts (table 1). Median MTX-PG2 concentrations were slightly, but significantly higher in the derivation cohort than in the validation cohort (p=0.015). In contrast, median MTX-PG3, MTX-PG4, MTX-PG5 and total MTX-PG were significantly lower in the derivation cohort than in the validation cohort (p<0.001 for MTX-PG3-5 and total MTX-PG, Figure 1a).
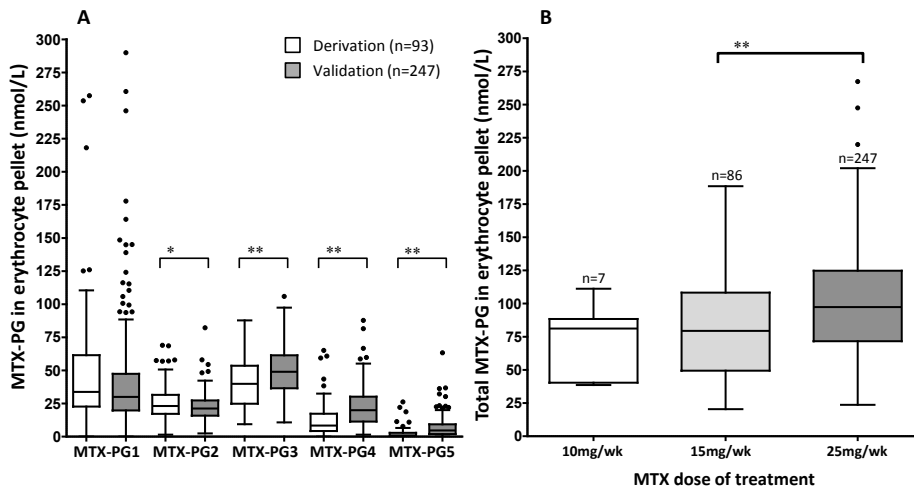
Figure 1. A) Concentrations of the separate MTX-PGs in the derivation (white bars, n=93) and validation (grey bars, n=247) cohorts. Brackets denote significant differences between cohorts, p-values are noted above the brackets. MTX-PG2 is significantly lower in the validation cohort, while MTX-PG3, MTX-PG4, and MTX-PG5 are higher in the validation cohort. Significant differences were tested with Mann-Whitney U-test.
B) Effect of MTX dosage on the concentration of total MTX-PG in the combined cohort. Increased dose of MTX leads to increased total MTX-PG. ANCOVA was adjusted for age, gender, erythrocyte folate, and rs4451422 in the FPGS gene. p-values are from the confounder adjusted data, boxplots are from unadjusted data.
*p<0.05, **p<0.001

Determinants of MTX-PGs

All variables listed in Table 1 were entered into a univariate linear regression model (Supplementary Table 1). Variables that obtained a p-value <0.2 in univariate linear regression were entered into a multivariate linear regression model (Table 2).

Chapter

6

Table 1. Baseline characteristics of MTX-R and tREACH cohorts.

	Derivation cohort	Validation cohort
Patient demographics	**N=93**	**N=247**
Age, years, mean (SD)	51.2 (16.1)	52.8 (14.2)
Female (%)	72.0	69.2
BSA, m^3, mean (SD)	n/a	1.9 (0.3)
DAS28esr, mean (SD)*	4.1 (1.4)	4.7 (1.2)
Rheumatoid factor positive (%)*	37.3	56.1
Anti-CCP positive (%)*	38.6	59.6
Days of treatment at study visit, mean (SD)	91.2 (11.8)	92.9 (9.0)
Medication		
Methotrexate Dose*	247	
10mg/wk (%)	7.5	0.0
15mg/wk (%)	91.6	0.4
25mg/wk (%)	1.1	99.6
Intramuscular administration of methotrexate (%)	7.1	0.0
Other DMARD use (%)	51.8	58.4
Hydroxychloroquine use (%)*	44.7	58.4
Sulfasalazine use (%)*	35.3	58.4
Biological use (%)	1.1	0.0
Corticosteroid use (%)*	12.9	89.1
Corticosteroid route of administration (%)		
No Corticosteroid	87.1	10.9
Subcutaneous	2.4	29.0
Oral	10.6	60.2
Laboratory parameters		
C- reactive protein, mg/L, median (IQR)	7.0 (3.0-13.0)	8.0 (4.0-20.0)
Erythrocyte sedimentation rate, mm/hr, mean (SD)	23.3 (18.9)	28.0 (21.4)
Albumin, (g/L), mean (SD)	44.1 (3.6)	43.6 (3.2)
Creatinine, mmol/L, mean (SD)	70.8 (16.5)	75.9 (16.8)
Erythrocyte folate, nmol/L, median (IQR)*	1075.7 (845.7-1323.0)	925.5 (679.8-1172.5)
Serum folate, nmol/L median (IQR)	17.3 (13.0-24.1)	17.3 (13.4-23.9)
eGFR-MDRD, ml/min/1.73m^2, mean (SD)*	88.1 (23.8)	80.7 (18.0)
Vitamin B12, pmol/L, median (IQR)	281.6 (225.5-376.8)	290.2 (234.5-403.9)
Vitamin B6, nmol/L, median (IQR)	76.0 (66.0-104.0)	81.0 (65.0-98.0)
Homocysteine umol /l, median (IQR)	12.4 (9.9-14.6)	11.3 (9.5-14.4)
Lifestyle parameters		
Alcohol consumption, drinks/month, median (IQR)	6.0 (0.0-32.0)	8.0 (2.0-32.0)
Cola consumption, drinks/month, median (IQR)	0.0 (0.0-24.0)	0.0 (0.0-8.0)
Coffee consumption, drinks/month, median (IQR)	56.0 (24.0-168.0)	112.0 (40.0-112.0)
Tea consumption, drinks/month, median (IQR)	56.0 (1.5-56.0)	40.0 (0.0-56.0)

* signifies a difference between groups that is p<0.05

In multivariate analysis, in the derivation cohort, there was a positive association between age at start of treatment and levels of MTX-PG1 (stβ 0.23; p=0.033), and total MTX-PGs (stβ 0.23; p=0.018), while exhibiting a trend for MTX-PG2 (stβ 0.18; p=0.098) and borderline significance for MTX-PG3 (stβ 0.21; p=0.052) (Table 2). This finding was replicated in the validation cohort for all MTX-PGs (MTX-PG1: stβ 0.13, p=0.04; MTX-PG2: stβ 0.21, p=0.001; MTX-PG3: stβ 0.22, p<0.001; MTX-PG4+5: stβ 0.25, p<0.001; and total MTX-PG: stβ 0.28, p<0.001) (Figure 2a). Erythrocyte folate was positively associated with levels of MTX-PG3 (stβ 0.32, p=0.021) and total MTX-PG (stβ 0.32, p=0.022), while exhibiting a trend for significance for MTX-PG4+5 (stβ 0.24, p=0.099) in the derivation cohort. This was replicated in the validation cohort for MTX-PG3 levels (stβ 0.15, p=0.04) and there was a trend towards significance for MTX-PG4+5 levels (stβ 0.13, p=0.087) and total MTX-PG levels (stβ 0.14, p=0.053) (Figure 2b). Also, in the derivation cohort, there were positive associations between serum folate concentration and MTX-PG1 levels (stβ 0.32, p=0.002), and between CRP concentration and levels of MTX-PG1 (stβ 0.29, p=0.043) and MTX-PG2 (stβ 0.32, p=0.022). These findings were not replicated in the validation cohort.

In the validation cohort, male patients had higher total MTX-PG levels than female patients (0.14, p=0.027), and homocysteine levels were positively associated MTX-PG4+5 levels (stβ 0.20, p=0.007). These findings were not found in the derivation cohort.

SNP analysis

A total of 28 SNPs in 18 MTX pathway genes were assessed for their contribution to MTX-PG levels (Table 3, Supplementary Table 2). With the exception of ABCB1 rs2032582 (χ^2=299.36, p<0.001) and MTHFR rs1801133 (χ^2=5.46, p=0.019), all SNPs were in Hardy-Weinberg-Equilibrium. SNPs not in Hardy-Weinberg-Equilibrium were entered into linear regressions as normal.

In the derivation cohort, patients with the FPGS rs4451422 wildtype genotype had significantly higher levels of MTX-PG3 (p=0.001), MTX-PG4+5 (p=0.004) and total MTX-PG (p<0.001; Table 3, Figure 2c). This was replicated in the validation cohort for MTX-PG3 (p=0.049), MTX-PG4+5 (p=0.043), and total MTX-PG (p=0.015) (Table 3, Figure 2c). Also, patients with the SLC46A1 rs2239907 wildtype or heterozygous genotype had significantly lower MTX-PG2 (p=0.031) levels in the derivation cohort; this was replicated in the validation cohort for MTX-PG1 (p=0.012).

In the derivation cohort, significant positive correlations were also found for ITPA rs1127354 and MTX-PG4+5 levels (p=0.024); ABCC5 rs2139560 and MTX-PG1 levels (p=0.001), and MTX-PG2 levels (p=0.022); ATIC rs2372536 and MTX-PG1 levels (p=0.008); ABCB1 rs1045642 and MTX-PG1 (p=0.029), MTX-PG2 (p=0.001), MTX-PG3 (p=0.012), and total MTX-PG (p=0.011) levels. A significant negative correlation was found for ABCC1 rs35592 and MTX-PG3 (p=0.021). None of these results were replicated in the validation cohort. In the validation cohort, significant positive associations were found for AMPD1 rs17602729 and MTX-PG2 levels (p=0.015); ABCC1 rs3784862 and MTX-PG1 levels

Table 2. Clinical, socio-demographic and biochemical determinants of erythrocyte MTX-PG levels at three months of treatment (multivariate analysis).

| Variable | Derivation Cohort | | | | | | Validation Cohort | | | | | |
| | N | st.B | | | | | N | st.B | | | | |
		MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4+5	Total MTX-PG		MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4+5	Total MTX-PG
Age (yr)	93	0.23*	0.18t	0.21#	0.17	0.23*	247	0.13*	0.21**	0.22***	0.25***	0.28***
Gender	93	0.05	-0.10	-0.09	0.03	-0.06	247	0.02	-0.08	-0.12#	-0.12#	-0.14*
DAS28esr	80	0.07	-0.01	0.18	0.22#	0.17	247	0.08	0.06	0.09	0.06	0.09
Anti-CCP positive	76	0.06	-0.09	-0.04	0.01	-0.06	198	-0.10	0.06	0.09	0.09	0.10
Days of treatment at study visit	87	-0.06	0.15	0.21#	-0.08	0.19#	234	0.06	0.06	0.017**	0.04	0.11#
Intramuscular administration of methotrexate	82	0.09	-0.22	-0.05	-0.07	-0.13	n/a	n/a	n/a	n/a	n/a	n/a
Other DMARD use	85	0.13	0.02	0.12	0.15	0.13	221	-0.12#	-0.01	-0.03	-0.04	-0.04
Hydroxychloroquine use	85	0.14	-0.05	0.06	0.15	0.08	221	-0p.12#	-0.01	-0.03	-0.04	-0.04
Sulfasalazine use	85	0.04	0.08	0.19#	0.13	0.18#	221	-0.12#	-0.01	-0.03	-0.04	-0.04
Corticosteroid use	85	-0.04	-0.05	0.01	0.01	-0.01	221	0.10	0.05	-0.05	-0.00	-0.01
Corticosteroid IM vs no Corticosteroid	85	-0.07	-0.07	-0.02	0.08	0.00	221	0.13	0.09	-0.17#	-0.12	-0.12
Corticosteroid Oral vs no Corticosteroid	85	-0.01	-0.02	0.02	-0.02	-0.01	221	0.18#	0.08	-0.05	-0.02	0.01
C- reactive protein, mg/l (ln)	76	0.29*	0.32*	0.07	-0.01	0.15	223	0.08	0.03	0.02	0.06	0.05
Erythrocyte sedimentation rate, >44 vs<44 mm/hr	91	-0.08	0.05	0.14	0.15	0.15	246	0.00	0.00	0.01	0.04	0.03
Albumin, g/l	86	0.07	0.02	-0.18	-0.08	-0.11	223	0.07	0.06	0.10	0.09	0.11
Creatinine, >78 vs <78 mmol/l	93	-0.03	0.01	0.03	0.02	0.03	98	0.15	0.18	0.20t	0.16	0.21#
Erythrocyte folate, nmol/l (ln)	88	-0.18	0.19	0.32*	0.24#	0.32*	218	0.08	0.02	0.15*	0.13#	0.14#
Serum folate, nmol/l	89	0.32**	-0.07	0.11	0.04	0.05	224	-0.05	-0.05	-0.07	-0.06	-0.08
eGFR-MDRD >88 vs<88 ml/min/1.73m^2	93	0.18	0.04	0.06	-0.11	0.00	98	-0.07	0.03	-0.08	-0.18	-0.12
Vitamin B12, pmol/l	88	0.10	-0.05	-0.06	-0.05	-0.07	218	-0.05	0.09	0.03	-0.08	-0.01
Homocysteine, µmol /l (ln)	86	0.16	0.04	0.17	0.14	0.16	213	0.03	0.03	0.05	0.20**	0.14#

Table 2. *(continued)*

Alcohol consumption, >4 vs <4 glasses per month	38	0.29	0.10	-0.19	-0.29	-0.20	230	0.03	0.06	0.09	0.04	0.08
Alcohol consumption, >32 vs <4 glasses per month	38	-0.04	-0.06	-0.22	-0.24	-0.30	230	0.02	0.03	0.12	0.04	0.07
Cola consumption, >8 vs <6 glasses per month	82	0.06	0.17	0.29*	0.16	0.27*	227	0.01	-0.15*	-0.04	0.09	-0.01
Coffee consumption, >120 vs <112 cups per month	82	-0.01	-0.12	-0.19#	-0.18	-0.21#	228	0.10	-0.01	0.07	0.02	0.04
Tea consumption, 8-168 cups/month vs rest	82	-0.08	-0.11	0.01	0.03	-0.02	225	0.03	-0.07	-0.01	0.01	-0.01
Tea consumption, >168 cups/month vs rest	82	0.12	0.05	0.06	0.10	0.09	225	-0.04	0.04	-0.04	0.01	0.00

* p<0.05
** p<0.01
*** p<0.001
#=p<0.1

Variables shown had p-values <0.1 in univariate analysis for at least one of the MTX-PGs. All variables have been adjusted for age and gender. In addition, C- reactive protein was also adjusted for DAS28esr and albumin; erythrocyte folate for serum folate; albumin for C- reactive protein; homocysteine for erythrocyte folate; parenteral administration of methotrexate for coffee; vitamin B12 for erythrocyte folate; anti-CCP for prednisone route.

ln: natural logarithm, DAS28esr: ESR based disease activity score 28, Anti-CCP: anti-cyclic citrullinated peptide antibody, DMARD: Disease modifying anti-rheumatic agent, ESR: erythrocyte sedimentation rate, GFR: Glomerular Filtration Rate.

Chapter

6

Table 3. SNP within cellular folate transport and metabolism routes in relation to methotrexate polyglutamate levels at 3 months of treatment in MTX-R and tREACH cohorts.

| | | | | Derivation | | | | | | | Validation | | | | | |
| | | | | Estimated marginal means (SE) | | | | | | | Estimated marginal means (SE) | | | | | |
			N	MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4+5	MTX-PGtotal		N	MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4+5	MTX-PGtotal
rs1127354 ITPA	C/A	Wt	81	44.8 (5.4)	27.1 (1.6)	39.1 (2.2)	13.2 (2.1)*	79.3 (4.6)		211	43.3 (3.4)	23.1 (0.7)	50.5 (1.3)	30.4 (1.5)	103.9 (2.8)
		Het/Var	12	62.0 (13.0)	27.5 (3.8)	49.6 (5.6)	25.1 (4.9)*	102.2 (11.0)		36	40.7 (7.8)	20.6 (1.5)	48.7 (2.8)	27.6 (3.4)	96.9 (6.3)
rs17602729 AMPD1	G/A	Wt	66	46.7 (6.3)	28.2 (1.8)	41.6 (2.5)	15.6 (2.4)	85.4 (5.3)		198	41.8 (3.6)	21.9 (0.7)*	49.4 (1.3)	29.9 (1.6)	101.2 (2.9)
		Het/Var	17	54.2 (11.2)	27.3 (3.2)	40.5 (4.5)	14.2 (4.3)	82.0 (9.4)		42	48.4 (7.3)	25.7 (1.4)*	52.6 (2.6)	30.3 (3.2)	108.6 (5.9)
rs35592 ABCC1	T/C	WT/Het	76	48.2 (5.8)	27.8 (1.7)	42.6 (2.3)*	16.1 (2.2)	86.5 (4.8)		229	43.3 (3.3)	22.7 (0.7)	50.0 (1.2)	30.2 (1.5)	102.9 (2.7)
		Var	7	49.9 (17.8)	30.1 (5.1)	25.9 (6.9)*	4.5 (6.7)	60.5 (14.6)		11	36.6 (14.5)	20.7 (2.9)	47.9 (5.2)	23.5 (6.4)	92.1 (11.6)
rs3784862 ABCC1	A/G	Wt	38	46.8 (7.9)	25.9 (2.2)	42.1 (3.2)	17.8 (3.0)	85.8 (6.7)		133	47.0 (4.2)*	23.6 (0.8)	49.9 (1.5)	28.0 (1.9)	101.5 (3.4)
		Het	34	52.9 (8.0)	30.9 (2.3)	40.9 (3.3)	12.2 (3.1)	84.0 (6.8)		90	32.7 (5.1)*	21.1 (1.0)	49.9 (1.9)	33.4 (2.3)	104.4 (4.2)
		Var	11	35.0 (15.0)	24.6 (4.3)	39.6 (6.1)	17.4 (5.8)	81.6 (12.7)		17	63.6 (11.3)*	22.5 (2.3)	50.6 (4.1)	28.2 (5.0)	101.4 (9.2)
rs868853 ABCC4	T/C	WT/Het	65	48.4 (6.1)	27.5 (1.7)	41.1 (2.3)	15.3 (2.3)	83.9 (4.9)		205	45.6 (3.5)*	22.8 (0.7)	49.9 (1.3)	29.4 (1.5)	102.0 (2.8)
		Var	15	51.1 (13.0)	32.2 (3.7)	46.2 (5.0)	16.8 (5.0)	95.3 (10.6)		34	27.5 (8.2)*	21.7 (1.6)	50.9 (2.9)	34.5 (3.6)	107.0 (6.6)
rs2139560 ABCC5	G/A	Wt	34	34.2 (7.7)**	24.3 (2.3)*	38.5 (3.3)	16.4 (3.2)	79.3 (7.0)		85	40.7 (5.2)	22.5 (1.0)	50.5 (1.8)	31.5 (2.3)	104.5 (4.1)
		Het	38	48.7 (7.1)**	28.7 (2.1)*	41.5 (3.1)	14.2 (3.0)	84.4 (6.4)		111	45.9 (4.7)	22.9 (0.9)	49.8 (1.7)	29.4 (2.1)	102.1 (3.8)
		Var	11	90.6 (13.0)**	36.6 (3.9)*	49.2 (5.6)	15.5 (5.5)	101.4 (11.7)		44	40.2 (7.5)	22.0 (1.5)	48.9 (2.7)	27.9 (3.3)	98.8 (6.0)
rs2372536 ATIC	C/G	Wt	37	37.0 (7.7)**	25.9 (2.4)	37.3 (3.2)	15.6 (3.2)	78.8 (6.8)		108	43.5 (4.7)	22.8 (0.9)	50.1 (1.7)	28.7 (2.1)	101.7 (3.7)
		Het	39	50.1 (7.2)**	29.5 (2.2)	43.1 (3.0)	14.3 (2.9)	86.9 (6.3)		98	42.3 (5.0)	23.0 (1.0)	51.0 (1.8)	32.3 (2.2)	106.3 (4.0)
		Var	7	94.0 (16.5)**	29.9 (5.0)	50.6 (50.6)	19.1 (6.8)	99.6 (14.6)		34	43.6 (8.2)	21.0 (1.6)	46.5 (2.9)	27.5 (3.6)	94.9 (6.5)
rs4451422 FPGS	A/C	Wt	61	55.8 (10.3)	31.6 (3.0)	52.3 (52.3)**	24.8 (3.8)**	108.7 (8.1)***		172	46.5 (5.8)	24.3 (1.2)	53.3 (2.1)*	34.3 (2.6)*	111.8 (4.6)*
		Het/Var	22	46.0 (6.3)	26.9 (1.8)	37.8 (2.4)**	12.2 (2.3)**	76.8 (4.9)***		68	41.6 (3.8)	21.9 (0.8)	48.6 (1.4)*	28.3 (1.7)*	98.8 (3.0)*
rs1045642 ABCB1	G/A	Wt	14	37.1 (12.7)*	20.6 (3.5)**	31.7 (5.1)*	10.3 (5.0)	62.6 (10.3)**		52	38.6 (6.8)	22.3 (1.4)	50.5 (2.4)	29.3 (3.0)	102.1 (5.4)
		Het	41	37.9 (7.3)*	25.0 (2.0)**	38.4 (2.9)*	13.3 (2.9)	76.7 (6.0)**		122	43.9 (4.5)	22.6 (0.9)	49.2 (1.6)	30.5 (2.0)	102.2 (3.6)
		Var	28	66.0 (8.5)*	34.5 (2.3)**	48.5 (3.4)*	19.5 (3.3)	102.5 (6.9)**		66	44.6 (6.0)	22.9 (1.2)	50.9 (2.1)	29.6 (2.6)	103.4 (4.8)

Table 3. *(continued)*

			n						n					
rs1801131 MTHFR	T/G	Wt	44	45.4 (7.2)	26.4 (2.1)	38.8 (2.9)	15.2 (2.8)	80.5 (6.0)	111	36.1 (4.7)*	22.1 (0.9)	50.9 (1.7)	32.3 (2.1)	105.3 (3.8)
		Het	29	54.1 (9.4)	29.3 (2.7)	44.8 (3.8)	15.8 (3.6)	90.0 (7.9)	107	45.5 (4.6)*	22.4 (0.9)	48.6 (1.7)	28.4 (2.1)	99.5 (3.7)
		Var	10	47.7 (14.7)	32.0 (4.2)	44.2 (5.9)	13.9 (5.7)	90.1 (12.3)	22	63.5 (10.0)*	25.8 (2.0)	51.8 (3.6)	26.7 (4.5)	104.4 (8.1)
Rs 3785911 ABCC3	A/C	Wt	43	49.0 (7.4)	27.1 (2.1)	40.9 (3.0)	13.8 (2.9)	81.8 (6.2)	126	46.4 (44.4)	22.9 (0.9)	48.3 (1.6)	27.5 (1.9)**	98.7 (3.5)*
		Het	30	44.7 (8.8)	28.0 (2.5)	41.8 (3.6)	16.8 (3.4)	86.5 (7.4)	93	40.5 (5.0)	22.4 (1.0)	50.6 (1.8)	30.1 (2.2)**	103.0 (4.0)*
		Var	10	59.4 (15.2)	33.6 (4.4)	41.8 (6.2)	17.4 (5.9)	92.8 (12.8)	21	34.6 (10.3)	21.9 (2.1)	56.8 (3.7)	43.5 (4.5)**	122.2 (8.2)*
rs4793665 ABCC3	T/C	Wt	22	32.3 (10.1)	23.8 (2.9)	41.8 (4.2)	18.6 (4.0)	84.4 (8.7)	79	44.2 (5.4)*	23.4 (1.1)	49.3 (1.9)	29.1 (2.4)	101.8 (4.4)
		Het	41	58.7 (7.8)	28.9 (2.3)	40.4 (3.2)	14.2 (3.1)	83.4 (6.7)	119	37.1 (4.4)*	21.8 (0.9)	49.3 (1.6)	30.0 (2.0)	101.1 (3.6)
		Var	20	46.4 (10.0)	30.3 (2.9)	42.3 (4.1)	14.0 (3.9)	86.6 (8.6)	42	58.6 (7.4)*	23.2 (1.5)	53.5 (2.7)	31.6 (3.3)	108.2 (6.0)
rs2239907 SLC46A1	C/T	Wt	26	46.9 (9.0)	26.9 (2.5)*	41.4 (3.6)	15.9 (3.5)	84.2 (7.6)	73	37.6 (5.6)*	23.8 (1.1)	51.2 (2.0)	30.7 (2.5)	105.7 (4.6)
		Het	47	46.4 (7.7)	26.1 (2.1)*	39.7 (3.1)	15.7 (3.0)	81.6 (6.5)	119	39.0 (4.4)*	21.2 (0.9)	48.9 (1.6)	30.5 (2.0)	100.6 (3.6)
		Var	10	59.7 (14.6)	38.0 (4.0)*	47.1 (5.9)	11.8 (5.6)	96.9 (12.2)	48	60.9 (6.8)*	24.2 (1.4)	50.7 (2.5)	27.6 (3.0)	102.5 (5.5)

* p<0.05
** p<0.01
*** p<0.001

Analysis was done using ANCOVA with correction for age and gender. WT = wildtype, Het = heterozygous, Var = homozygous variant. Rs = reference SNP number. Only SNPs with a significant correlation with any MTX-PG in the derivation or validation cohort are shown, full table in supplementary table 2. ABCB1/ABCC1/ABCC2/ABCC3/ABCC4/ABCC5/ABCG2: adenosine triphosphate-binding cassette transporter subfamily B/C/G member 1/2/3/4/; FPGS: folylpolyglutamate synthetase; FOLR1/FOLR2: folate receptor 1/2; GGH: gamma glutamyl hydrolase; SLC 46A1/SLC19A:solute carrier 46A1/19A1.

Chapter

6
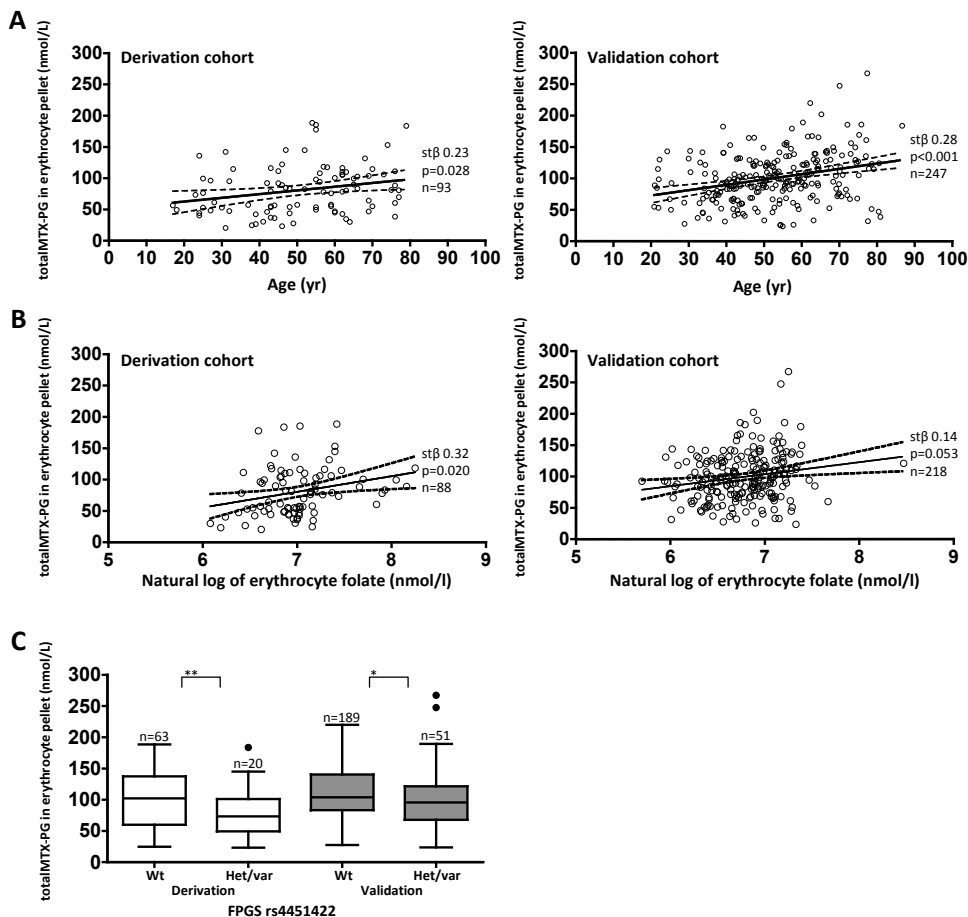
Figure 2. A) Linear regression of age and total MTX-PG. Solid line represents a trendline with its 95% confidence interval (dotted line). In both cohorts age is positively associated with total MTX-PG. Regression analysis is plotted from the unadjusted data, Stβ and p-values are from the confounder adjusted data.

B) Linear regression of erythrocyte folate and total MTX-PG. Solid line represents a trendline with its 95% confidence interval (dotted line). In both cohorts, age is positively associated with total MTX-PG. Regression analysis is plotted from the unadjusted data, Stβ and p-values are from the confounder adjusted data.

C) Effect of the FPGS rs4451422 variant allele on the concentration of total MTX-PG. Patients of the FPGS rs4451422 heterozygous and homozygous genotype have significantly lower concentrations of total MTX-PG. ANCOVA was adjusted for age and gender. Wt: wildtype carriers, het/var: heterozygous combined with homozygous variant carriers. Brackets denote significant differences groups, p-values are noted above the brackets. *p<0.05, **p<0.001

(p=0.014); MTHFR rs1801131 and MTX-PG1 levels (p=0.031); ABCC3 rs3785911 and MTX-PG4+5 (p=0.004), and total MTX-PG levels (p=0.029); ABCC3 rs4793665 and MTX-PG1 levels (p=0.038). A significant negative correlation was found for ABCC4 rs868853 and MTX-PG1 levels (p=0.038). These results were not observed in the derivation cohort.

Table 4. Multivariate regression model of the three strongest determinants.

	MTX-PG1		MTX-PG2		MTX-PG3		MTX-PG4+5		MTX-PG2-5	
	St.β	p	St.β	p	St.β	p	St.β	p	St.β	p
Derivation Cohort										
Age	0.21	0.06	0.20	0.09	0.24	0.03	0.20	0.08	0.28	0.01
Erythrocyte folate	-0.19	0.13	0.04	0.75	0.21	0.08	0.18	0.15	0.19	0.10
FPGS rs4451422 wt vs. het/var	-0.08	0.47	-0.17	0.13	-0.36	<0.001	-0.33	0.003	-0.38	<0.001
R^2	0.14		0.04		0.21		0.11		0.21	
Validation Cohort										
Age	0.13	0.08	0.22	0.002	0.23	0.001	0.22	0.002	0.28	<0.001
Erythrocyte folate	0.05	0.53	0.03	0.66	0.12	0.09	0.10	0.16	0.11	0.099
FPGS rs4451422 wt vs. het/var	0.01	0.84	-0.07	0.30	-0.08	0.25	-0.09	0.18	-0.10	0.12
R^2	0		0.03		0.07		0.07		0.10	
Combined Cohort										
Dose	-0.07	0.26	-0.22	<0.001	0.23	<0.001	0.30	<0.001	0.2	<0.001
Age	0.15	0.013	0.20	0.001	0.23	<0.001	0.21	<0.001	0.27	<0.001
Erythrocyte folate	0.03	0.65	0.03	0.66	0.15	0.01	0.11	0.06	0.13	0.024
FPGS rs4451422 wt vs. het/var	-0.01	0.80	-0.09	0.106	-0.16	0.003	-0.15	0.005	-0.18	<0.001
R^2	0.01		0.08		0.15		0.15		0.16	

Variables which, after correction for confounders, had a significant correlation in both cohorts were included in one multivariate regression model. FPGS (folylpolyglutamate synthetase) rs4451422 was dichotomized into wild type versus heterozygous/variant. Correlations with p<0.05 are considered significant. Confounders are not shown and were serum folate (for erythrocyte folate) and gender (for FPGS rs4451422).

Combined multivariate model

The significant variables present in both cohorts and their confounders were included in one multivariate regression model. The included variables were age, erythrocyte folate and FPGS rs4451422. Confounders included were gender and serum folate. In the derivation cohort, this combined model explained 14% of MTX-PG1 variability, 4% of MTX-PG2 variability, 21% of MTX-PG3 variability, 11% of MTX-PG4+5 variability, and 21% of total MTX-PG variability (Table 4). However, in the validation cohort, the model only explained 0% of MTX-PG1 variability, 3% of MTX-PG2 variability, 7% of MTX-PG3 variability, 7% of MTX-PG4+5 variability, and 10% of total MTX-PG variability.

MTX Dose

As the variation in MTX dosage in each cohort was insufficient to adequately determine the influence of treatment dose on MTX-PG concentration, the effect of dosage was studied by grouping both cohorts and comparing the different treatment doses. Multivariate regression

analysis was performed using age, gender, erythrocyte folate and FPGS rs4451422 as co-variables. Treatment dose did not have a significant association with MTX-PG1. However, treatment dose had a positive association with MTX-PG3 levels (stβ 0.23; p<0.001), MTX-PG4+5 levels (stβ 0.30; p<0.001), and total MTX-PG levels (stβ 0.20; p=0.002; Figure 1b). Strikingly, there was a negative association with MTX-PG2 levels (stβ -0.22; p<0.001). The model including dose explained 1% of MTX-PG1 variability, 8% of MTX-PG2 variability, 15% of MTX-PG3 variability, 15% of MTX-PG4+5 variability, and 16% of total MTX-PG variability in the combined cohort.

Discussion

To the best of our knowledge, we are the first to report clinical, genetic, socio-demographic and biochemical determinants of erythrocyte MTX-PG accumulation at three months of low-dose MTX treatment in a prospective study, using a derivation and validation cohort. In our study, we found age, MTX dosage, erythrocyte folate content and the FPGS rs4451422 SNP as the major determinants of MTX-PG levels in both cohorts.

MTX is the 'anchor drug' in the treatment of pediatric and adult arthritis due to its high efficacy, low cost and good safety profile. Its use is hampered because 20-40% of patients are non-responsive to treatment and 30% of patients suffer from adverse events.

To further improve efficacy and reduce toxicity personalized treatment is mandatory by prescribing patients the right drug in the right concentration. [2, 35] The dosage of MTX, required to suppress disease activity, varies between patients and as of yet therapeutic drug monitoring of low-dose MTX therapy is not possible because plasma MTX is rapidly cleared and is unrelated to response. [4, 36] This has led to a trial and error approach in finding the right MTX dose for RA patients. However, intracellular MTX can be measured [15, 37, 38] and we have shown previously that erythrocyte MTX-PG levels predict reponse in the first nine months of treatment in three prospective cohort studies in RA and JIA. [39, 40] Knowing the determinants of MTX-PG accumulation and the cutoff concentration that predicts good response with good sensitivity and specificity might help with targeting treatment at the individual patient in order to reach the optimal MTX-PG level. TDM of MTX therapy may also help to identify patients that do not or partially comply with treatment.

In concordance with previous findings [18], we found age as the predominant determinant for erythrocyte MTX-PG levels with increasing age leading to increased concentrations of long-chain and total MTX-PGs (Figure 2a). Although reduced renal function is likely an important part of this complex interaction, eGFR-MDRD and creatinine levels did not have a significant effect on MTX-PG levels in either of our cohorts. More extensive research is needed to find out the underlying interactions.

Previous studies have shown that dose is a driving factor for the accumulation of MTX-PGs [18, 19, 41]. In our study, the validation cohort had elevated MTX-PG3, MTX-PG4 and MTX-PG5 levels, but lower MTX-PG2 levels than the derivation cohort (Figure 1a). This difference in MTX-PG levels between cohorts is likely caused by the difference in dose as the

cohorts had significantly different dosing regimes (Table 1).

However, in our cohorts, there was too little variation in dosage to be able to demonstrate and validate a relation between MTX dosage and erythrocyte MTX-PG accumulation in each cohort. Therefore, we studied the effect of MTX dosage on erythrocyte MTX-PG accumulation by grouping both cohorts and comparing the treatment doses. Using multivariate analysis we confirmed that the differences in MTX-PG concentration between our cohorts were largely due to the difference in dose. Patients treated with 25 mg/wk had 61% higher concentrations of long-chain MTX-PGs, and 18% higher concentrations of total MTX-PG (Figure 1b) than patients treated with 15 mg/wk or less. Interestingly, the group that was treated with higher MTX dosage had 21% lower short-chain MTX-PG levels, possibly indicating that the addition of glutamate groups occurs at a higher rate than the removal of the glutamate groups, which would lead the high exposure to MTX to push the equilibrium towards long-chain MTX-PGs.

In our validation cohort, co-medication was strictly protocolized (Table 1). Therefore, we cannot conclusively dismiss the effect of co-medication on the accumulation of MTX-PG. Corticosteroid supplementation especially was very strongly correlated to MTX dose. However, none of the co-medications had significant associations with MTX-PG levels and when entered as co-variable in multivariate linear regression they had no effect on the association.

In addition to dose of treatment, route of administration has been shown to effect MTX-PG levels as well.[12, 19] The effective dose of subcutaneous administration would be substantially higher because of the increased bio-availability. In our cohorts, only 7% of the derivation cohort and none of the patients from the validation cohort received subcutaneous methotrexate and we did not see an effect of route of administration on the MTX-PG levels. This is likely due to the small amount of patients that received subcutaneous methotrexate and we expect this to have a stronger effect when more patients are treated with subcutaneous methotrexate.

Folic acid use during MTX treatment has no, or negative effects on MTX efficacy. [42, 43] suggesting that higher concentrations of folate during MTX treatment facilitate lower effectiveness of MTX due to competition with folate for transporter proteins, polyglutamylation proteins and target enzymes. However, we showed in two prospective RA cohorts that high baseline erythrocyte folate was related to response to MTX.[44] We speculated that patients with higher concentrations of baseline erythrocyte folate may be more effective in accumulating intracellular MTX because of the high structural similarity of MTX to folate; MTX uses the same cellular machinery for uptake, transport and metabolism. Baseline erythrocyte folate might be viewed as a functional marker for the capacity to take up and accumulate folates, thereby predicting MTX accumulation during treatment. In support of this hypothesis, we show that higher baseline erythrocyte folate levels are associated with higher levels of MTX-PGs. We also found that higher baseline serum folate levels were associated with higher MTX-PG1 levels in the derivation cohort, although not

in the validation cohort, which may reflect improved take-up of MTX from the intestine. It might be speculated that the observed relation between age and MTX-PG levels is caused by changes in folate with age. However, although age and baseline erythrocyte folate levels are correlated in both cohorts (derivation cohort r=0.229, validation cohort r=0.177), the relation between age and MTX-PG did not change when erythrocyte folate or serum folate was included in the model as variable, suggesting that age has a distinct effect on MTX-PG accumulation. In the present study, baseline erythrocyte folate was significantly lower in the validation cohort whereas this cohort accumulated the highest MTX-PG levels due to the much higher dosis (25 mg/week). The difference in baseline erythrocyte folate between cohorts might be explained by the slightly higher disease activity in the validation cohort; the higher activity of the immune system in patients with higher disease activity might lead to higher folate use leading to a lower baseline erythrocyte folate in the validation cohort.

Folylpolyglutamate synthetase (FPGS) has a central role in the metabolism of methotrexate, as it is the enzyme that attaches the glutamate groups to methotrexate, creating the methotrexate polyglutamates. Any changes in function might therefore dramatically decrease the longer chain MTX-PGs, thereby leading to a slower build-up of MTX-PGs and lower steady-state concentrations, which in turn can lead to decreased response to medication [45]. In our study, we see this effect most strongly in the derivation cohort, where medium chain and long chain MTX-PGs, as well as total MTX-PGs are significantly lower (Table 3) in patients with the heterozygous or homozygous variant genotype of FPGS rs4451422. In the validation cohort, the effect is less prominent, with smaller differences in concentrations between genotypes. The higher MTX dosage in the validation cohort might partially override the genetic determinants [46], leading to a decreased influence of this genotype. This could indicate that patients with the homozygous variant genotype would benefit from an increase in MTX dose, thereby possibly lowering the time needed for patients to achieve optimal treatment and might prevent needless switching to the more expensive biological.

We also found SLC46A1 rs2239907 to be significantly associated with MTX-PGs in both cohorts. SLC46A1 is a proton-coupled folate transporter that is responsible for the intestinal uptake of folate. Patients with the SLC46A1 rs2239907 homozygous variant allele have significantly higher concentrations of short-chain MTX-PGs than patients with the wildtype allele. This could correspond to an increased uptake of MTX, which would lead to higher plasma MTX levels and increased exposure of the bone marrow to MTX. Similar to previous studies the associations of SNPs to MTX-PGs are weak. In other studies, polymorphisms in the GGH and SLC19 genes have been found to influence the long chain MTX-PG levels [6, 8]. In our cohorts, there was no significant association of GGH or SLC19 SNPs in the derivation cohort, although there was an effect on long-chain MTX-PGs in the validation cohort. To our knowledge we are the first to publish an extensive overview of the effect of SNPS in the MTX pathway on intracellular MTX accumulation using a prospective derivation and validation cohort and linking FPGS rs4451422 and SLC46A1 rs2239907 to

MTX-PG accumulation in both cohorts.

Previous research also found dose of MTX, route of administration, age and renal function [15, 18, 19, 41] to be strongly associated with MTX-PG levels. In concordance with this data, we found MTX dose and age to be strong determinants. However, renal function was not significantly associated with MTX-PG levels in our cohorts. The discrepancy between results from previous studies and our study can be partially explained by the cross-sectional cohorts that were used in previous studies. In our study, patients were prospectively followed while other studies used patients that were treated with MTX for up to 19 years [6, 8, 18, 19, 41]. The MTX-PG accumulation over such a long period of time would be very different, mostly in steady state, and possibly controlled by different determinants. Previous studies also used patients treated with a relatively low dose of MTX, comparable to the derivation cohort in our study. The validation cohort uses an almost two-fold higher MTX dose, which might override some of the biological and genetic determinants, thereby leading to other significant determinants of MTX-PGs. Despite strong correlations, the determinants found in this study only explained up to 21% of the variability in the derivation cohort, and even less in the validation cohort (up to 10%). This was also seen in the combined cohort, where only up to 16% of variability (MTX-PG2-5) was explained by the model including dose of treatment. This indicates that there are other, as of yet undiscovered factors that influence the MTX-PG status. One possibility could be alternative splicing of FPGS, which has been shown to influence response to MTX in leukemia cell lines [47]. Alternative splicing leads to loss of function of FPGS, resulting in a different polyglutamation status and loss of MTX retention in the cell. Another possibility could be differences in methylation, causing differences in expression of the folate pathway genes thereby leading to variation in MTX uptake, or polyglutamation.

In conclusion, our study is the first prospective study investigating the determinants of intracellular MTX-PGs using a derivation and validation cohort. We found that higher age, higher erythrocyte folate concentration, higher MTX dose and the FPGS rs4451422 wildtype variant all lead to higher accumulation of medium and long chain MTX-PGs. Knowing these determinants might help targeting treatment at the individual patient.

Chapter

6

References

1   Strand V, Cohen S, Schiff M, Weaver A, Fleischmann R, Cannon G, et al. Treatment of active rheumatoid arthritis with leflunomide compared with placebo and methotrexate. Leflunomide Rheumatoid Arthritis Investigators Group. Arch Intern Med. 1999 Nov 22;159(21):2542-50.

2   Wessels JA, van der Kooij SM, le Cessie S, Kievit W, Barerra P, Allaart CF, et al. A clinical pharmacogenetic model to predict the efficacy of methotrexate monotherapy in recent-onset rheumatoid arthritis. Arthritis Rheum. 2007 Jun;56(6):1765-75.

3   Bannwarth B, Pehourcq F, Schaeverbeke T, Dehais J. Clinical pharmacokinetics of low-dose pulse methotrexate in rheumatoid arthritis. Clin Pharmacokinet. 1996 Mar;30(3):194-210.

4   Chabner BA, Allegra CJ, Curt GA, Clendeninn NJ, Baram J, Koizumi S, et al. Polyglutamation of methotrexate. Is methotrexate a prodrug? J Clin Invest. 1985 Sep;76(3):907-12.

5   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Caldwell J, et al. Pharmacogenetic and metabolite measurements are associated with clinical status in patients with rheumatoid arthritis treated with methotrexate: results of a multicentred cross sectional observational study. Ann Rheum Dis. 2005 Aug;64(8):1180-5.

6   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Walsh M, et al. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum. 2004 Sep;50(9):2766-74.

7   Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum. 2006 Oct;54(10):3095-103.

8   Dervieux T, Kremer J, Lein DO, Capps R, Barham R, Meyer G, et al. Contribution of common polymorphisms in reduced folate carrier and gamma-glutamylhydrolase to methotrexate polyglutamate levels in patients with rheumatoid arthritis. Pharmacogenetics. 2004 Nov;14(11):733-9.

9   Angelis-Stoforidis P, Vajda FJ, Christophidis N. Methotrexate polyglutamate levels in circulating erythrocytes and polymorphs correlate with clinical efficacy in rheumatoid arthritis. Clin Exp Rheumatol. 1999 May-Jun;17(3):313-20.

10 Kremer JM, Lee JK. The safety and efficacy of the use of methotrexate in long-term therapy for rheumatoid arthritis. Arthritis Rheum. 1986 Jul;29(7):822-31.

11 Hroch M, Tukova J, Dolezalova P, Chladek J. An improved high-performance liquid chromatography method for quantification of methotrexate polyglutamates in red blood cells of children with juvenile idiopathic arthritis. Biopharm Drug Dispos. 2009 Apr;30(3):138-48.

12 Becker ML, van Haandel L, Gaedigk R, Lasky A, Hoeltzel M, Stobaugh J, et al. Analysis of intracellular methotrexate polyglutamates in patients with juvenile idiopathic arthritis: effect of route of administration on variability in intracellular methotrexate polyglutamate concentrations. Arthritis Rheum. 2010 Jun;62(6):1803-12.

13 Becker ML, Gaedigk R, van Haandel L, Thomas B, Lasky A, Hoeltzel M, et al. The effect of genotype on methotrexate polyglutamate variability in juvenile idiopathic arthritis and association with drug response. Arthritis Rheum. 2011 Jan;63(1):276-85.

14 den Boer E, Meesters RJ, Van Zelst BD, Luider TM, Hazes JMW, Heil SG, et al. Measuring methotrexate polyglutamates in red blood cells: a new LC-MS/MS based method. Analytical and Bioanalytical Chemistry. 2012:in press.

15 Dervieux T, Orentas Lein D, Marcelletti J, Pischel K, Smith K, Walsh M, et al. HPLC determination of erythrocyte methotrexate polyglutamates after low-dose methotrexate therapy in patients with rheumatoid arthritis. Clin Chem. 2003 Oct;49(10):1632-41.

16   van Haandel L, Becker ML, Leeder JS, Williams TD, Stobaugh JF. A novel high-performance liquid chromatography/mass spectrometry method for improved selective and sensitive measurement of methotrexate polyglutamation status in human red blood cells. Rapid Commun Mass Spectrom. 2009 Dec;23(23):3693-702.

17   Dalrymple JM, Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Barclay ML. Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. Arthritis Rheum. 2008 Nov;58(11):3299-308.

18   Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Frampton C, James J, et al. Determinants of red blood cell methotrexate polyglutamate concentrations in rheumatoid arthritis patients receiving long-term methotrexate treatment. Arthritis Rheum. 2009 Aug;60(8):2248-56.

19   Dervieux T, Zablocki R, Kremer J. Red blood cell methotrexate polyglutamates emerge as a function of dosage intensity and route of administration during pulse methotrexate therapy in rheumatoid arthritis. Rheumatology (Oxford). 2010 Dec;49(12):2337-45.

20   Claessen SJ, Hazes JM, Huisman MA, van Zeben D, Luime JJ, Weel AE. Use of risk stratification to target therapies in patients with recent onset arthritis; design of a prospective randomized multicenter controlled trial. BMC Musculoskelet Disord. 2009;10:71.

21   Society DR. NVR Medicijnen MTX richtlijn 2009-update 2011. http://wwwnvrnl/uploads/ki/tO/kitOmw9WmZQBHEq61H4YMQ/NVR-Medicijnen-MTX-richtlijn-2009-update-2011pdf. 2011.

22   Weigand M, Frei E, Graf N, Wiessler M. Comparative analysis of methotrexate polyglutamates in lymphoblast preparations from bone marrow and blood, and the contribution of residual red blood cells. J Cancer Res Clin Oncol. 2000 Jul;126(7):407-11.

23   23International Hapmap Project. Internet. Accessed June 7, 2012..

24   24National Center for Biotechnology Information. dbSNP short genetic variations. Internet. Accessed June 7, 2012..

25   Ranganathan P, Culverhouse R, Marsh S, Mody A, Scott-Horton TJ, Brasington R, et al. Methotrexate (MTX) pathway gene polymorphisms and their effects on MTX toxicity in Caucasian and African American patients with rheumatoid arthritis. J Rheumatol. 2008 Apr;35(4):572-9.

26   Ranganathan P. An update on methotrexate pharmacogenetics in rheumatoid arthritis. Pharmacogenomics. 2008 Apr;9(4):439-51.

27   Gervasini G. Polymorphisms in methotrexate pathways: what is clinically relevant, what is not, and what is promising. Curr Drug Metab. 2009 Jul;10(6):547-66.

28   Gradhand U, Tegude H, Burk O, Eichelbaum M, Fromm MF, Konig J. Functional analysis of the polymorphism -211C>T in the regulatory region of the human ABCC3 gene. Life Sci. 2007 Mar 27;80(16):1490-4.

29   Ostergaard M, Ernst A, Labouriau R, Dagiliene E, Krarup HB, Christensen M, et al. Cyclooxygenase-2, multidrug resistance 1, and breast cancer resistance protein gene polymorphisms and inflammatory bowel disease in the Danish population. Scand J Gastroenterol. 2009;44(1):65-73.

30   Boyles AL, Wilcox AJ, Taylor JA, Shi M, Weinberg CR, Meyer K, et al. Oral facial clefts and gene polymorphisms in metabolism of folate/one-carbon and vitamin A: a pathway-wide association study. Genet Epidemiol. 2009 Apr;33(3):247-55.

31   DeVos L, Chanson A, Liu Z, Ciappio ED, Parnell LD, Mason JB, et al. Associations between single nucleotide polymorphisms in folate uptake and metabolizing genes with blood folate, homocysteine, and DNA uracil concentrations. Am J Clin Nutr. 2008 Oct;88(4):1149-58.

32   de Jonge R, Tissing WJ, Hooijberg JH, Jansen G, Kaspers GJ, Lindemans J, et al. Polymorphisms in folate-related genes and risk of pediatric acute lymphoblastic leukemia. Blood. 2009 Mar 5;113(10):2284-9.

33   Ansari M, Sauty G, Labuda M, Gagne V, Laverdiere C, Moghrabi A, et al. Polymorphisms in multidrug resistance-associated protein gene 4 is associated with outcome in childhood acute lymphoblastic leukemia. Blood. 2009 Aug 13;114(7):1383-6.

34   de Rotte MC, Bulatovic M, Heijstek MW, Jansen G, Heil SG, van Schaik RH, et al. ABCB1 and ABCC3 gene polymorphisms are associated with first-year response to methotrexate in juvenile idiopathic arthritis. J Rheumatol. 2012 Oct;39(10):2032-40.

Chapter

6

35   Bulatovic M, Heijstek MW, Van Dijkhuizen EH, Wulffraat NM, Pluijm SM, de Jonge R. Prediction of clinical non-response to methotrexate treatment in juvenile idiopathic arthritis. Ann Rheum Dis. 2012 Sep;71(9):1484-9.

36   Hornung N, Ellingsen T, Attermann J, Stengaard-Pedersen K, Poulsen JH. Patients with rheumatoid arthritis treated with methotrexate (MTX): concentrations of steady-state erythrocyte MTX correlate to plasma concentrations and clinical efficacy. J Rheumatol. 2008 Sep;35(9):1709-15.

37   den Boer E, Meesters RJ, van Zelst BD, Luider TM, Hazes JM, Heil SG, et al. Measuring methotrexate polyglutamates in red blood cells: a new LC-MS/MS-based method. Anal Bioanal Chem. 2013 Feb;405(5):1673-81.

38   van Haandel L, Becker ML, Williams TD, Leeder JS, Stobaugh JF. Measurement of methotrexate polyglutamates in human erythrocytes by ion-pair UPLC-MS/MS. Bioanalysis. 2011 Dec;3(24):2783-96.

39   Bulatovic Calasan M, den Boer E, de Rotte MC, Vastert SJ, Kamphuis S, de Jonge R, et al. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in juvenile idiopathic arthritis patients. Ann Rheum Dis. 2013 Nov 28.

40   de Rotte MC, den Boer E, de Jong PH, Pluijm SM, Bulatovic Calasan M, Weel AE, et al. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in patients with rheumatoid arthritis. Ann Rheum Dis. 2013 Dec 5.

41   Stamp LK, Barclay ML, O'Donnell JL, Zhang M, Drake J, Frampton C, et al. Effects of changing from oral to subcutaneous methotrexate on red blood cell methotrexate polyglutamate concentrations and disease activity in patients with rheumatoid arthritis. J Rheumatol. 2011 Dec;38(12):2540-7.

42   Ortiz Z, Shea B, Suarez Almazor M, Moher D, Wells G, Tugwell P. Folic acid and folinic acid for reducing side effects in patients receiving methotrexate for rheumatoid arthritis. Cochrane Database Syst Rev. 2000(2):CD000951.

43   Khanna D, Park GS, Paulus HE, Simpson KM, Elashoff D, Cohen SB, et al. Reduction of the efficacy of methotrexate by the use of folic acid: post hoc analysis from two randomized controlled studies. Arthritis Rheum. 2005 Oct;52(10):3030-8.

44   de Rotte MC, de Jong PH, Pluijm SM, Calasan MB, Barendregt PJ, van Zeben D, et al. Association of low baseline levels of erythrocyte folate with treatment nonresponse at three months in rheumatoid arthritis patients receiving methotrexate. Arthritis Rheum. 2013 Nov;65(11):2803-13.

45   Owen SA, Hider SL, Martin P, Bruce IN, Barton A, Thomson W. Genetic polymorphisms in key methotrexate pathway genes are associated with response to treatment in rheumatoid arthritis patients. Pharmacogenomics J. 2013 Jun;13(3):227-34.

46   French D, Yang W, Cheng C, Raimondi SC, Mullighan CG, Downing JR, et al. Acquired variation outweighs inherited variation in whole genome analysis of methotrexate polyglutamate accumulation in leukemia. Blood. 2009 May 7;113(19):4512-20.

47   Stark M, Wichman C, Avivi I, Assaraf YG. Aberrant splicing of folylpolyglutamate synthetase as a novel mechanism of antifolate resistance in leukemia. Blood. 2009 Apr 30;113(18):4362-9.

Chapter

6

Supplementary table 1. Clinical, socio-demographic and biochemical determinants of erythrocyte MTX-PG levels at three months of treatment (univariate analysis).

Variable	Derivation Cohort						Validation Cohort					
				St.B						St.B		
	N	MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4+5	Total MTX-PG	N	MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4+5	Total MTX-PG
Age, years, mean (SD)	93	0,22*	0,20#	0,22*	0,16	0,25*	247	0,13*	0,22***	0,24***	0,27***	0,30***
Female (%)	93	0,00	-0,13	-0,13	0,00	-0,11	247	-0,01	-0,11#	-0,16*	-0,16*	-0,18**
BSA, m3, mean (SD)	0	n/a	n/a	n/a	n/a	n/a	245	0,057	-0,05	-0,07	-0,05	-0,07
DAS28esr, mean (SD)	80	0,12	0,03	0,22#	0,24*	0,22#	247	0,10	0,10	0,13*	0,11#	0,14**
Rheumatoid factor positive (%)	83	-0,01	0,01	0,10	0,09	0,09	223	-0,03	0,13#	0,06	-0,02	0,05
Anti-CCP positive (%)	83	0,10	-0,02	0,03	0,01	0,01	223	-0,08	0,05	0,05	0,08	0,08
Days of treatment at study visit, mean (SD)	87	-0,08	0,11	0,16	0,05	0,14	234	0,05	0,06	0,16*	0,03	0,10
Intramuscular administration of methotrexate (%)	85	0,11	-0,16	0,02	-0,02	-0,05	0	n/a	n/a	n/a	n/a	n/a
Other DMARD use (%)	85	0,16	0,04	0,14	0,17	0,16	221	-0,12#	0,00	-0,02	-0,03	-0,03
Hydroxychloroquine use (%)	85	0,17	-0,04	0,07	0,17	0,09	221	-0,12#	0,00	-0,02	-0,03	-0,03
Sulfasalazine use (%)	85	0,03	0,07	0,18#	0,12	0,17	221	-0,12#	0,00	-0,02	-0,03	-0,03
Biological use (%)	85	0,00	0,00	-0,08	-0,08	-0,07	0	n/a	n/a	n/a	n/a	n/a
Corticosteroid use	85	-0,03	-0,03	0,03	0,02	0,01	221	0,09	0,05	-0,06	-0,01	-0,02
Corticosteroid IM vs no Corticosteroid	85	-0,02	-0,03	0,03	0,11	0,05	221	0,13	0,10	-0,16	-0,10	-0,10
Corticosteroid Oral vs no Corticosteroid	85	-0,02	-0,02	0,02	-0,03	-0,01	221	0,18#	0,06	-0,07	0,00	-0,02
C- reactive protein, mg/L, median (IQR)	91	0,22*	0,22*	0,14	0,01	0,15	246	0,09	0,09	0,08	0,10	0,11#
Erythrocyte sedimentation rate , >44 vs<44 mm/hr	92	0,05	0,13	0,18#	0,15	0,20#	247	0,06	0,06	0,08	0,12#	0,11#
Albumin, (g/L), mean (SD)	88	-0,09	-0,11	-0,23	-0,11	-0,20#	224	-0,01	-0,02	0,01	-0,01	-0,01
Creatinine, >78 vs<78 mmol/l	93	0,03	0,09	0,12	0,06	0,12	98	0,11	0,18#	0,21	0,23*	0,25
Erythrocyte folate, nmol/L, median (IQR)	88	0,08	0,10	0,30**	0,21#	0,27*	218	0,07	0,08	0,18**	0,16*	0,18**
Serum folate, nmol/L median (IQR)	89	0,35***	-0,04	0,15	0,07	0,09	224	-0,03	-0,01	-0,02	-0,02	-0,02
eGFR-MDRD >88 vs<88 ml/min/ 1.73m²	93	0,02	-0,02	-0,02	-0,15	-0,09	98	-0,11	-0,03	-0,12	-0,22*	-0,17#

Supplementary table 1. (continued)

Vitamin B12, pmol/L, median (IQR)	88	0,14	-0,02	0,04	0,03	0,03	224	-0,01	0,14*	0,11	-0,01	0,08
Vitamin B6, nmol/L, median (IQR)	89	-0,10	-0,10	0,08	0,06	0,03	199	0,01	0,01	-0,01	-0,04	-0,03
Homocysteine umol /l, median (IQR)	87	0,16	0,09	0,14	0,10	0,14	225	0,03	0,05	0,06	0,20**	0,15*
Alcohol consumption, >4 vs<4 glasses per month	38	0,27	0,07	-0,21	-0,29	-0,21	230	0,04	0,10	0,14#	0,09	0,13#
Alcohol consumption , >32 vs<4 glasses per month	38	-0,01	-0,05	-0,15	-0,16	-0,16	230	0,04	0,02	0,18*	0,11	0,14#
Cola consumption, >8 vs<6 glasses per month	82	-0,02	0,13	0,23*	0,09	0,19#	227	-0,02	-0,17**	-0,08	0,04	-0,06
Coffee consumption, >120 vs<112 cups per month	82	-0,01	-0,09	-0,16	-0,17	-0,18	228	0,11#	0,02	0,11#	0,07	0,09
Tea consumption, 8-168 cups/month vs rest	82	-0,11	-0,14	-0,02	0,01	-0,05	225	0,02	-0,09	-0,05	-0,03	-0,06
Tea consumption, >168 cups/month vs rest	82	0,13	0,03	0,04	0,10	0,08	225	-0,03	0,05	-0,03	0,03	0,01

* p<0.05
** p<0.01
*** p<0.001
#=p<0.1

In: natural logarithm, DAS28esr: ESR based disease activity score 28, Anti-CCP: anti-cyclic citrullinated peptide antibody, DMARD: Disease modifying anti-rheumatic agent, ESR: erythrocyte sedimentation rate, GFR: Glomerular Filtration Rate.

Chapter

6

Supplementary table 2. SNP within cellular folate transport and metabolism routes in relation to methotrexate polyglutamate levels at 3 months of treatment in derivation and validation cohort.

SNP			Derivation						Validation					
				Estimated marginal means (SE)						Estimated marginal means (SE)				
			N	MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4+5	MTX-PGtotal	N	MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4+5	MTX-PGtotal
rs73598374 ADA[†]	g>a	Wt	68	50.3 (6.1)	28.8 (1.7)	40.9 (2.4)	14.6 (2.3)	84.4 (5.1)	210	43.1 (3.5)	22.6 (0.7)	49.7 (1.3)	30.1 (1.6)	102.4 (2.8)
		Het	15	38.9 (12.2)	24.2 (3.5)	43.2 (4.9)	18.3 (4.7)	85.7 (10.3)	30	42.8 (8.6)	22.8 (1.7)	51.4 (3.1)	29.3 (3.8)	103.4 (6.9)
rs1127354 ITPA	c>a	Wt	81	44.8 (5.4)	27.1 (1.6)	39.1 (2.2)	13.2 (2.1)*	79.3 (4.6)	211	43.3 (3.4)	23.1 (0.7)	50.5 (1.3)	30.4 (1.5)	103.9 (2.8)
		Het/Var	12	62.0 (13.0)	27.5 (3.8)	49.6 (5.6)	25.1 (4.9)*	102.2 (11.0)	36	40.7 (7.8)	20.6 (1.5)	48.7 (2.8)	27.6 (3.4)	96.9 (6.3)
rs17602729 AMPD1	c>t	Wt	66	46.7 (6.3)	28.2 (1.8)	41.6 (2.5)	15.6 (2.4)	85.4 (5.3)	198	41.8 (3.6)	21.9 (0.7)*	49.4 (1.3)	29.9 (1.6)	101.2 (2.9)
		Het/Var	17	54.2 (11.2)	27.3 (3.2)	40.5 (4.5)	14.2 (4.3)	82.0 (9.4)	42	48.4 (7.3)	25.7 (1.4)*	52.6 (2.6)	30.3 (3.2)	108.6 (5.9)
rs10106587 GGH	a>c	Wt	36	48.8 (8.3)	28.2 (2.4)	40.7 (3.4)	13.0 (3.2)	81.9 (7.0)	120	44.8 (4.4)	22.0 (0.9)	49.5 (1.6)	30.7 (1.9)	102.2 (3.5)§
		Het	42	52.1 (7.6)	28.4 (2.2)	41.4 (3.1)	15.7 (2.9)	85.5 (6.4)	106	43.2 (4.9)	23.6 (1.0)	51.3 (1.7)	29.1 (2.2)	104.0 (3.9)
		Var	5	27.2 (20.7)	25.1 (6.0)	43.6 (8.4)	22.7 (8.0)	91.3 (17.5)	14	24.1 (12.7)	20.4 (2.5)	44.3 (4.5)	29.9 (5.6)	94.6 (10.2)
rs3758149 GGH	c>t	Wt	44	45.2 (7.2)	27.4 (2.1)	42.3 (2.9)	17.8 (2.8)	87.5 (6.1)	112	42.2 (4.7)	22.2 (0.9)	48.6 (1.7)	29.6 (2.1)	100.5 (3.8)
		Het	32	47.9 (8.5)	28.0 (2.5)	38.8 (3.4)	11.9 (3.3)	78.6 (7.2)	113	42.3 (4.6)	23.2 (0.9)	50.5 (1.6)	29.5 (2.0)	103.1 (3.7)
		Var	7	75.7 (17.6)	32.8 (5.1)	48.0 (7.1)	13.5 (6.8)	94.3 (14.9)	15	52.3 (12.2)	21.5 (2.4)	54.7 (4.3)	34.9 (5.4)	111.1 (9.7)
rs3784862 ABCC1	a>g	Wt	38	46.8 (7.9)	25.9 (2.2)	42.1 (3.2)	17.8 (3.0)	85.8 (6.7)	133	47.0 (4.2)*	23.6 (0.8)	49.9 (1.5)	28.0 (1.9)	101.5 (3.4)
		Het	34	52.9 (8.0)	30.9 (2.3)	40.9 (3.3)	12.2 (3.1)	84.0 (6.8)	90	32.7 (5.1)*	21.1 (1.0)	49.9 (1.9)	33.4 (2.3)	104.4 (4.2)
		Var	11	35.0 (15.0)	24.6 (4.3)	39.6 (6.1)	17.4 (5.8)	81.6 (12.7)	17	63.6 (11.3)*	22.5 (2.3)	50.6 (4.1)	28.2 (5.0)	101.4 (9.2)
rs35592 ABCC1	t>c	Wt/Het	76	48.2 (5.8)	27.8 (1.7)	42.6 (2.3)*	16.1 (2.2)	86.5 (4.8)	229	43.3 (3.3)	22.7 (0.7)	50.0 (1.2)	30.2 (1.5)	102.9 (2.7)
		Var	7	49.9 (17.8)	30.1 (5.1)	25.9 (6.9)*	4.5 (6.7)	60.5 (14.6)	11	36.6 (14.5)	20.7 (2.9)	47.9 (5.2)	23.5 (6.4)	92.1 (11.6)
rs2274407 ABCC4	g>t	Wt	71	48.1 (5.9)	27.8 (1.7)	40.5 (2.4)	14.3 (2.3)	82.7 (4.9)	212	42.7 (3.5)	22.5 (0.7)	49.7 (1.2)	29.7 (1.5)	101.9 (2.8)
		Het/Var	12	49.9 (13.7)	29.5 (4.0)	46.8 (5.5)	21.4 (5.2)	97.7 (11.4)	28	45.8 (9.1)	23.0 (1.8)	52.3 (3.2)	32.5 (4.0)	107.7 (7.3)
rs868853 ABCC4	g>a	Wt/Het	65	48.4 (6.1)	27.5 (1.7)	41.1 (2.3)	15.3 (2.3)	83.9 (4.9)	205	45.6 (3.5)*	22.8 (0.7)	49.9 (1.3)	29.4 (1.5)	102.0 (2.8)
		Var	15	51.1 (13.0)	32.2 (3.7)	46.2 (5.0)	16.8 (5.0)	95.3 (10.6)	34	27.5 (8.2)*	21.7 (1.6)	50.9 (2.9)	34.5 (3.6)	107.0 (6.6)
rs1801394 MTRR	a>g	Wt	23	39.5 (9.9)	26.9 (2.9)	39.7 (4.0)	15.4 (3.9)	82.0 (8.4)	48	43.2 (7.0)	24.6 (1.4)	54.8 (2.5)	33.3 (3.1)	112.7 (5.6)
		Het	39	51.5 (7.7)	29.8 (2.2)	42.2 (3.1)	14.5 (3.0)	86.4 (6.5)	109	46.4 (4.7)	22.2 (0.9)	48.4 (1.7)	29.4 (2.1)	100.0 (3.7)
		Var	21	51.8 (10.4)	25.8 (3.0)	41.4 (4.2)	16.6 (4.0)	83.8 (8.8)	83	38.6 (5.3)	22.0 (1.0)	49.3 (1.9)	28.9 (2.3)	100.3 (4.2)
rs2139560 ABCC5	t>c	Wt	34	34.2 (7.7)**	24.3 (2.3)*	38.5 (3.3)	16.4 (3.2)	79.3 (7.0)	85	40.7 (5.2)	22.5 (1.0)	50.5 (1.8)	31.5 (2.3)	104.5 (4.1)
		Het	38	48.7 (7.1)**	28.7 (2.1)*	41.5 (3.1)	14.2 (3.0)	84.4 (6.4)	111	45.9 (4.7)	22.9 (0.9)	49.8 (1.7)	29.4 (2.1)	102.1 (3.8)
		Var	11	90.6 (13.0)**	36.6 (3.9)*	49.2 (5.6)	15.5 (5.5)	101.4 (11.7)	44	40.2 (7.5)	22.0 (1.5)	48.9 (2.7)	27.9 (3.3)	98.8 (6.0)

Supplementary table 2. (continued)

SNP	allele	genotype	n						n					
rs2372536 ATIC	c>g	Wt	37	37.0 (7.7)**	25.9 (2.4)	37.3 (3.2)	15.6 (3.2)	78.8 (6.8)	108	43.5 (4.7)	22.8 (0.9)	50.1 (1.7)	28.7 (2.1)	101.7 (3.7)
		Het	39	50.1 (7.2)**	29.5 (2.2)	43.1 (3.0)	14.3 (2.9)	86.9 (6.3)	98	42.3 (5.0)	23.0 (1.0)	51.0 (1.8)	32.3 (2.2)	106.3 (4.0)
		Var	7	94.0 (16.5)**	29.9 (5.0)	50.6 (50.6)	19.1 (6.8)	99.6 (14.6)	34	43.6 (8.2)	21.0 (1.6)	46.5 (2.9)	27.5 (3.6)	94.9 (6.5)
rs4451422 FPGS	t>g	Wt	61	55.8 (10.3)	31.6 (3.0)	52.3 (52.3)**	24.8 (3.8)**	108.7 (8.1)***	172	46.5 (5.8)	24.3 (1.2)	53.3 (2.1)*	34.3 (2.6)*	111.8 (4.6)*
		Het/Var	22	46.0 (6.3)	26.9 (1.8)	37.8 (2.4)**	12.2 (2.3)**	76.8 (4.9)***	68	41.6 (3.8)	21.9 (0.8)	48.6 (1.4)*	28.3 (1.7)*	98.8 (3.0)*
rs514933 FOLR2	t>c	Wt	25	41.6 (9.6)	26.1 (2.8)	41.4 (3.9)	16.2 (3.7)	83.7 (8.1)	90	46.8 (5.1)	22.3 (1.0)	48.1 (1.8)	27.1 (2.2)	97.6 (4.1)
		Het	46	53.9 (7.1)	29.3 (2.1)	40.0 (2.9)	13.3 (2.7)	82.6 (6.0)	113	39.3 (4.6)	21.8 (0.9)	50.9 (1.6)	32.2 (2.0)	104.9 (3.7)
		Var	12	39.0 (13.7)	26.6 (4.0)	47.1 (5.5)	21.5 (5.3)	95.1 (11.6)	37	44.8 (7.8)	25.8 (1.6)	51.6 (2.8)	30.2 (3.4)	107.7 (6.3)
rs5751876 ADORA2A	t>c	Wt	35	43.8 (8.1)	28.3 (2.3)	40.7 (3.3)	14.7 (3.1)	83.7 (6.8)	77	38.5 (5.5)	22.5 (1.1)	49.9 (2.0)	30.8 (2.4)	103.2 (4.5)
		Het	40	54.5 (7.7)	28.2 (2.2)	42.9 (3.1)	17.2 (2.9)	88.3 (6.4)	115	46.4 (4.6)	22.8 (0.9)	49.2 (1.6)	28.9 (2.0)	101.0 (3.7)
		Var	8	37.9 (16.4)	25.8 (4.8)	35.8 (6.6)	7.5 (6.3)	69.1 (13.8)	48	42.1 (6.9)	22.2 (1.4)	51.8 (2.5)	31.2 (3.0)	105.1 (5.5)
rs2032582 ABCB1	g>t/a	gg	25	40.8 (9.9)	26.1 (2.9)	39.7 (4.1)	12.5 (3.9)	78.3 (8.4)	72	39.2 (5.7)	22.8 (1.1)	51.1 (2.0)	29.8 (2.5)	103.7 (4.6)
		gt	38	58.8 (7.7)	30.3 (2.2)	41.9 (3.1)	14.1 (3.0)	86.3 (6.5)	112	42.4 (4.6)	21.7 (0.9)	48.2 (1.6)	30.4 (2.0)	100.3 (3.7)
		ga	1	24.4 (45.9)	16.4 (13.3)	33.2 (18.8)	9.8 (17.8)	59.3 (38.9)	5	26.2 (21.1)	20.6 (4.2)	52.1 (7.5)	32.6 (9.4)	105.2 (16.9)
		ta	4	30.4 (23.5)	21.3 (6.8)	30.3 (9.6)	10.3 (9.1)	62.0 (19.9)	6	25.6 (19.4)	20.7 (3.9)	51.5 (6.8)	28.3 (8.6)	100.5 (15.5)
		aa	0	n/a	n/a	n/a	n/a	n/a	44	54.4 (7.3)	24.8 (1.5)	51.4 (2.6)	29.0 (3.3)	105.2 (5.9)
		tt	15	38.2 (11.8)	26.7 (3.4)	44.2 (4.8)	22.2 (4.6)	93.2 (10.0)	1	47.6 (47.5)	36.5 (9.4)	93.6 (16.8)	42.8 (21.1)	172.9 (38.1)
rs1128503 ABCB1	c>t	Wt	27	41.0 (9.6)	25.7 (2.8)	41.2 (4.5)	12.2 (3.7)	76.8 (8.1)	75	39.9 (5.6)	22.6 (1.1)	51.9 (2.0)	30.9 (2.5)	105.5 (4.5)
		Het	39	56.5 (7.7)	30.6 (2.2)	41.2 (4.5)	14.7 (3.0)	88.1 (6.5)	118	40.2 (4.5)	21.7 (0.9)	48.2 (1.6)	29.8 (2.0)	99.7 (3.6)
		Var	17	40.7 (11.1)	25.5 (3.2)	41.2 (4.5)	19.9 (4.3)	86.6 (9.4)	47	55.2 (7.0)	24.9 (1.4)	51.5 (2.5)	28.9 (3.1)	105.2 (5.6)
rs1045642 ABCB1	t>c	Wt	14	37.1 (12.7)*	20.6 (3.5)**	31.7 (5.1)*	10.3 (5.0)	62.6 (10.3)**	52	38.6 (6.8)	22.3 (1.4)	50.5 (2.4)	29.3 (3.0)	102.1 (5.4)
		Het	41	37.9 (7.3)*	25.0 (2.0)**	38.4 (2.9)*	13.3 (2.9)	76.7 (6.0)**	122	43.9 (4.5)	22.6 (0.9)	49.2 (1.6)	30.5 (2.0)	102.2 (3.6)
		Var	28	66.0 (8.5)*	34.5 (2.3)**	48.5 (3.4)*	19.5 (3.3)	102.5 (6.9)**	66	44.6 (6.0)	22.9 (1.2)	50.9 (2.1)	29.6 (2.6)	103.4 (4.8)
rs1801131 MTHFR	a>c	Wt	44	45.4 (7.2)	26.4 (2.1)	38.8 (2.9)	15.2 (2.8)	80.5 (6.0)	111	36.1 (4.7)*	22.1 (0.9)	50.9 (1.7)	32.3 (2.1)	105.3 (3.8)
		Het	29	54.1 (9.4)	29.3 (2.7)	44.8 (3.8)	15.8 (3.6)	90.0 (7.9)	107	45.5 (4.6)*	22.4 (0.9)	48.6 (1.7)	28.4 (2.1)	99.5 (3.7)
		Var	10	47.7 (14.7)	32.0 (4.2)	44.2 (5.9)	13.9 (5.7)	90.1 (12.3)	22	63.5 (10.0)*	25.8 (2.0)	51.8 (3.6)	26.7 (4.5)	104.4 (8.1)
rs1801133 MTHFR	c>t	Wt	50	44.8 (7.1)	27.3 (2.0)	40.0 (2.9)	14.9 (2.8)	82.2 (5.9)	107	44.3 (4.8)	22.1 (1.0)	48.9 (1.7)	29.2 (2.1)	100.3 (3.8)
		Het	26	58.1 (9.1)	30.3 (2.6)	44.7 (3.7)	16.4 (3.6)	91.4 (7.7)	97	44.8 (4.9)	23.1 (1.0)	50.6 (1.7)	29.9 (2.2)	103.6 (3.9)
		Var	7	34.7 (17.4)	23.8 (5.0)	37.1 (7.0)	13.0 (6.8)	73.9 (14.6)	36	33.9 (8.0)	22.6 (1.6)	51.3 (2.9)	32.4 (3.5)	106.3 (6.4)
rs717620 ABCC2	g>a	Wt	58	51.2 (6.4)	28.1 (1.9)	41.9 (2.6)	16.0 (2.5)	86.0 (5.4)	174	39.9 (3.7)	22.4 (0.7)	49.5 (1.3)	29.7 (1.6)	101.6 (3.0)
		Het/Var	25	41.1 (9.7)	27.7 (2.8)	39.9 (3.9)	13.4 (3.8)	81.1 (8.2)	65	52.2 (6.1)	23.2 (1.2)	50.9 (2.2)	30.6 (2.7)	104.6 (4.9)

Chapter

6

Supplementary table 2. (continued)

SNP		Genotype	n						n					
rs4118396 ABCC2	t>c	Wt	29	54.0 (8.7)	29.1 (2.5)	41.8 (3.6)	15.3 (3.4)	86.1 (7.4)	104	44.3 (4.7)	22.5 (0.9)	48.1 (1.7)	27.8 (2.1)	98.5 (3.8)
		Het	47	42.0 (7.1)	27.2 (2.1)	41.2 (2.9)	15.8 (2.8)	84.1 (6.1)	109	40.8 (4.8)	22.7 (1.0)	50.2 (1.7)	32.2 (2.1)	106.9 (3.9)
		Var	7	70.0 (17.5)	29.4 (5.1)	40.4 (7.2)	10.6 (6.9)	80.4 (15.0)	27	46.1 (9.2)	22.7 (1.8)	49.6 (3.3)	30.5 (4.1)	102.7 (7.4)
Rs2231142 ABCG2	a>c	Wt/Het	69	44.0 (6.2)	26.8 (1.8	42.5 (2.5)	16.8 (2.4)	86.1 (5.3)	186	43.3 (3.8)	22.3 (0.7)	49.7 (1.3)	29.9 (1.7)	101.9 (3.0)
		Var	14	65.7 (12.1)	32.7 (3.5)	36.5 (4.9)	9.2 (4.7)	78.5 (10.3)	54	42.0 (6.4)	23.6 (1.3)	50.9 (2.3)	30.2 (2.8)	104.6 (5.2)
Rs13120400 ABCG2	c>t	Wt/Het	78	48.5 (5.8)	28.0 (1.7)	42.1 (2.3)	15.9 (2.2)	86.1 (4.8)	223	42.7 (3.4)	22.4 (0.7)	49.8 (1.2)	30.0 (1.5)	102.2 (2.7)
		Var	5	46.0 (20.8)	28.3 (6.0)	27.0 (8.2)	3.1 (7.9)	58.4 (17.2)	17	48.1 (11.8)	25.2 (2.3)	52.1 (4.2)	30.2 (5.2)	107.5 (9.4)
Rs 3785911 ABCC3	c>t	Wt	43	49.0 (7.4)	27.1 (2.1)	40.9 (3.0)	13.8 (2.9)	81.8 (6.2)	126	46.4 (44.4)	22.9 (0.9)	48.3 (1.6)	27.5 (1.9)**	98.7 (3.5)*
		Het	30	44.7 (8.8)	28.0 (2.5)	41.8 (3.6)	16.8 (3.4)	86.5 (7.4)	93	40.5 (5.0)	22.4 (1.0)	50.6 (1.8)	30.1 (2.2)**	103.0 (4.0)*
		Var	10	59.4 (15.2)	33.6 (4.4)	41.8 (6.2)	17.4 (5.9)	92.8 (12.8)	21	34.6 (10.3)	21.9 (2.1)	56.8 (3.7)	43.5 (4.5)**	122.2 (8.2)*
rs4793665 ABCC3	c>t	Wt	22	32.3 (10.1)	23.8 (2.9)	41.8 (4.2)	18.6 (4.0)	84.4 (8.7)	79	44.2 (5.4)*	23.4 (1.1)	49.3 (1.9)	29.1 (2.4)	101.8 (4.4)
		Het	41	58.7 (7.8)	28.9 (2.3)	40.4 (3.2)	14.2 (3.1)	83.4 (6.7)	119	37.1 (4.4)*	21.8 (0.9)	49.3 (1.6)	30.0 (2.0)	101.1 (3.6)
		Var	20	46.4 (10.0)	30.3 (2.9)	42.3 (4.1)	14.0 (3.9)	86.6 (8.6)	42	58.6 (7.4)*	23.2 (1.5)	53.5 (2.7)	31.6 (3.3)	108.2 (6.0)
rs1051266 SLC19A1	g>a	Wt/Het	67	51.5 (6.1)	28.7 (1.8)	42.6 (2.4)	15.5 (2.4)	86.8 (5.1)	203	44.5 (3.5)	23.0 (0.7)	49.7 (1.3)	28.9 (1.6)	101.6 (2.8)
		Var	16	34.8 (11.7)	25.0 (3.4)	35.7 (4.7)	14.3 (4.5)	75.0 (9.8)	37	34.9 (7.8)	20.5 (1.6)	51.5 (2.8)	35.8 (3.4)	107.9 (6.3)
rs2239907 SLC46A1	a>g	Wt	26	46.9 (9.0)	26.9 (2.5)*	41.4 (3.6)	15.9 (3.5)	84.2 (7.6)	73	37.6 (5.6)*	23.8 (1.1)	51.2 (2.0)	30.7 (2.5)	105.7 (4.6)
		Het	4	46.4 (7.7)	26.1 (2.1)*	39.7 (3.1)	15.7 (3.0)	81.6 (6.5)	119	39.0 (4.4)*	21.2 (0.9)	48.9 (1.6)	30.5 (2.0)	100.6 (3.6)
		Var	10	59.7 (14.6)	38.0 (4.0)*	47.1 (5.9)	11.8 (5.6)	96.9 (12.2)	48	60.9 (6.8)*	24.2 (1.4)	50.7 (2.5)	27.6 (3.0)	102.5 (5.5)

* p<0.05
** p<0.01
*** p<0.001

Analysis was done using ANCOVA with correction for age and gender. WT = wildtype, Het = heterozygous, Var = homozygous variant. Rs = reference SNP number. ABCB1/ABCC1/ABCC2/ABCC3/ABCC4/ABCC5/ABCG2: adenosine triphosphate-binding cassette transporter subfamily B/C/G member 1/2/3/4/; FPGS: folylpolyglutamate synthetase; FOLR1/FOLR2: folate receptor 1/2; GGH: gamma glutamyl hydrolase; SLC 46A1/SLC19A:solute carrier 46A1/19A1.

Chapter

6

Chapter 7

Effect of two dosing regimens on intracellular MTX polyglutamate accumulation in rheumatoid arthritis patients

E. den Boer
M.C.F.J. de Rotte
B.C.P. Koch
J.M.W. Hazes
R. de Jonge

ABSTRACT

BACKGROUND: Low-dose methotrexate (MTX) is the gold standard in the treatment of rheumatoid arthritis (RA). Response to MTX is very variable and is related to the intracellular MTX-polyglutamate (MTX-PG) levels. Similar to response, the intracellular MTX-PG concentrations are very variable. There is little information on the effect of different MTX dosing schemes on the speed of accumulation and concentration and intracellular distribution of intracellular MTX-PGs.

OBJECTIVES: The aim of this study was to measure the speed of erythrocyte MTX-PG accumulation as well as the concentration and distribution of MTX-PG species in RA patients on two different MTX dosing regimens.

METHODS: Erythrocyte MTX-PG concentrations were prospectively measured at 3, 6 and 9 months of treatment. Adult RA patients from two longitudinal cohorts were included. Patients in the MTX-R received 15 mg/week MTX, whereas patients from the tREACH received 25 mg/week MTX. Patients were included if they were treated with MTX during the whole observation period and had at least one MTX-PG measurement. A recently developed LC-MS/MS assay was used for the measurement of the separate MTX-PG levels.

RESULTS: The largest part of the accumulation of the MTX-PGs occurred during the first 3 months of treatment with only marginal increase in the months thereafter. Steady state levels for all MTX-PGs were reached after 6 months of treatment in both cohorts. Patients from the MTX-R needed a longer time to accumulate similar levels of MTX-PGs than patients from the tREACH and after 9 months of treatment the MTX-R group still had lower levels of the long-chain MTX-PGs. Strikingly, during the early phase of treatment, patients from the MTX-R had higher levels of the short-chain MTX-PGs (p=0.02), but lower levels of long-chain MTX-PGs (p=0.006). In the MTX-R cohort, the distribution of MTX-PGs was biased towards short-chain MX-PGs whereas in the tREACH cohort the distribution was biased towards long-chain MTX-PGs even though the total MTX-PG levels were not different.

CONCLUSIONS Higher treatment dose of MTX led to a faster accumulation and higher concentrations of long-chain MTX-PGs resulting in a selective distribution towards long-chain MTX-PGs. As the long-chain MTX-PGs are considered to be the more active MTX-species this study suggests that more intensive treatment may be more efficient. This is supported by a shift towards higher treatment doses and extended co-medication over time in the MTX-R, whereas in the tREACH medication stayed more or less stable. In contrast to previous findings, the main bulk of MTX-PG accumulation takes place during the first three months of treatment and steady states are reached at six months of treatment.

INTRODUCTION

Because of its high efficacy and good safety profile the folate antagonist methotrexate (MTX) is the medicine of choice for the treatment of multiple diseases. High-dose MTX treatment is used for proliferative diseases such as acute lymphoblastic leukemia, whereas low-dose methotrexate is used for arthritic diseases such as rheumatoid arthritis (RA). In low-dose treatment, MTX is eliminated from the plasma within 24 hours and plasma MTX is unrelated to response.[1] Therefore, plasma MTX is not a reliable tool for the monitoring of treatment in low-dose MTX treatment. However, MTX polyglutamates accumulate intracellularly over time and these are more stable than plasma MTX, making intracellular MTX an interesting target for therapeutic drug monitoring (TDM).[2-7]

After MTX is transported into the cell, it is rapidly converted to MTX polyglutames (MTX-PGs) by the sequential addition of glutamic acid residues. In a competing reaction, the MTX-PGs are deconjugated, leading to a variety of chain-lengths (MTX-PG2-7), the concentration and distribution of which varies widely per patient and is influenced by multiple factors such as dose, treatment duration and genetics.[2, 5, 6, 8-12] In low-dose MTX treatment, the pentaglutamate (MTX-PG5) is the highest order of glutamylation detected, while the triglutamate form (MTX-PG3) of MTX predominates.[8-10, 12, 13] Polyglutamylation retains MTX in the cell because the MTX-PGs are a poor substrate for the MTX efflux proteins.

The time to reach steady-state intracellular MTX levels is highly variable between patients and increases with the number of PGs attached to MTX.[9, 11, 14] In low dose MTX, the median time to reach steady-state erythrocyte-MTX levels is highly variable between patients and increases with the number of PGs attached to MTX.[9, 14] While the short-chain MTX-PGs quickly reach steady state levels, the time to steady state for the medium and long-chain MTX-PGs ranges from 3 months to multiple years.[9] Steady-state levels also are highly variable between patients and can differ up to four-fold.[3, 6, 9, 10, 15] The mechanisms behind the highly variable intracellular MTX-PG levels and accumulation have still not been completely elucidated. Previous research has shown that increased age, higher treatment dose, route of administration and decreased renal function are associated with higher MTX-PG levels, as well as multiple single nucleotide polymorphisms (SNP) in MTX pathway genes.[7, 8, 12, 14, 16]

The erythrocyte MTX-PGs have been shown to correlate with response in multiple studies. In fact, the efficacy of MTX is thought to increase with the elongation of the polyglutamate chain, confirming their potential use in TDM.[2, 11, 17, 18] In a cross-sectional study in RA patients, a cut-off value for response of >60 nmol/L MTX-PG3 has been proposed,[2] and in a recent prospective study in two RA cohorts, we determined a cut-off value for response of >74 nmol/L total MTX-PG.[17]

Although erythrocyte MTX-PG levels have been shown to be a valid target for TDM in RA patients, little is known about the effect of different dosing regimens on the accumulation and previous studies are either cross-sectional[6, 8, 19] or consist of small

Chapter

7

sample groups.[9, 14] The use of these cut-off values would be greatly increased if it is clear how to reach them.

In this study, we aim to prospectively measure the speed of erythrocyte MTX-PG accumulation as well as the concentration and distribution of MTX-PG species over the course of treatment in RA patients on two different MTX dosing regimens.

METHODS

Patients

This study includes data of RA patients treated with MTX from two prospective cohorts:

1) the methotrexate in Rotterdam, Netherlands cohort (MTX-R), a longitudinal prospective cohort of patients diagnosed with RA who started MTX between January 2006 and March 2011 in the department of Rheumatology, University Medical Center Rotterdam (Erasmus MC), Netherlands. 2) the treatment in Rotterdam Early Arthritis Cohort tREACH), a clinical multicentre, stratified single-blinded trial (ISRCTN26791028) and was described earlier.[20] Patients were included in the tREACH if they met the 2010 ACR/EULAR criteria for RA.[21]

The medical ethics committee of the Erasmus MC approved both studies and patients gave written informed consent before inclusion. For both cohorts, an extra EDTA tube was drawn from the patient at 3, 6 and 9 months of treatment. This sample was immediately put on ice after collection and centrifuged for 10 min at 1700g and 4 °C. Plasma and erythrocyte cell-pellet aliquots were stored at -80 °C.

Patients from the MTX-R and tREACH cohorts were included in this study if they were prescribed MTX at baseline, 3 and 6 months of treatment, and had at least one MTX-PG measurement at either 3, 6 or 9 months of treatment. Patients were excluded if they were prescribed parenteral MTX, had MTX-PG levels lower than 15 nmol/L erythrocytes, which is our threshold for compliance to treatment. Also, we chose to include only patients whose blood had been drawn within 4 weeks of the time-points. All patients were MTX naïve at inclusion and were newly diagnosed with RA according to the 2010 classification criteria.

MTX dosage

In the MTX-R cohort, dosage and co-medication were decided by the physician at each visit. Patients from the tREACH cohort started with 17.5 mg/week MTX per os, which was escalated to 25 mg/week in 3 weeks. Patients in the tREACH were randomized to treatment with or without sulfasalazine (2 g/day), hydroxychloroquine (400 mg/day) and intramuscular (methylprednisolone 120 mg or triamcinolone 80 mg) or oral (tapering scheme; week 1-4: 15 mg/day, week 5-6: 10 mg/day, week 7-8 5 mg/day, week 9-10: 2.5 mg/day) glucocorticosteroids,[20] which were In both cohorts, patients received folic acid (10 mg/week) during MTX treatment. All patients were assessed at baseline, after 3 months, 6 months and 9 months of treatment.

MTX-PG quantification

MTX-PGs were measured in the cell-pellet aliquots using a recently developed LC-MS/MS method.[22] MTX-PG1 and MTX-PG2 are considered as short-chain MTX-PGs; MTX-PG3 is considered as medium-chain MTX-PG; MTX-PG4 and MTX-PG5 are considered as long-chain MTX-PGs.

Statistics

Comparison of patient characteristics between cohorts was made by Student t-test, X2 test or the Mann-Withney u-test where appropriate. Statistical analyses were done with SPSS PASW 20.0.0.1 for Windows (SPSS Inc., Chicago, IL, USA) unless stated otherwise. P-values less than 0.05 were considered significant. Cubic splines were made in R version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Patient characteristics

Out of all patients, there were 98, 81 and 73 patients where MTX-PG levels had been measured in the MTX-R at t3, t6 and t9 respectively. In the tREACH, MTX-PG levels had been measured in 270, 232, 222 patients at t3, t6 and t9 respectively. Of these 76, 58 and 34 patients of the MTX-R could be included in the study at t3, t6 and t9, respectively, and 222, 133 and 99 patients of the tREACH could be included in the study at t3, t6 and t9, respectively.

The remaining patients were excluded because patients did not use MTX at all the time points prior to sampling, did not have an MTX-PG measurement, or patients did not have MTX-PG levels above our threshold for compliance (15 nmol/L total MTX-PG). Patients receiving parenteral MTX were also excluded.

At baseline, both cohorts were very similar (Table 1). In the MTX-R, disease activity was slightly lower (DAS: 4.2 vs 4.7; p<0.01), and less patients were positive for rheumatoid factor (38.7% vs 62.5%, p<0.001) and anti-CCP (40.0% vs 67.3%; p<0.001). Also, patients in the MTX-R had slightly higher eGFR-MDRD (88.4 ml/min/1.73m^2 vs 81.2 ml/min/1.73m^2; p<0.05) and erythrocyte folate content (1041.0 nmol/L vs 892.4 nmol/L; p<0.001) than the tREACH cohort.

MTX dose

Treatment dose of MTX was significantly different between both cohorts (p<0.001, Table 2) at baseline. Patients in the MTX-R were treated with median 15 mg/wk (range 10-25 mg/week) and patients in the tREACH were treated with median 25 mg/wk (range 15-25 mg/week) as per study protocol. Also, in the MTX-R, a smaller percentage of patients used hydroxychloroquine (45.6% vs 58.5%; p<0.05), sulfasalazine (36.7% vs 58.5%; p<0.001) and corticosteroids (12.2% vs 89.5%; p<0.001).

Chapter

7

Table 1. Baseline characteristics of MTX-R and tREACH cohorts.

		MTX-R		tREACH
	N		N	
Patient demographics				
Age, years, mean (SD)	82	50.7 (16.3)	232	52.7 (14.0)
Female (%)	82	72.0	232	66.8
BSA, m3, mean (SD)	n/a		229	1.9 (0.3)
DAS28esr, mean (SD)**	75	4.2 (1.3)	232	4.7 (1.3)
Rheumatoid factor positive (%)****	75	38.7	208	62.5
Anti-CCP positive (%)***	75	40.0	208	67.3
Days of treatment at study visit, mean (SD)[t]	80	91.6 (12.2)	219	93.3 (8.5)
Medication				
Methotrexate Dose***	82		232	
10mg/wk (%)		4.9		0.0
15mg/wk (%)		93.9		0.4
25mg/wk (%)		1.2		99.6
Other DMARD use (%)	79	53.2	229	58.5
Hydroxychloroquine use (%)*	79	45.6	229	58.5
Sulfasalazine use (%)***	79	36.7	229	58.5
Biological use (%)t	79	1.3	229	0.0
Corticosteroid use (%)***	79	12.7	229	89.5
Corticosteroid route of administration (%)***	79			
No Corticosteroid		87.3	229	10.5
IM		2.4		27.9
Oral		10.1		61.6
Laboratory parameters				
C- reactive protein, mg/L, median (IQR)t	82	7.0 (3.0-13.0)	231	8.0 (4.0-19.0)
Erythrocyte sedimentation rate, mm/hr, mean (SD)	82	23.5 (19.5)	232	27.7 (20.6)
Albumin, (g/L), mean (SD)	78	44.0 (3.6)	213	43.7 (3.2)
Creatinine, mmol/L, mean (SD)[t]	82	70.7 (16.7)	96	75.6 (17.0)
Erythrocyte folate, nmol/L, median (IQR)***	78	1041.0 (837.5-1288.3)	207	892.4 (666.2-1157.1)
eGFR-MDRD, ml/min/1.73m^2, mean (SD)*	82	88.4 (24.2)	96	81.2 (17.9)
Lifestyle parameters				
Alcohol consumption, drinks/month, median (IQR)	37	4.0 (0.0-32.0)	219	10.0 (2.0-32.0)
Cola consumption, drinks/month, median (IQR)	76	0.0 (0.0-24.0)	217	2.0 (0.0-8.0)
Coffee consumption, drinks/month, median (IQR)	76	96.0 (40.0-168.0)	217	112.0 (40.0-140.0)
Tea consumption, drinks/month, median (IQR)	79	56.0 (0.5-56.0)	215	24.0 (0.0-56.0)

*p<0.05; **p<0.01; ***p<0.001; [t]p<0.1

At t3, patients in the MTX-R study received a lower dose of MTX (median: 15, range 5-25 mg/week vs 25, range 12.5-25 mg/week p<0.001) and a smaller percentage of patients used biologicals (0% vs 19.5%; p<0.001).

At t6, patients in the MTX-R study were treated with a lower dose of MTX (median 20 range 10-25 mg/week vs median 25 range 2.5-25 mg/week, p<0.001) and biological use was lower in the MTX-R group (2.9% vs 28.6%; p<0.001).

Over the course of treatment, patients in the MTX-R were switched to higher dosages of MTX, with approximately 30% of patients receiving more than 15 mg/week from t3 and approximately 60% receiving more than 15 mg/week MTX from t6. Co-medication was increased over time for MTX-R patients as was the percentage of patients that received intramuscular MTX (Table 5).

In the tREACH cohort, medication was slowly decreased over time, with patients receiving 25 mg/week decreasing from approximately 100% at t0 to approximately 90% at t3 and approximately 80% at t6. Co-medication was also decreased over time in the tREACH (Table 2), except for biological, which increased to up to 28.6% over the course of treatment.

Table 2. Medication over time in both cohorts.

	MTX-R			tREACH		
	t0	t3	t6	t0	t3	t6
	N=82	N=65	N=34	N=222	N=175	N=110
Methotrexate Dose (mg/wk, %)	***	***	***			
2.5						1.0
5		1.6				
7.5		1.6				1.0
10	4.9	8.2	14.7	0.4	0.7	
12.5					0.7	4.0
15	93.9	59.0	29.4		4.4	7.1
17.5					2.2	5.1
20		8.2	14.7		2.2	3.0
22.5						2.0
25	1.2	21.3	41.2	99.6	89.7	76.8
Other DMARD use (%)	53.2	62.3	67.6	58.5	57.1	54.1
Hydroxychloroquine use (%)	45.6*	54.1	58.8	58.5	57.1	51.0
Sulfasalazine use (%)	36.7***	41.0	41.2	58.5	51.1	46.9
Biological use (%)	1.3	0***	2.9**	0.0	19.5	28.6
Corticosteroid use (%)	12.7***	4.9	5.9	89.5	6.0	7.1
Corticosteroid route of administration (%)						
No Corticosteroid	87.3***	95.1	94.1	10.5	94.0	92.9
Intramuscular	2.5	0.0	0.0	27.9	2.3	4.1
Oral	10.1	4.9	5.9	61.6	3.8	3.1

* p<0.05; ** p<0.01; *** p<0.001

Chapter 7

Disease activity

Disease activity was measured using the DAS28esr and is presented in table 3 and Figure 1. Patients in the MTX-R started treatment with significantly lower DAS28esr than patients in the tREACH (p=0.002). However, at the other time-points the DAS28esr did not differ. In the MTX-R, the DAS28esr lowered significantly between t0 and t3 (p<0.001), but not between t3 and t6 or t6 and t9, although a trend towards lower DAS28esr was observed from t6 to t9 (p=0.063). In the tREACH the DAS28esr lowered from t0 to t3 (p<0.001) and from t6 to t9 (p=0.002). From t3 to t6 the decrease in DAS28 was almost significant (p=0.050).

The change in DAS28esr (deltaDAS28esr), relative to t0, was less pronounced in the MTX-R and differed significantly between both cohorts at all time-points (p<0.005). In both individual cohorts, the change in DAS28esr was largest from t0 to t3. In the MTX-R, the change in DAS28esr was significant from t0 to t3 (p<0.001) and from t6 to t9 (p=0.046). In the tREACH, the change in the DAS28esr was significant from t0 to t3 (p<0.001) and from t6 to t9 (p=0.002).

Table 3. Disease activity during treatment

	t0	t3	t6	t9
MTX-R	n=75	n=76	n=58	n=33
DAS28esr (median [IQR])	4.15** [3.07-5.09]	2.93# [1.98-3.85]	2.59 [1.84-3.69]	2.64¥ [1.69-3.35]
deltaDAS28esr (median [IQR])		-1.03# [-1.75 -0.67]	-1.09 [-2.14 -0.02]	-1.26# [-2.69 -0.18]
tREACH	n=232	n=222	n=133	n=99
DAS28esr (median [IQR])	4.67 [3.75-5.49]	2.81# [2.15-3.89]	2.79 [1.98-3.54]	2.20# [1.50-3.25]
deltaDAS28esr (median [IQR])		-1.66**# [-2.45 -0.74]	-1.84**¥ [-2.76 -1.02]	-2.29**# [-3.34 -1.2]

DAS, disease activity score; IQR, inter quartile range; deltaDAS28esr, change in DAS28esr relative to t0
* p<0.05, ** p<0.01, *** p<0.001, between cohorts
# p<0.05, ¥ p<0.1, compared to previous time-point

Erythrocyte MTX-PG levels and differences between cohorts

The erythrocyte MTX-PG concentrations in both cohorts are presented in Table 4 and Figure 1. When looking at the difference in MTX-PG levels between both cohorts, patients in the MTX-R cohort accumulated higher levels of short-chain MTX-PG1+2 at t3 (p=0.033), t6 (p=0.020) and t9 (p=0.012). However, medium-chain MTX-PG3 was lower in the MTX-R at t3 (p=0.001), but not at t6 (p=0.056) or t9 (p=0.399). Long-chain MTX-PG4+5 levels were lower in the MTX-R at t3 (p=0.000) and t6 (p=0.006) but not at t9 (p=0.303). The total MTX-PG1-5 levels did not differ significantly between both cohorts.

Accumulation in each cohort during treatment

In the MTX-R cohort, medium-chain MTX-PG3 concentration increased from t3 to t6 (p<0.001), but not from t6 to t9 (p=0.256). Similarly, long-chain MTX-PG4+5 concentrations increased over time from t3 to t6 (p<0.001), but not from t6 to t9 (p=0.248). Total MTX-PG1-5 levels also increased from t3 and t6 (p=0.010), but not from t6 to t9 (p=0.153).

In the tREACH, the medium-chain MTX-PG3 levels increased significantly from t3 to t6 (p<0.001), but not from t6 to t9 (p=0.051). Long-chain MTX-PG4+5 levels increased significantly from t3 to t6 (p=0.031) but not from t6 to t9 (p=0.263). The total MTX-PG1-5 levels increased significantly between t3 and t6 (p=0.012), but not between t6 and t9 (p=0.390). The short-chain MTX-PG1+2 levels did not differ significantly between time-points in either cohort.

Table 4. Erythrocyte MTX-PG concentrations and distribution in patients using oral methotrexate

	MTX-R			tREACH		
	Concentration nmol/L [IQR]			Concentration nmol/L [IQR]		
	t3	**t6**	**t9**	**t3**	**t6**	**t9**
	N=76	N=58	N=34	N=222	N=133	N=99
Short-chain MTX-PG1+2	61.4 [42.1-96.3]*	68.8 [47.9-99.4]*	71.1 [51.3-124.2]**	51.7 [35.9-75.1]	53.8 [38.9-78.0]	56.7 [39.7-85.3]
Intermediate MTX-PG3	40.6 [24.6-53.7] ***¥	50.2 [36.3-65.8]	56.4 [36.4-81.6]	48.8 [36.4-61.5] ¥	58.1 [46.7-70.7]#	66.2 [45.9-77.5]
Long-chain MTX-PG4+5	8.7 [4.4-21.8]*** ¥	18.1 [6.5-34.0]**	25.3 [14.1-45.3]	23.9 [13.2-38.3] ¥	26.1 [14.8-44.5]	31.7 [16.3-48.6]
Total MTX	118.4 [79.1-158.7] ¥	153.2 [110.6-196.0]	169.8 [127.7-217.4]	132.8 [98.5-168.1] ¥	147.2 [113.5-194.6]	159.0 [119.4-225.0]
	Percentage of total MTX-PG [IQR]			Percentage of total MTX-PG [IQR]		
Short-chain MTX-PG1+2	57 [43-68]*** ¥	53 [37-63]**	45 [37-59]**	42 [31-53] ¥	40 [30-50]	39 [28-48]
Intermediate MTX-PG3	33 [27-40]*** ¥	36 [29-41]**	35 [24-44]	38 [33-42] ¥	40 [35-43]	40 [35-44]
Long-chain MTX-PG4+5	8 [5-16]*** ¥	13 [6-22]**	16 [9-23]*	18 [12-28]	19 [12-26]	20 [13-27]

* p<0.05; ** p<0.01; *** p<0.001

Distribution of MTX-PGs

Because it is the long-chain MTX-PGs that are thought to mediate the effect of MTX, we were interested in the distribution of the MTX-PG in the cell and the effect of treatment dose and treatment duration on the distribution of erythrocyte MTX over time (Table 4). Patients in the MTX-R had a larger percentage of short-chain MTX-PG1+2 at t3 (p<0.001), t6 (p=0.001) and t9 (p=0.008). However, patients in the MTX-R had a smaller percentage of the medium-chain MTX-PG3 at t3 (p<0.001) and t6 (p=0.004). The percentage of long-chain MTX-PG4+5 was also significantly lower in the MTX-R at t3 (p<0.001), t6 (p=0.001) and t9

Chapter

7

(p=0.034). In the MTX-R, the distribution of all MTX-PGs changed significantly from t3 to t6 (p<0.001) with the percentage of short-chain MTX-PG1+2 decreasing and the percentage of medium-chain MTX-PG3 and long-chain MTX-PG4+5 increasing. Between t6 and t9, no change in intracellular MTX-PG distribution was observed.

In the tREACH, the percentage of short-chain MTX-PG1+2 decreased from t3 to t6 (p<0.001) and the percentage of medium-chain MTX-PG3 increased from t3 to t6 (p<0.001). From t6 to t9 no changes in intracellular MTX-PG distribution were observed. The percentage of long-chain MTX-PG4+5 did not change during treatment (Table 4).

Parenteral administration of MTX

Table 5. Erythrocyte MTX-PG concentrations and distribution in patients using parenteral methotrexate

	MTX-R			tREACH		
	Concentration nmol/L [IQR]			Concentration nmol/L [IQR]		
	t3	t6	t9	t3	t6	t9
	N=6	N=7	N=7	N=0	N=14	N=12
Short-chain MTX-PG1+2	44.1 [33.2-130.4]	56.6 [40.0-119.7]	62.5 [32.5-131.9]		48.7 [23.2-63.7]	50.7 [38.2-61.9]
Intermediate MTX-PG3	43.9 [26.1-56.6]	84.5 [63.1-99.6]	77.3 [51.2-96.4]		64.6 [38.3-81.0]	71.7 [63.5-82.5]
Long-chain MTX-PG4+5	15.3 [7.8-22.5]	42.0 [39.0-74.8]	31.9 [6.0-59.5]		40.5 [13.8-60.5]	48.1 [23.6-59.7]
Total MTX	98.7 [75.8-214.3]	196.6 [150.9-251.7]	180.9 [119.9-299.9]		149.1 [95.3-205.6]	164.3 [137.6-203.3]
	Percentage of total MTX-PG [IQR]			Percentage of total MTX-PG [IQR]		
Short-chain MTX-PG1+2	49 [43-72]	29 [23-48]	44 [29-58]		31 [24-36]	25 [23-42]
Intermediate MTX-PG3	36 [27-44]	43 [36-46]	41 [31-48]		41 [38-49]	43 [36-47]
Long-chain MTX-PG4+5	12 [4-17]	22 [17-35]	20 [5-25]		27 [19-32]	31 [18-34]

A small amount of people in our cohorts received parenteral instead of orally administrated MTX. In the MTX-R there were 6 patients at baseline, 7 patients at t3 and 7 patients at t6 that received parental MTX. In the tREACH, no patients received parenteral MTX at baseline, 14 patients at t3 and 12 patients at t6 received parenteral MTX. In the MTX-R, the patients that received parenteral MTX did not have different MTX-PG levels at t3 from patients that received oral MTX. At t6, patients with parenteral administered MTX had significantly higher levels of medium-chain MTX-PG3 (p=0.002) and long-chain MTX-PG4 (p=0.003). At t9, no differences in MTX-PG levels were observed. (table 5). In the tREACH, no significant differences in MTX-PG levels were found between patients receiving parenteral or oral MTX.
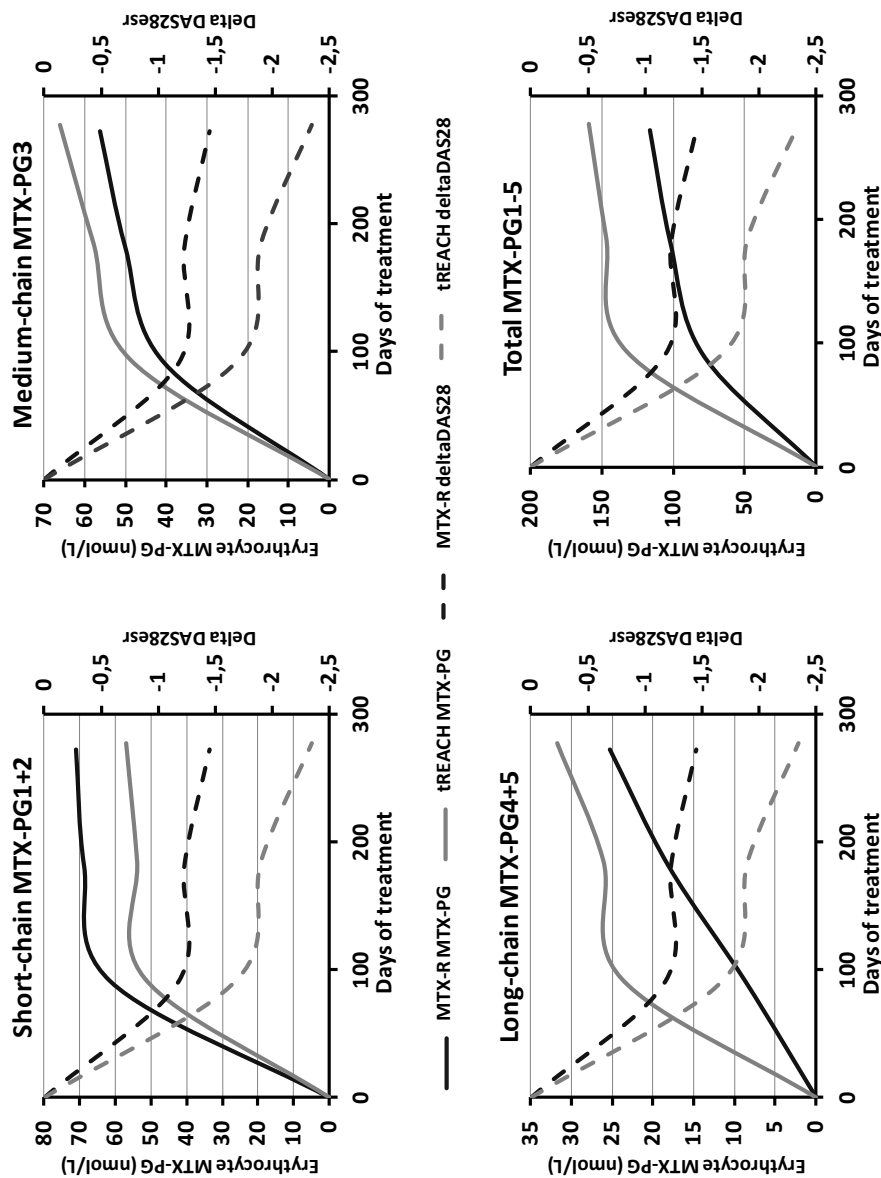
Figure 1. Concentration of MTX-PGs over the course of treatment versus the change in disease activity during treatment (deltaDAS28esr). Although an upwards trend is visible in the figures, there is no significant difference between t6 and t6 for any of the MTX-PGs. deltaDAS28esr was calculated by subtracting the DAS28esr at later time-point from the DAS28esr at t0.

No difference in MTX-PG levels or distribution of MTX-PG species was observed between patients receiving parenteral MTX from each cohort. However, within the MTX-R the fraction of long-chain MTX-PG4+5 was higher at t6 (p=0.019) in patients that received parenteral MTX.

In the tREACH, despite no differences in MTX levels between administration routes, short-chain MTX-PG1+2 accounted for a lower percentage of the total MTX-PG content in patients that received parenteral MTX at t6 (p=0.014) and t9 (p=0.019). At t9, the percentage of long-chain MTX-PG4+5 was significantly higher in patients receiving parenteral MTX (p=0.034).

DISCUSSION

This report shows the build-up of MTX-PG in erythrocytes over the first 9 months of treatment. To our knowledge we are the first to show similar data in two separate prospectively collected cohorts treated with MTX. As both cohorts have been treated with different dosages of MTX this study enabled us to determine the dosage dependent differences in MTX build-up.

We show that the main bulk of MTX-PG accumulation happens during the first 3 months of treatment with only marginal increases in the months thereafter, regardless of the dose of treatment in our cohorts or the extend of polyglutamylation. Despite the fact that patients in the MTX-R needed more time to reach steady-state for the long-chain MTX-PGs, in both cohorts the predominant concentration gain was during the first 3 months of treatment and from 6 months of treatment the median MTX-PG levels were essentially stable. Other studies have found that while MTX-PG1 and MTX-PG2 reached steady state early in treatment (<12 weeks), the other MTX species took significantly longer (>20 weeks). This discrepancy might be due to the dosages of MTX that were given in the different studies. These studies were done with dosages around 10 mg/week and reported significantly longer times to reach steady state.[9, 14] In our study, the cohort treated with a lower dose of MTX (MTX-R, mean 15 mg/week) also needed a slightly longer treatment period to reach steady state. A large part of the patients in this cohort received an increased MTX dosage during treatment, possibly shortening the time needed to reach steady state, leading to the big discrepancy in results.

We have previously shown that intensive treatment of RA leads to quicker and better decline of disease activity without extra risk of adverse events.[17, 23] We also showed that erythrocyte MTX-PG levels are correlated with response to treatment and that the erythrocyte MTX-PG levels can predict how well patients respond to treatment. [17, 18] As the higher treatment dose of MTX leads to the faster accumulation of MTX-PGs in erythrocytes and to a selective redistribution towards longer chain (and therefore longer intracellular retention) MTX-PGs, patients might benefit from receiving more intensive treatment in order to reach higher intracellular levels of MTX-PGs earlier in treatment. Interestingly, the biggest change in disease activity happens during the first three months of treatment, coinciding with the largest increase of MTX-PGs. Also, the patient group that

received the highest dose of MTX (tREACH) and accumulated the long-chain MTX-PGs fastest showed a faster improvement in disease activity than the patient group that received the lower dose of MTX (Figure 1).

Considering that the main bulk of the accumulation of MTX-PGs happens during the first three months of treatment, the optimal point of MTX prediction might lie within these first three months, when accumulation is more likely to be in a linear phase.

Compliance can be a big problem in chronic diseases and poses a large risk when measuring drug levels. We tried to prevent including non/very bad-compliers in our study population with by using a cut-off value for compliance of 15 nmol/L total MTX at each time-point. We will probably still have an amount of bad/partial compliers in our study, which could be represented by patients with high levels of short-chain MTX-PG1 but little to no levels of the longer chain MTX-PGs. We did not have a validated measure for compliance and can therefore only speculate on the degree of compliance of the patients in our study. We acknowledge that this potential weakness could be influencing our results, and we hope that future studies will be able to include a measurement of compliance in order to realize a 'true' picture of drug exposure. Considering that dosage of MTX, and therefore exposure to MTX is an important determinant of the erythroctye MTX-PG levels, compliance could be the most important factor in exposure, next to dosage.

Another important factor of exposure is the route of administration. A small percentage of the patients of our cohorts received parenteral administered MTX due to adverse events or because response was judged to be too low. In line with previous studies on the effect of parenteral administered MTX,[6, 14, 19] we found that parenteral MTX led to higher levels of MTX-PGs. Interestingly parenteral MTX users had lower amounts of short chain MTX, and there was no difference in MTX-PG levels between the parenteral MTX users of both cohorts, despite the difference in administered dose.

We found that the distribution of MTX-PGs was also strongly dependent on dose and route of administration. There was a selective redistribution towards the (more active) long-chain MTX-PGs in patients that were treated with higher dosages of MTX or when parenteral administration was used. Even patients that switched to parenteral treatment during treatment displayed this difference in distribution of MTX-PGs. These patients also reached higher concentrations of MTX-PGs early in treatment, suggesting that treatment with higher dosage MTX is more effective at treating RA. This hypothesis is supported by the fact that almost half of the MTX-R patients (the cohort that left treatment to the decision of the doctor) were started on 20-25 mg/week MTX by 6 months of treatment, whereas they started with 15 mg/week (Table 2).

The accumulation of long-chain MTX-PGs took longer in low-dose treated and orally treated patients and short-chain MTX-PGs were higher in these patients. Although the total MTX-PG concentrations did not differ between cohorts after six months of treatment, the distribution of the MTX species remained different in both cohorts over nine months of treatment. This may be caused by changed expression of the MTX metabolism in response

Chapter

7

to MTX exposure.[24]

In conclusion, we found that dosage and route of administration significantly alter the speed of accumulation and the distribution of MTX species in the cell. From a metabolite perspective, this finding favours the use of higher dosages of MTX or parentally administered MTX in the treatment of RA.

Chapter

7

References

1   Bannwarth B, Pehourcq F, Schaeverbeke T, Dehais J. Clinical pharmacokinetics of low-dose pulse methotrexate in rheumatoid arthritis. Clin Pharmacokinet. 1996 Mar;30(3):194-210.

2   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Caldwell J, et al. Pharmacogenetic and metabolite measurements are associated with clinical status in patients with rheumatoid arthritis treated with methotrexate: results of a multicentred cross sectional observational study. Ann Rheum Dis. 2005 Aug;64(8):1180-5.

3   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Walsh M, et al. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum. 2004 Sep;50(9):2766-74.

4   Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum. 2006 Oct;54(10):3095-103.

5   Angelis-Stoforidis P, Vajda FJ, Christophidis N. Methotrexate polyglutamate levels in circulating erythrocytes and polymorphs correlate with clinical efficacy in rheumatoid arthritis. Clin Exp Rheumatol. 1999 May-Jun;17(3):313-20.

6   Becker ML, van Haandel L, Gaedigk R, Lasky A, Hoeltzel M, Stobaugh J, et al. Analysis of intracellular methotrexate polyglutamates in patients with juvenile idiopathic arthritis: effect of route of administration on variability in intracellular methotrexate polyglutamate concentrations. Arthritis Rheum. 2010 Jun;62(6):1803-12.

7   Becker ML, Gaedigk R, van Haandel L, Thomas B, Lasky A, Hoeltzel M, et al. The effect of genotype on methotrexate polyglutamate variability in juvenile idiopathic arthritis and association with drug response. Arthritis Rheum. 2011 Jan;63(1):276-85.

8   Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Frampton C, James J, et al. Determinants of red blood cell methotrexate polyglutamate concentrations in rheumatoid arthritis patients receiving long-term methotrexate treatment. Arthritis Rheum. 2009 Aug;60(8):2248-56.

9   Dalrymple JM, Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Barclay ML. Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. Arthritis Rheum. 2008 Nov;58(11):3299-308.

10   Dervieux T, Orentas Lein D, Marcelletti J, Pischel K, Smith K, Walsh M, et al. HPLC determination of erythrocyte methotrexate polyglutamates after low-dose methotrexate therapy in patients with rheumatoid arthritis. Clin Chem. 2003 Oct;49(10):1632-41.

11   Hobl EL, Jilma B, Erlacher L, Duhm B, Mustak M, Broll H, et al. A short-chain methotrexate polyglutamate as outcome parameter in rheumatoid arthritis patients receiving methotrexate. Clin Exp Rheumatol. 2012 Mar-Apr;30(2):156-63.

12   den Boer E, de Rotte MCFJ, Pluijm SMF, Heil SG, Hazes JMW, de Jonge R. Determinants of erythrocyte methotrexate polyglutamate levels in rheumatoid arthritis. Accepted for publication.

13   van Haandel L, Becker ML, Leeder JS, Williams TD, Stobaugh JF. A novel high-performance liquid chromatography/mass spectrometry method for improved selective and sensitive measurement of methotrexate polyglutamation status in human red blood cells. Rapid Commun Mass Spectrom. 2009 Dec;23(23):3693-702.

14   Dervieux T, Zablocki R, Kremer J. Red blood cell methotrexate polyglutamates emerge as a function of dosage intensity and route of administration during pulse methotrexate therapy in rheumatoid arthritis. Rheumatology (Oxford). 2010 Dec;49(12):2337-45.

15   Hornung N, Ellingsen T, Attermann J, Stengaard-Pedersen K, Poulsen JH. Patients with rheumatoid arthritis treated with methotrexate (MTX): concentrations of steady-state erythrocyte MTX correlate to plasma concentrations and clinical efficacy. J Rheumatol. 2008 Sep;35(9):1709-15.

16   Dervieux T, Kremer J, Lein DO, Capps R, Barham R, Meyer G, et al. Contribution of common polymorphisms in reduced folate carrier and gamma-glutamylhydrolase to methotrexate polyglutamate levels in patients with rheumatoid arthritis. Pharmacogenetics. 2004 Nov;14(11):733-9.

17   de Rotte MC, den Boer E, de Jong PH, Pluijm SM, Bulatovic Calasan M, Weel AE, et al. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in patients with rheumatoid arthritis. Ann Rheum Dis. 2013 Dec 5.

18   Bulatovic Calasan M, den Boer E, de Rotte MC, Vastert SJ, Kamphuis S, de Jonge R, et al. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in juvenile idiopathic arthritis patients. Ann Rheum Dis. 2013 Nov 28.

19   Stamp LK, Barclay ML, O'Donnell JL, Zhang M, Drake J, Frampton C, et al. Effects of changing from oral to subcutaneous methotrexate on red blood cell methotrexate polyglutamate concentrations and disease activity in patients with rheumatoid arthritis. J Rheumatol. 2011 Dec;38(12):2540-7.

20   Claessen SJ, Hazes JM, Huisman MA, van Zeben D, Luime JJ, Weel AE. Use of risk stratification to target therapies in patients with recent onset arthritis; design of a prospective randomized multicenter controlled trial. BMC Musculoskelet Disord. 2009;10:71.

21   Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010 Sep;62(9):2569-81.

22   den Boer E, Meesters RJ, Van Zelst BD, Luider TM, Hazes JMW, Heil SG, et al. Measuring methotrexate polyglutamates in red blood cells: a new LC-MS/MS based method. Analytical and Bioanalytical Chemistry. 2012:in press.

23   de Jong PH, Hazes JM, Barendregt PJ, Huisman M, van Zeben D, van der Lubbe PA, et al. Induction therapy with a combination of DMARDs is better than methotrexate monotherapy: first results of the tREACH trial. Ann Rheum Dis. 2013 Jan;72(1):72-8.

24   Blits M, Jansen G, Assaraf YG, van de Wiel MA, Lems WF, Nurmohamed MT, et al. Methotrexate normalizes up-regulated folate pathway genes in rheumatoid arthritis. Arthritis Rheum. 2013 Nov;65(11):2791-802.

Chapter

7

Chapter 8

Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in rheumatoid arthritis patients

M.C.F.J. de Rotte
E. den Boer
P.H.P. de Jong
S.M.F. Pluijm
M. Bulatovic-Calasan
A.E. Weel AE
A.M. Huisman
A.H. Gerards
B. van Schaeybroeck
N.M. Wulffraat
J. Lindemans
J.M.W. Hazes
R. de Jonge

Abstract

OBJECTIVE: To investigate if erythrocyte methotrexate-polyglutamate (MTX-PG) concentrations in rheumatoid arthritis (RA) patients are associated with disease activity or adverse events.

METHODS: We used a longitudinal study-design with two cohorts. The derivation cohort included 102 and the validation cohort included 285 RA patients on MTX. We measured erythrocyte MTX-PG with 1 to 5 glutamate residues at 3, 6 and 9 months after MTX-start with an LC-MS/MS assay. Outcomes were DAS28 and adverse events. Longitudinal associations of MTX-PG concentrations after 3, 6, and 9 months with DAS28 were tested with a linear mixed model adjusted for age, gender, baseline DAS28, MTX-dose and co-medication.

RESULTS: In the derivation cohort, mean DAS28 decreased from 4.26 (SE=0.14) at baseline to 2.72 (SE=0.13) after 9 months. Thirty percent of patients in the derivation cohort experienced more than 3 adverse events after 3 months, which decreased to 18% after 9 months. In the validation cohort, DAS28 and adverse events were comparable with the derivation cohort. In the derivation cohort, MTX-PG1 (ß=-0.005), MTX-PG2 (ß=-0.022), MTX-PG3 (β=-0.007) and total MTX-PG (ß=-0.004) were associated (p<0.05) with lower DAS28 over 9 months. In the validation cohort, MTX-PG2 (ß=-0.015), MTX-PG3 (ß=-0.010), MTX-PG4 (ß=-0.008) and total MTX-PG (ß=-0.003) were associated with lower DAS28 over 9 months. None of the MTX-PGs was associated with adverse events.

CONCLUSION: In this first longitudinal study, we showed that an increase in erythrocyte MTX-PG concentrations were associated with a decreased DAS28 over 9 months in two cohorts, and are therefore a potential tool for therapeutic drug monitoring of MTX in RA.

Introduction

Methotrexate (MTX) is the cornerstone disease-modifying anti-rheumatic drug (DMARD) in the treatment of patients with rheumatoid arthritis (RA). However, significant numbers of patients fail to achieve adequate suppression of disease activity or experience adverse events causing refusal of dose increase or treatment continuation. [1] In those who are non-responsive, increasing MTX-dose can be an alternative. Dosage of MTX, required to suppress disease activity, varies between patients and is unpredictable. Until now, the decision to increase dosage is dependent on assessment of disease activity, accepted upper limit of drug dosing, and occurrence of adverse events. [2] If patients fail to respond to MTX, even after dosage increase, or develop severe adverse events within 3 to 6 months, additional treatment with biologicals is instituted. [3] Therapeutic drug monitoring (TDM) of intracellular MTX concentrations in erythrocytes may help identifying refractoriness patients with non-response and high concentration and patients with a difficulty in accumulating MTX or non-compliance who may benefit from a dose increase or treatment of compliance issues.

Plasma MTX is eliminated from plasma within 24 hours [4] and is unrelated to response [5] and therefore, is not a reliable tool for TDM. [6] MTX is transported intracellularly and retained within cells long after it has been eliminated from plasma. [5] Circulating MTX contains 1 glutamate moiety (MTX-PG1). Once inside cells, up to 4 additional glutamates (PG2-PG5) are added to retain intracellular MTX, which in turn increases its affinity for target enzymes in one-carbon metabolism, thus promoting MTX's anti-inflammatory effects. Higher MTX-dose leads to higher intracellular erythrocyteMTX polyglutamate (MTX-PG) concentrations. [7, 8] Summarizing, erythrocyte MTX-PGs could have a promising role as biomarkers of patients' response to MTX and in turn could be potentially used as TDM tool.

Erythrocyte MTX-PGs have been related to response in several studies in adult RA. [4, 9-12] In addition, we showed in an accompanying paper that in juvenile idiopathic arthritis (JIA) long-chain erythrocyteMTX-PGs were associated with lower disease activity at 3 months and during one year of MTX treatment. [13] However, there have been reports with contrasting results in RA and in JIA. [2, 14, 15] Most of these studies used cross-sectional analyses [2, 9, 10, 14, 15] in which patients were in different stages of MTX-treatment varying from 3 months to >10 years. Disadvantages of cross-sectional analysis are that you cannot distinguish between those treated for weeks or years and that you cannot make causal inference. [16] Additionally, comparison between patients is complicated because MTX is stopped in obstinate non-responders and because MTX-PG accumulation is a function of time. [5]

The aim of this prospective, longitudinal study was to investigate if intracellular erythrocyteMTX-PG concentrations are related to disease activity or adverse events in RA patients on MTX and thus if MTX-PGs could be a tool for TDM.

Chapter

8

Patients and Methods

Study design and patients

The derivation cohort was the 'Methotrexate in Rotterdam' cohort (MTX-R). The MTX-R is a longitudinal prospective cohort of patients who started MTX between January 2006 and March 2011 at the Rheumatology Department, Erasmus University Medical Center, Rotterdam (Erasmus MC), Netherlands. The validation cohort was the 'Treatment in Rotterdam Early Arthritis Cohort' (tREACH). The tREACH is a clinical multicentre, stratified single-blinded trial (ISRCTN26791028), as described earlier. [17, 18]. The medical ethics committee from the Erasmus MC approved both studies and patients gave written informed consent before inclusion.

Derivation cohort patients were included if diagnosed with RA by the physician. Validation cohort patients were included in if they fulfilled the 2010 ACR/EULAR criteria for RA [19]. Patients on biologicals at baseline were excluded.

In the derivation cohort, clinicians chose MTX-dosage and co-medication for every visit. In the validation cohort, MTX starting dose was set at 25 mg/week (reached after 3 weeks). If patients had DAS28<2.6 for 2 consecutive visits MTX-dose was decreased with 2.5 mg/month until stop. Patients were randomized to treatment with or without sulfasalazine, hydroxychloroquine and glucocorticoids. Patients in both cohorts received folic acid (10 mg/week) during MTX treatment. In both cohorts, patients were assessed at baseline, and after 3, 6 and 9 months.

Biochemical parameters

One additional EDTA blood sample-tube was obtained from patients during every study visit besides routine EDTA and serum samples for erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Alanine-aminotransferase (ALAT), rheumatoid factor, anti-cyclic citrullinated peptide antibody, leukocytes, trombocytes and hemoglobine. The additional EDTA tube was immediately put on ice after collection, centrifuged for 10 min at 1700 × g at 4 °C. Plasma and cell-pellet aliquots were stored at -80 °C. MTX-PGs were analysed from the cell-pellet aliquots with a liquid chromatography-electrospray ionization-tandem mass spectrometry-based assay using stable-isotope-labelled internal standards, as described recently by us. [20] Concentrations of MTX-PGs were reported in nmol/L packed erythrocytes.

Disease activity and adverse events

Disease activity outcome was the disease activity score 28 (DAS28). [21] Adverse event outcome was categorized into: one or more (versus none) and three or more (versus two or less). Adverse events included gastrointestinal complaints, malaise, psychological complaints, hepatotoxicity, bone marrow depression and other complaints. Gastrointestinal complaints involved diarrhoea, vomiting, sickness and abdominal pain. Malaise involved fatigue, dizziness, headache, sleeplessness and not feeling well. Psychological complaints involved depression and personality changes. Other complaints involved dyspnoea,

alopecia, infection, mucositis, epistaxis, skin related complaints and other. Gastrointestinal complaints, malaise, psychological complaints and other complaints were assessed with a questionnaire every visit and scored by a researcher. Hepatotoxicity was defined as ALAT, 3 times upper level of normal. Bone marrow depression was defined as leucocytes$<3.0\times10^9$/l or thrombocytes$<100\times10^9$/l.

Statistical analyses

Comparisons of patient characteristics between derivation and validation cohorts were made by Student's t-test, χ^2-test, Mann-Whitney U test or Friedman's two-way analysis of variance by ranks as appropriate. Correlations were tested with Spearman's correlation-test. Multiple linear regression analyses was used for cross-sectional analyses of MTX-PG concentrations measured at 3, 6 and 9 months with continuous outcomes (DAS28) at corresponding visits. Multivariate logistic regression analyses was used for dichotomous outcomes (adverse events). Longitudinal analyses of association of MTX-PG concentrations, measured at 3, 6, and 9 months, with DAS28 at corresponding study visits were performed with a linear mixed model for continuous outcomes. All analyses were corrected for potential confounders: age, gender, baseline DAS28, MTX-dose and use of other DMARDs, non-steroidal anti-inflammatory drugs (NSAID), glucocorticoids and biologicals. Confounders were added as covariates to regression analyses. Co-medication and MTX-dose observed 3 months prior to the visit analysed were added as covariates.

Finally, for those MTX-PGs that had significant association with DAS28, cut-off concentrations for moderate/good-response versus non-response according to EULAR response criteria [22] were determined in the derivation cohort using receiver operating characteristic curves. Cut-off concentrations were chosen to have optimal sensitivity and specificity. EULAR response criteria allow only patients with baseline DAS28≥3.3. For these cut-of concentrations diagnostic parameters: sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were subsequently determined. Statistics were performed with SPSS Statistics Version 20.0.0.1 (SPSS Inc. Chicago, IL, USA).

Results

Patients and MTX-PG concentrations

At baseline, 102 patients were included in the derivation cohort and 285 patients were included in the validation cohort (Figure 1). MTX-PGs in the derivation cohort were measured in 79 patients after 3 months of treatment, 67 patients after 6 months and 59 patients after 9 months. In the validation cohort, MTX-PGs were measured in 228 patients after 3 months, 183 patients after 6 months and 177 patients after 9 months.

Disease activity at MTX start was lower in the derivation cohort compared with the validation cohort (table 1). MTX-dose was higher in the validation cohort (25 mg/week) compared with the derivation cohort (15 mg/week). Patients in the derivation cohort used more NSAIDs, less steroids and more parenteral MTX than patients in the validation cohort.
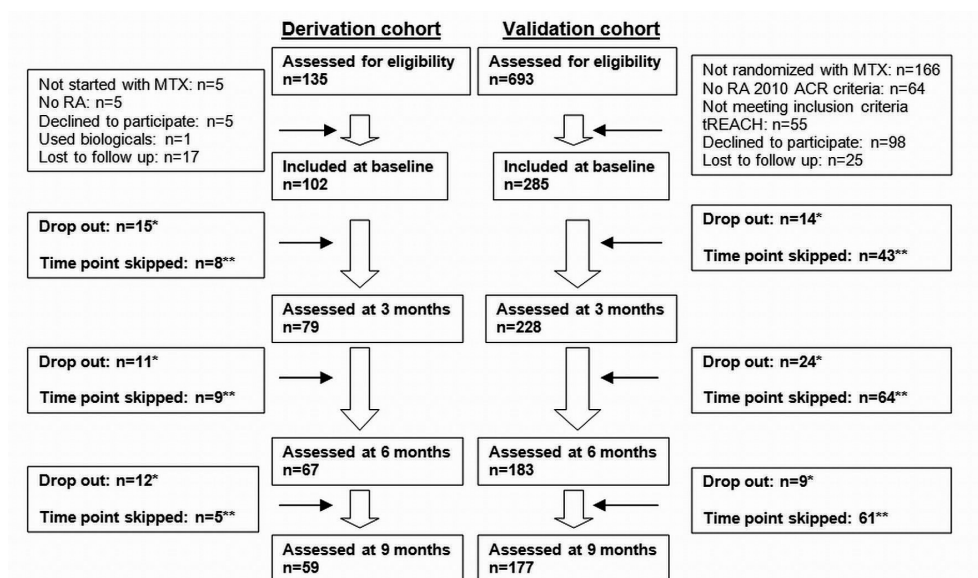
Chapter

8

Figure 1. Flow chart of patient follow up for both cohorts. *Reasons for drop out were: patient refusal, adverse events, communication problems, no compliance, lost to follow up, and MTX stopped; **Reasons for time point skipped were: there was insufficient material available for determinations of MTX-PG concentrations, and patients did not show up. tREACH, treatment in Rotterdam Early Arthritis Cohort; RA, rheumatoid arthritis; ACR, American College of Rheumatology; MTX-PG, methotrexate-polyglutamate.

Table 1. Baseline characteristics per cohort.

Laboratory parameters	Derivation cohort n=102	Validation cohort n=285	p-value
Rheumatoid factor positive	41%	66%	<0.001
Anti-cyclic citrullinated peptide antibody positive	41%	70%	<0.001
Erythrocyte sedimentation rate mm/h, median (IR)	19 (9-33)	23 (13-40)	0.011
Clinical parameters			
Gender, male	29%	30%	0.991
Age, mean (SD)	52 (16)	54 (14)	0.299
VAS mm, mean (SD)	54 (26)	53 (22)	0.704
28 tender joint count (SD), median (IR)	4 (1-8)	6 (3-10)	<0.001
28 swollen joint count (SD), median (IR)	3 (1-7)	6 (3-10)	<0.001
DAS28, mean (SD)	4.26 (1.43)	4.94 (1.15)	<0.001
Medication			
Methotrexate dose, mean (SD)	15 (2)	25 (1)	<0.001
NSAIDs	36%	14%	<0.001
Other DMARDs	57%	62%	0.408
Oral corticosteroids	11%	62%	<0.001
Parenteral corticosteroids	3%	32%	<0.001
Parenteral methotrexate	6%	0%	<0.001

IR, interquartile range; SD, standard deviation; VAS, patient global assessment of general health on a visual analogue scale; DAS, disease activity score; NSAID, non-steroid anti-inflammatory drug; DMARD, disease modifying anti-rheumatic drug.

Table 2 shows medians and ranges of erythrocyte MTX-PG concentrations at 3, 6 and 9 months in both cohorts. Supplementory table 1 shows dosing adjustments. In both cohorts constant concentration was achieved for MTX-PG1 and MTX-PG2 after 3 months. However in the derivation and validation cohort, MTX-PG3 (p<0.001, p<0.001), MTX-PG4 (p<0.001, p=0.009), MTX-PG5 (p<0.001, p=0.003) and total MTX-PG (p=0.024, p<0.001) had higher concentrations after 6 months compared to 3 months. The concentrations at 6 months and 9 months were the same for all MTX-PGs in both cohorts.

Supplementory table 2a/b shows the correlations between all MTX-PGs at 3 months. After 3 months, MTX-PG3 (40 versus 48 nmol/L; p=0.001), MTX-PG4 (8 versus 19 nmol/L; p<0.001) and MTX-PG5 (1 versus 5 nmol/L; p<0.001) concentrations were lower in the derivation cohort compared with the validation cohort. After 6 months, MTX-PG5 (3 versus 4 nmol/L; p=0.003) concentrations were lower in the derivation cohort. After 9 months there were no differences in MTX-PG concentrations between cohorts.

Table 2. Erythrocyte-MTX-PG concentrations, median (minimum-maximum), in nmol/L packed erythrocytes over time in both cohorts.

Derivation cohort	3 months	6 months	9 months
MTX-PG1	35 (0-258)	36 (0-248)	38 (0-199)
MTX-PG2	23 (0-69)	26 (0-65)	23 (0-77)
MTX-PG3	40 (0-88)	54 (0-116)	59 (0-125)
MTX-PG4	8 (0-61)	16 (0-79)	18 (0-76)
MTX-PG5	1 (0-26)	3 (0-31)	4 (0-50)
Total MTX-PG	117 (0-396)	153 (0-396)	158 (0-396)
Validation cohort	3 months	6 months	9 months
MTX-PG1	28 (0-337)	30 (0-186)	29 (0-166)
MTX-PG2	21 (0-82)	21 (0-69)	21 (0-105)
MTX-PG3	48 (0-97)	56 (0-136)	56 (0-173)
MTX-PG4	19 (0-88)	20 (0-100)	20 (0-103)
MTX-PG5	5 (0-64)	4 (0-82)	4 (0-48)
Total MTX-PG	130 (0-476)	144 (0-413)	139 (0-559)

MTX-PG, methotrexate-polyglutamate

Disease activity

In the derivation cohort, mean DAS28 decreased from 4.26 (SE=0.14) at baseline to 2.92 (SE=0.13) after 3 months (p<0.001), to 2.83 (SE=0.15) after 6 months and to 2.72 (SE=0.13) after 9 months. In the validation cohort, mean DAS28 decreased from 4.95 (SE=0.07) at baseline to 3.12 (SE=0.07) after 3 months (p<0.001), to 2.93 (SE=0.08) after 6 months and to 2.66 (SE=0.08) after 9 months. Supplementory table 3 shows numbers of patients switching between moderate/good-response and non-response.

Table 3 shows results for both cohorts of cross-sectional analyses for associations

Chapter

8

between each MTX-PG measured after 3, 6 or 9 months with DAS28 determined at the corresponding study visit. In the derivation cohort after 3 months, higher MTX-PG1 (β=-0.006; SE=0.002), MTX-PG2 (β=-0.021; SE=0.008), MTX-PG3 (β=-0.016; SE=0.006), MTX-PG4 (β=-0.021; SE=0.010) and total MTX-PG (β=-0.006; SE=0.002) were associated with lower DAS28. After 9 months MTX-PG2 (β=-0.019; SE=0.009) and MTX-PG5 (β=0.037; SE=0.016) were associated with DAS28. In the validation cohort, after 6 months, MTX-PG2 (β=-0.015; SE=0.007), MTX-PG3 (β=-0.011; SE=0.003) and after 9 months, MTX-PG2, (β=-0.012; SE=0.006), MTX-PG3 (β=-0.007; SE=0.003) and total MTX-PG (β=-0.002; SE=0.001) were associated with lower DAS28.

Table 3. Cross-sectional and longitudinal analysis of MTX-PG concentrations in nmol/L packed erythrocytes and DAS28.

	Cross-sectional analysis, β (SE)			Longitudinal analysis, β (SE)
	3 months	6 months	9 months	0-9 months
MTX-PG1	-0.006 (0.002)*	-0.004 (0.004)	-0.005 (0.003)	-0.005 (0.002)*
MTX-PG2	-0.021 (0.008)*	-0.020 (0.011)	-0.019 (0.009)*	-0.022 (0.005)**
MTX-PG3	-0.016 (0.006)*	0.000 (0.007)	0.002 (0.005)	-0.007 (0.003)*
MTX-PG4	-0.021 (0.010)*	0.007 (0.009)	-0.002 (0.008)	-0.006 (0.004)
MTX-PG5	-0.055 (0.029)	0.015 (0.025)	0.037 (0.016)*	0.006 (0.012)
Total MTX-PG	-0.006 (0.002)*	-0.002 (0.002)	-0.002 (0.002)	-0.004 (0.001)**
Validation cohort	3 months	6 months	9 months	0-9 months
MTX-PG1	-0.002 (0.002)	0.000 (0.002)	-0.004 (0.002)	-0.002 (0.001)
MTX-PG2	-0.014 (0.007)	-0.015 (0.007)*	-0.012 (0.006)*	-0.015 (0.003)**
MTX-PG3	-0.004 (0.004)	-0.011 (0.003)*	-0.007 (0.003)*	-0.010 (0.002)**
MTX-PG4	-0.004 (0.005)	-0.007 (0.005)	-0.006 (0.004)	-0.008 (0.002)*
MTX-PG5	-0.006 (0.009)	-0.007 (0.008)	-0.007 (0.009)	-0.008 (0.005)
Total MTX-PG	-0.002 (0.001)	-0.002 (0.001)	-0.002 (0.001)*	-0.003 (0.001)**

*p<0.05. **p<0.001.
Results are corrected for baseline DAS, gender, age, MTX-dose, other DMARDs use, NSAID use, glucocorticoid use and biological use. In the cross-sectional analysis, each MTX-PG concentration at each timepoint was associated with the DAS28 at the same time point using linear regression. For the longitudinal analyses a mixed model was used during the first 9 months treatment. MTX-PG, methotrexate-polyglutamate; DAS, disease activity score; DMARD, disease modifying anti-rheumatic drug; NSAID, non-steroid anti-inflammatory drug.

In the derivation cohort, longitudinal analyses showed that MTX-PG1 (β=-0.005, SE=0.002), MTX-PG2 (β=-0.022, SE=0.005), MTX-PG3 (β=-0.007; SE=0.003) and total MTX-PG (β=-0.004, SE=0.001) were associated with lower DAS28 over the first 9 months. In the validation cohort, MTX-PG2 (β=-0.015, SE=0.003), MTX-PG3 (β=-0.010, SE=0.002), MTX-PG4 (β=-0.008; SE=0.002) and total MTX-PG (β=-0.003, SE=0.001) were longitudinally associated with lower DAS28. For an increase in 1 nmol/L MTX-PG2 there is a decrease of 0.02 in DAS28.

For an increase in 1 nmol/L MTX-PG3 there is a decrease of 0.01 in DAS28. For an increase in 1 nmol/L total MTX-PG there is a decrease of 0.003 in DAS28.
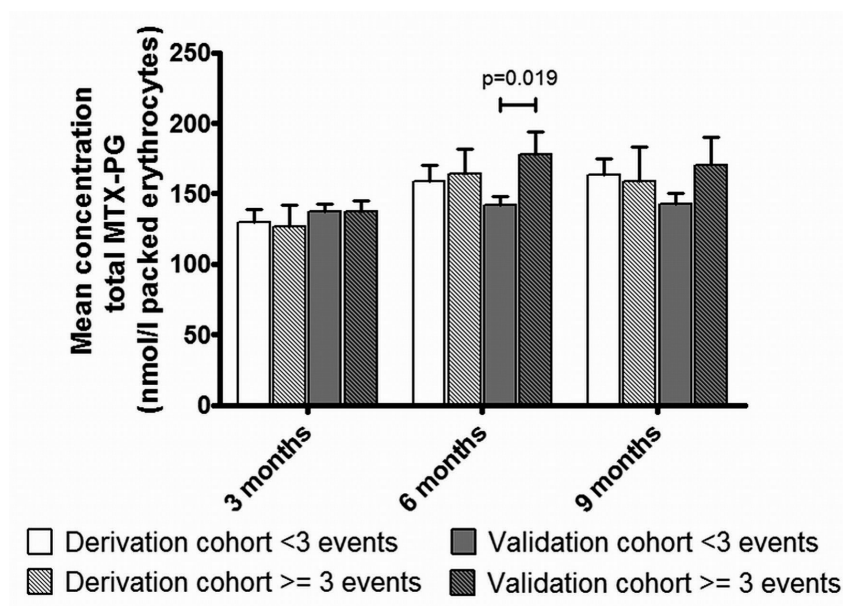

Figure 2. Mean concentration total erythrocyteMTX-PG in both cohorts for patients with < 3 adverse events and patients with ≥ 3 adverse events after 3, 6 and 9 months. MTX-PG, methotrexate-polyglutamate.

Adverse events

In the derivation cohort after 3 months 20% of the patients (n=16) had no adverse events, 42% (n=33) gastrointestinal complaints, 49% (n=39) malaise, 13% (n=10) psychological complaints, 2% (n=2) hepatotoxicity, 2% (n=2) bone marrow depression, 26% (n=21) other adverse events and 30% (n=24) 3 or more adverse events. After 9 month 15% (n=9) had no adverse events, 31% (n=18) gastrointestinal complaints, 23% (n=14) malaise, 4% (n=2) psychological complaints, 4% (n=2) hepatotoxicity, 1% (n=1) bone marrow depression, 28% (n=17) other adverse events and 18% (n=11) 3 or more adverse events. In the validation cohort, percentages of patients with adverse events were comparable with the derivation cohort. After 3 months 31% (n=71) experienced 3 or more adverse events, which decreased to 15% (n=27) after 9 months.

Figure 2 shows the concentrations of total MTX-PGs after 3, 6 and 9 months in both cohorts, stratified for patients with 3 or more adverse events and patients with 2 or fewer adverse events. Patients with >3 adverse events had higher MTX-PG concentrations than patients with ≤2 adverse events after 6 months in the validation cohort (142 versus 178 nmol/L, p=0.019). In the derivation cohort after 3 months, total MTX-PG was not associated with no adverse events (OR=1.00; 95%CI=0.99-1.01), gastrointestinal complaints (OR=1.00; 95%CI=0.99-1.00), malaise (OR=1.01; 95%CI=1.00-1.01), psychological complaints (OR=1.00; 95%CI=0.98-1.01), hepatotoxicity (OR=0.92; 95%CI=0.80-1.05), bone marrow depression

Chapter

8

(OR=0.96; 95%CI=0.86-1.07), other adverse events (OR=1.00; 95%CI=0.99-1.01) and 3 or more adverse events (OR=1.00; 95%CI=0.99-1.01). Same results were obtained for cross sectional analyses after 6 and 9 months and for all individual MTX-PGs. In the validation cohort, all results from the cross sectional analyses were comparable to the derivation cohort. Thus, no significant associations were found in this study between any of the MTX-PGs and adverse events.

Cut-off concentrations

MTX-PG2, MTX-PG3, MTX-PG4 and total MTX-PG were longitudinally associated with lower DAS28 during 9 months treatment. Therefore, cut-off concentrations for EULAR moderate/good-response and their diagnostic parameters were determined. Table 4 shows that cut-off concentrations of ≥22 nmol/L for MTX-PG2 and ≥74 nmol/L for total MTX-PG could discriminate well between patients with moderate/good versus non-response.

Table 4. Cut-off values in nmol/L packed erythrocytes, sensitivity and specificity of erythrocyte MTX-PGs to predict EULAR moderate/good-response at 3 months.

	MTX-PG2	MTX-PG3	MTX-PG4	Total MTX-PG
AUC	72%	68%	64%	71%
95% CI for AUC	56%-88%	48%-87%	42%-85%	53%-89%
p-value for AUC	0.025	0.072	0.163	0.034
Cut-off concentration	22 nmol/L	32 nmol/L	6 nmol/L	74 nmol/L
Sensitivity	65%	67%	74%	87%
Specificity	82%	73%	64%	64%
Positive predictive value	78%	71%	67%	71%
Negative predictive value	70%	69%	71%	83%

Determined in the derivation cohort after 3 months with receiver operating characteristic curves using EULAR moderate/good-response as determinant for good response. EULAR response criteria allow only patients with baseline DAS28≥3.3 (n=57). MTX-PG, methotrexate-polyglutamate; AUC, area under the curve.

Discussion

We investigated in this first longitudinal study whether erythrocyteMTX-PG concentrations at 3, 6 and 9 months after MTX start were associated with disease activity and adverse events in two RA cohorts. In both cohorts, an increase in MTX-PG concentrations is associated with a decrease in DAS28 during the first 9 months. Associations were strongest for MTX-PG2 and MTX-PG3. Cut-off concentrations (total MTX-PG: ≥74 nmol/L) could be used to identify patients with moderate/good-response to MTX treatment. In this study, we did not find an association between MTX-PG concentrations and adverse events. Besides our results from the present study in adult RA, we also show in an accompanying paper that MTX-PG3, MTX-PG4, MTX-PG5 and total MTX-PG concentrations are related to lower disease activity in JIA. [13]

MTX-PG concentrations in erythrocytes have been associated with response to

MTX in arthritis patients before. [4, 9-12] A study showed that erythrocyte MTX-PGs in responders and partial responders were significantly higher than in non-responders. [9] Others showed that lower MTX-PG levels were associated with higher disease activity and lower decrease in DAS28 [10] and that patients with less decrease in DAS28 had lower MTX-PG levels. [11] Others showed that erythrocyte MTX levels were significantly higher in patients responding to MTX therapy than in patients classified as non-responders. [4] MTX-PG2 was found to have positive correlation with improvement in DAS28 over the first 16 weeks. [12] Contrary to all these studies, MTX-PG4, MTX-PG5, MTX-PG3-5 and total MTX-PG were found higher in patients with high disease activity. [2] These results were based on cross-sectional analyses with independent t-tests, nonparametric Mann-Whitney U-tests and chi-square tests for patient divided into non-responders (DAS28>3.2) and responders (DAS28≤3.2). Furthermore, Stamp et al. [2] analysed patients who received MTX for a period of 3 months to 19 years (median 3 years). In the present study, however, disease activity was determined in patients starting MTX treatment using a continuous outcome variable (DAS28), which provided more power. Also, this enabled us to perform analyses with linear multivariate models so that we could adjust for a variety of possible confounders. Moreover in our cross-sectional approach all patients used MTX for the same length of time and the cross-sectional approach was repeated at 3 study visits. We also performed longitudinal analyses to determine the association of MTX-PGs with disease activity during the entire 9-month follow-up. Taken together, the cross-sectional analyses in a heterogeneous population may have caused discrepant results compared with the present study.

In line with other studies [2, 9, 13, 23], we did not find any association between MTX-PG concentrations and adverse events. However, relationships between MTX-adverse events and higher concentrations of MTX-PG4 and MTX-PG5 have been reported. [24] Also, in JIA an association between elevated liver function tests and gastrointestinal adverse events and high MTX-PG3-5 concentrations has been found. [14] In our cohorts, all patients were treated with folic acid. This treatment has been proven to reduce MTX adverse events in RA patients. [25] This could have diluted the relationship between MTX-PG concentrations and adverse events.

As others have shown before [7, 8], also in our cohorts MTX-dose seems to have an effect on individual MTX-PGs. The higher MTX-dose in the validation cohort caused higher MTX-PG3, MTX-PG4 and MTX-PG5 concentrations after 3 months (p≤0.001). After 9 months there were no differences in MTX-PG concentrations between cohorts. This may be explained because 28% of patients in the derivation cohort used 25 mg MTX/week after 9 months. Also, the longer MTX use could have caused higher MTX-PG concentrations. [5] Maximum dose for MTX in RA is 25 mg/week. Performing TDM is most important in patients with lower MTX-dose short after MTX start. We therefore determined cut-off values for MTX-PGs for achieving EULAR moderate/good-response in the derivation cohort at 3 months. Patients with total MTX-PG concentration <74 nmol/L after 3 months MTX may need dose increase to achieve lower disease activity. In the derivation cohort, 11 (14%) and in the

Chapter

8

validation cohort 35 (15%) patients achieved total MTX-PG concentrations ≥74 nmol/L after 3 months and were non-responder. This group of patients probably has no benefit from MTX despite an adequate total MTX-PG concentration and may need additional medication.

There are some inconsistencies between the cohorts in the associations of each PG with disease activity (Table 3). This could be a dose effect since higher dose in the validation cohort drives the formation of longer MTX-PGs. This is visible in our study because MTX-PG3, MTX-PG4 and MTX-PG5 have higher concentrations after 3 months in the validation cohort. Based on our study, MTX-PG2 and MTX-PG3 would be the best candidates for TDM or prediction of clinical response. MTX-PG2 was slightly superior to MTX-PG3 in terms of the effect size (Beta's Table 3) and diagnostic test accuracy (Table 4). On the other hand, MTX-PG3 is more abundant and therefore, can be measured with more (analytical) precision (SEs Table 3). Additionally, due to the kinetics of MTX-PG accumulation, the variability in the accumulation half-life of MTX-PG2 is larger than that of MTX-PG3 [5] making MTX-PG3 a more suitable predictor to measure. However, from a clinical point of view, it would be even better to predict response and to optimize MTX dose much earlier than 3 months. To this aim, MTX-PG2 would be a better candidate because of its much shorter accumulation half-time than MTX-PG3 [5] (see also Table 2). Future prospective studies should investigate the predictive power of MTX-PGs measured much earlier after the start of MTX treatment. In the accompanying study, especially long chain MTX-PGs were associated with lower disease activity in JIA. [13]

The hypothesis in this study was based on MTX working mechanism and therefore MTX monotherapy would have been ideal. However, more than half of patients in this study received other DMARDs, NSAIDs and corticosteroids besides MTX. These drugs also have an impact on disease activity and can cause similar adverse events. Therefore, all analyses were corrected for co-medication. Corrected results were not significantly different from uncorrected results. This was not a pharmacokinetic study. However, we compared MTX-PG concentrations between 3, 6 and 9 months. MTX-PG1 and MTX-PG2 achieved a constant concentration after 3 months and MTX-PG3,4,5 and total MTX-PG achieved constant concentration after 6 months. Dervieux et al. [7] reported steady-state after 7 weeks for MTX-PG1-5. Others [5] showed that median times to reach steady state were 6.2, 10.6, 41.2, 149.0 and 139.8 weeks, respectively, for MTX-PG1,2,3,4 and 5. For MTX-PG4 and MTX-PG5 in our study it took less time (6 months) to reach constant concentration. Differences after 6 months may be too small to pick up with simple statistics. There were many differences in baseline characteristics of both cohorts. But, in the way we collected the data methodologically, both cohorts are almost identical. Having longitudinal data of MTX-PGs for 3 visits in first 9 months of MTX treatment in two different cohorts is unique. Because we find similar relationships between MTX-PGs and DAS28 in both cohorts, despite these differences between cohorts, supports the conclusion that erythrocyteMTX-PG levels are related to clinical response.

In conclusion, higher erythrocyte MTX-PG concentrations were associated with lower DAS28 during 9 months MTX treatment in RA patients in two independent cohorts. MTX-PGs were not associated with adverse events. Erythrocyte MTX-PG concentrations are a potential tool for therapeutic drug monitoring of MTX therapy in RA patients.

Chapter

8

References

1   Stamp LK, Roberts RL. Effect of genetic polymorphisms in the folate pathway on methotrexate therapy in rheumatic diseases. Pharmacogenomics. 2011 Oct;12(10):1449-63.

2   Stamp LK, O'Donnell JL, Chapman PT, Zhang M, James J, Frampton C, et al. Methotrexate polyglutamate concentrations are not associated with disease control in rheumatoid arthritis patients receiving long-term methotrexate therapy. Arthritis Rheum. 2010 Feb;62(2):359-68.

3   Smolen JS, Aletaha D, Bijlsma JW, Breedveld FC, Boumpas D, Burmester G, et al. Treating rheumatoid arthritis to target: recommendations of an international task force. Ann Rheum Dis. 2010 Apr;69(4):631-7.

4   Hornung N, Ellingsen T, Attermann J, Stengaard-Pedersen K, Poulsen JH. Patients with rheumatoid arthritis treated with methotrexate (MTX): concentrations of steady-state erythrocyte MTX correlate to plasma concentrations and clinical efficacy. J Rheumatol. 2008 Sep;35(9):1709-15.

5   Dalrymple JM, Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Barclay ML. Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. Arthritis Rheum. 2008 Nov;58(11):3299-308.

6   Bannwarth B, Pehourcq F, Schaeverbeke T, Dehais J. Clinical pharmacokinetics of low-dose pulse methotrexate in rheumatoid arthritis. Clin Pharmacokinet. 1996 Mar;30(3):194-210.

7   Dervieux T, Zablocki R, Kremer J. Red blood cell methotrexate polyglutamates emerge as a function of dosage intensity and route of administration during pulse methotrexate therapy in rheumatoid arthritis. Rheumatology (Oxford). 2010 Dec;49(12):2337-45.

8   Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Frampton C, James J, et al. Determinants of red blood cell methotrexate polyglutamate concentrations in rheumatoid arthritis patients receiving long-term methotrexate treatment. Arthritis Rheum. 2009 Aug;60(8):2248-56.

9   Angelis-Stoforidis P, Vajda FJ, Christophidis N. Methotrexate polyglutamate levels in circulating erythrocytes and polymorphs correlate with clinical efficacy in rheumatoid arthritis. Clin Exp Rheumatol. 1999 May-Jun;17(3):313-20.

10   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Caldwell J, et al. Pharmacogenetic and metabolite measurements are associated with clinical status in patients with rheumatoid arthritis treated with methotrexate: results of a multicentred cross sectional observational study. Ann Rheum Dis. 2005 Aug;64(8):1180-5.

11   Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum. 2006 Oct;54(10):3095-103.

12   Hobl EL, Jilma B, Erlacher L, Duhm B, Mustak M, Broll H, et al. A short-chain methotrexate polyglutamate as outcome parameter in rheumatoid arthritis patients receiving methotrexate. Clin Exp Rheumatol. 2012 Mar-Apr;30(2):156-63.

13   Bulatovic Calasan M, Den Boer E, De Rotte MCFJ, Vastert SJ, Kamphuis S, De Jonge R, et al. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in juvenile idiopathic arthrits patients. Ann Rheum Dis. 2013:In press.

14   Becker ML, Gaedigk R, van Haandel L, Thomas B, Lasky A, Hoeltzel M, et al. The effect of genotype on methotrexate polyglutamate variability in juvenile idiopathic arthritis and association with drug response. Arthritis Rheum. 2011 Jan;63(1):276-85.

15   Dolezalova P, Krijt J, Chladek J, Nemcova D, Hoza J. Adenosine and methotrexate polyglutamate concentrations in patients with juvenile arthritis. Rheumatology (Oxford). 2005 Jan;44(1):74-9.

16   de Rotte MC, Luime JJ, Bulatovic M, Hazes JM, Wulffraat NM, de Jonge R. Do snapshot statistics fool us in MTX pharmacogenetic studies in arthritis research? Rheumatology (Oxford). 2010 Jun;49(6):1200-1.

17   Claessen SJ, Hazes JM, Huisman MA, van Zeben D, Luime JJ, Weel AE. Use of risk stratification to target therapies in patients with recent onset arthritis; design of a prospective randomized multicenter controlled trial. BMC Musculoskelet Disord. 2009;10:71.

18   de Jong PH, Hazes JM, Barendregt PJ, Huisman M, van Zeben D, van der Lubbe PA, et al. Induction therapy with a combination of DMARDs is better than methotrexate monotherapy: first results of the tREACH trial. Ann Rheum Dis. 2012 Jun 7.

19   Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis. 2010 Sep;69(9):1580-8.

20   den Boer E, Meesters RJ, Van Zelst BD, Luider TM, Hazes JMW, Heil SG, et al. Measuring methotrexate polyglutamates in red blood cells: a new LC-MS/MS based method. Analytical and Bioanalytical Chemistry. 2012:in press.

21   Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum. 1995 Jan;38(1):44-8.

22   van Gestel AM, Prevoo ML, van 't Hof MA, van Rijswijk MH, van de Putte LB, van Riel PL. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis. Comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism Criteria. Arthritis Rheum. 1996 Jan;39(1):34-40.

23   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Walsh M, et al. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum. 2004 Sep;50(9):2766-74.

24   Brooks AJ, Begg EJ, Zhang M, Frampton CM, Barclay ML. Red blood cell methotrexate polyglutamate concentrations in inflammatory bowel disease. Ther Drug Monit. 2007 Oct;29(5):619-25.

25   Ortiz Z, Shea B, Suarez Almazor M, Moher D, Wells G, Tugwell P. Folic acid and folinic acid for reducing side effects in patients receiving methotrexate for rheumatoid arthritis. Cochrane Database Syst Rev. 2000(2):CD000951.

Chapter

8

Supplementory table 1. Number of patients with MTX-dose adjustments and mean MTX-dose per visit.

Derivation cohort	Baseline	3 months	6 months
MTX-dose increase to 25 mg/week	1 (1%)	18 (18%)	29 (28%)
Mean MTX-dose (SD)	15 (2)	16 (6)	16 (8)
Validation cohort			
MTX-dose decrease to 15 mg/week	1 (0.4%)	14 (5%)	23 (8%)
Mean MTX-dose (SD)	25 (1)	22 (7)	19 (8)

The MTX-dose observed at the visits 3 months prior to the visits were MTX-PG was measured are given, because this was the dose were the patients were exposed to in the 3 months prior to the visit were MTX-PG concentrations were measured. MTX, methotrexate; SD standard deviation.

Supplementory table 2a. Spearman's correlations in the derivation cohort at 3 months.

		Total MTX-PG	MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4	MTX-PG5
Total MTX-PG	Correlation Coefficient	1,000	,795**	,755**	,825**	,587**	,441**
	Sig. (2-tailed)	.	,000	,000	,000	,000	,000
MTX-PG1	Correlation Coefficient	,795**	1,000	,821**	,370**	,059	-,032
	Sig. (2-tailed)	,000	.	,000	,001	,602	,780
MTX-PG2	Correlation Coefficient	,755**	,821**	1,000	,448**	,098	-,068
	Sig. (2-tailed)	,000	,000	.	,000	,382	,546
MTX-PG3	Correlation Coefficient	,825**	,370**	,448**	1,000	,888**	,726**
	Sig. (2-tailed)	,000	,001	,000	.	,000	,000
MTX-PG4	Correlation Coefficient	,587**	,059	,098	,888**	1,000	,914**
	Sig. (2-tailed)	,000	,602	,382	,000	.	,000
MTX-PG5	Correlation Coefficient	,441**	-,032	-,068	,726**	,914**	1,000
	Sig. (2-tailed)	,000	,780	,546	,000	,000	.

** Correlation is significant at the 0.01 level (2-tailed).

Supplementory table 2b. Spearman's correlations in the validation cohort at 3 months.

		Total MTX-PG	MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4	MTX-PG5
Total MTX-PG	Correlation Coefficient	1,000	,762**	,692**	,875**	,737**	,652**
	Sig. (2-tailed)	.	,000	,000	,000	,000	,000
MTX-PG1	Correlation Coefficient	,762**	1,000	,719**	,450**	,249**	,167*
	Sig. (2-tailed)	,000	.	,000	,000	,000	,011
MTX-PG2	Correlation Coefficient	,692**	,719**	1,000	,581**	,256**	,146*
	Sig. (2-tailed)	,000	,000	.	,000	,000	,028
MTX-PG3	Correlation Coefficient	,875**	,450**	,581**	1,000	,858**	,771**
	Sig. (2-tailed)	,000	,000	,000	.	,000	,000
MTX-PG4	Correlation Coefficient	,737**	,249**	,256**	,858**	1,000	,979**
	Sig. (2-tailed)	,000	,000	,000	,000	.	,000
MTX-PG5	Correlation Coefficient	,652**	,167*	,146*	,771**	,979**	1,000
	Sig. (2-tailed)	,000	,011	,028	,000	,000	.

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed).

Supplementory table 3. Switching between moderate/good-response and non-response according to EULAR response criteria.

Derivation cohort	Between 3 and 6 months	Between 6 and 9 months
Moderate/good-responder to non-responder	9 (19%)	0 (0%)
Stayed non-responder	3 (6%)	5 (12%)
Non-responder to moderate/good-responder	5 (10%)	4 (10%)
Stayed moderate/good-responder	31 (65%)	32 (78%)
Validation cohort		
Moderate/good-responder to non-responder	19 (8%)	13 (6%)
Stayed non-responder	8 (3%)	4 (2%)
Non-responder to moderate/good-responder	30 (13%)	21 (10%)
Stayed moderate/good-responder	173 (75%)	170 (82%)

Chapter

8

Chapter 9

Discussion

Discussion

Because of its high efficacy and good safety profile the folate antagonist methotrexate (MTX) is the 'anchor drug' in the treatment of rheumatoid arthritis and other arthritic diseases. However, its use is hampered by high inter-patient variation in response and 20-40% do not respond well to treatment. Furthermore, about 30% of patients develop adverse events leading some to discontinue treatment. [1]

Methotrexate is administered in a fixed (non-individualized) dose and treatment regime is adapted according to how the patient reacts, leading to an unpredictable search by trial and error for the correct dosage and treatment regime. A tool to predict how and if patients will respond to initial methotrexate treatment would greatly enhance clinical practice and help tailor treatment to the individual patient.

It has been shown that the intracellular metabolites of methotrexate, the methotrexate-polyglutamates, are associated with response to treatment. [2-8] These MTX-PGs might be a valuable target for therapeutic drug monitoring.

The aims of this thesis were (see *Chapter 1*);

1) To develop a quantitative method for the measurement of erythrocyte MTX-PG.

2) To examine which clinical, genetic, socio-demographic, and biochemical factors influence the MTX-PG levels in patients treated with low-dose MTX .

3) To examine the prospective accumulation of MTX-PGs in erythrocytes during low-dose pulse MTX treatment and its association with response

To achieve this we developed a method for the quantative determination of methotrexate polyglutamates in erythrocytes. Using this method, we measured the MTX-PG levels in erythrocytes from patients of two independent prospective cohorts and we examined the factors that influence MTX-PG accumulation as well as studying the speed and distribution of MTX-PG accumulation. Finally, we correlated MTX-PG levels to reponse to treatment in RA. The results of this thesis validate the use of erythrocyte MTX-PG for TDM and shed new light on the factors that influence the MTX-PG levels. The knowledge gained during this research will aid in the development of personalized treatment of RA.

Main findings of this thesis:

1. An ultrafast method for the measurement of MTX-PGs has been developed using novel MALDI-MS/MS technology. This method will be of great value for large scale studies because of the speed of measurement. (*chapter 2*)

2. A new LC-MS/MS/based method has been developed for the quantitation of MTX-PGs. This method is the first to use stable isotope labeled standards and is easily applicable in the clinical lab due to non-complicated sample preparation and short runtimes. (*chapter 3*)

3. A new method for the measurement of MTX in plasma has been developed, which will be of great use for potential pharmacokinetic studies and for the measurement of MTX in ALL patients, especially in the setting of carboxylase-G2 rescue. (*chapter 4*)

4. We found that an immune-assay based method is a promising low-tech and easy to use assay for the determination of total MTX-PG content in erythrocytes. (*chapter 5*)

5. We determined that age, MTX dosage, erythrocyte folate content and the FPGS rs4451422 SNP are the major factors that influence MTX-PG levels in two independent cohorts. (*chapter 6*)

6. We showed that MTX-PG accumulation and distribution in the erythrocyte is driven by dosage and that higher dose not only leads to higher MTX-PG concentration, but also that distribution shifts towards long-chain MTX-PGs. (*chapter 7*)

7. The accumulation of MTX-PGs in erythrocytes occurs mainly in the first three months of treatment and steady state is generally achieved before 6 months of treatment. (*chapter 7*)

8. We show that the MTX-PGs levels in erythrocytes are related to response and that the MTX-PG levels at 3 months of treatment can predict how well patients respond during the first 9 month of treatment. (*chapter 8*)

Aim 1: To develop a quantitative method for the measurement of erythrocyte MTX-PG

Because the measurement of MTX-PGs is difficult and not many methods have been published that are clinically applicable, we developed multiple assays for the quantitation of MTX-PGs in erythrocytes.

In *chapter 2*, we described the development of a MALDI-QqQ-MS/MS method for the quantitation of erythrocyte MTX-PGs. This method uses relatively new and advanced technology that combines a high-repetition matrix-assisted laser desorption/ionization (MALDI) source with a triple quadruple mass analyzer, thereby negating the time-consuming chromatography step utilized in most MS based assays to separate the analytes of interest. This new technology has been shown to aproach the performance of LC-MS/MS [9] and has been successfully used in multiple studies. [10-16]

This method was able to measure accurately and sensitively within the clinically relevant range of erythrocyte MTX-PGs from patients receiving low-dose MTX therapy and uses fast and relatively simple solid-phase extraction for the isolation of the MTX-PGs from the erythrocyte. Accuracy and precision were well within the FDA-guidelines [17] approved range and the MTX-PG levels that were measured in patient samples were in good agreement with previously reported concentrations. Because of the high speed of the analysis (10 seconds per analysis) this method is well suited for large studies where many samples need to be measured in a batch-type design.

Chapter

9

155

A major advantage of the MALDI-QqQ-MS/MS instrumentation is that it is directly available for analysis of other substances without downtime of the instrumentation compared with common LC-MS instrumentation due to exchange of separation columns and equilibration of the applied instrumentation. The method compares well with other reported methods that generally require >20 minutes per sample because of the chromatography step required for the adequate separation of MTX-PGs.

Although the method described in *chapter 2* is fast and precise, because of the novel nature of the technology, the routine clinical lab is not likely to possess such a machine and an assay on a more accessible machine would be preferred. Because of this we developed a simple LC-MS/MS-based method for the separate quantitation of individual MTX-PGs in human erythrocytes, which is described in *chapter 3*.

This is the first method described in the literature using stable-isotope-labelled internal standards for the measurement of the intracellular MTX-PGs. It is greatly improved over previously reported assays by a shorter and simpler sample-preparation, the use of mass spectrometry and a relatively short analysis time. Because of the MS based nature of this assay and the use of stable isotope internal standards this method is not disadvantaged by interference from compounds similar to MTX, such as endogenous folates or MTX breakdown products such as DAMPA and 7-OH-MTX.

The described method has a linear range that encompasses the known clinical range of the different MTX-PGs and the LLOQ for the different MTX-PGs is comparable to or lower than previously reported methods. This is especially important for the longer-chain MTX-PGs, as these are generally present in lower concentrations than the short-chain MTX-PGs. The LLOQ could however still be improved by more extensive sample clean-up such as SPE, but this would increase the cost and preparation time of the method. Because this method is relatively simple and uses equipment found in most clinical laboratories it is easy to transfer to routine measurement.

We used our method to analyse the erythrocyte MTX-PG content of 50 samples obtained from MTX-treated RA patients. The concentrations and distribution of MTX PG1–5 as measured by our method were in line with those previously reported.

Theoretically, MTX-PG6 and MTX-PG7 are also present in the erythrocytes. However, the presence of these MTX-PGs has not been demonstrated conclusively. As analysis of multiple patients in different studies reported in literature as well as our own patient samples did not reveal detectable amounts of MTX-PG6 and MTX-PG7 in low-dose MTX treatment. Therefore, and because of the prohibitive cost of the MTX-PG6 and MTX-PG7 stable-isotope-labelled internal standards, we decided not to focus on MTX-PG6 and MTX-PG7 in our studies. Although the transition of MTX-PG6 and MTX-PG7 was always included in our analyses, we never found a signal about the background for these analytes. Possibly, in long-term high-dose (ALL) patients these MTX-PGs will be present in measureable quantities.

As shown in our validation experiments, recovery and matrix-effects differ strongly for each MTX-PG and between different erythrocyte pellets. Concomitantly, the use of a stable-isotope-labelled internal standard is mandatory for a reliable quantitation in a complex matrix such as erythrocytes.

In conclusion, we describe a LC-MS/MS-based method to measure MTX in erythrocytes with minimal sample pre-treatment, relatively short analysis time, and good diagnostic performance that can be applied in routine clinical TDM. The method is validated for the clinical lab and is currently the only method that measures MTX-PG1–5 separately using stable-isotope-labelled internal standards.

Plasma levels of MTX are low to non-existent in low-dose MTX treatment such as used in RA. However, for pharmacokinetic studies where samples are collected quickly after taking the drug and plasma levels are still present, an accurate and specific method for the quantitation of MTX in plasma would be of great value. Moreover, a reliable method to measure plasma MTX levels in high-dose MTX treatment such as ALL would also be of clinical value. Most clinical laboratories use an immunoassay for the detection of MTX in plasma. However, immuno-assays generally have less specificity and higher LLOQs than LC-MS/MS-based assays. As the plasma MTX levels are used to guide clinical decision making and considering the quick clearance of MTX and the low levels that are reached a more sensitive, interference-free method would be very valuable.

In *chapter 4* we describe the development and validation of a straightforward and robust LC–MS/MS–based method for the measurement of MTX in plasma. This method is adapted from our erythrocyte MTX-PG method. The presented method does not require expensive and time-consuming sample preparation, such as solid-phase extraction, and because of the use of a stable isotope-labelled internal standard, it is a very useful method for implementation within the clinical laboratory. The short analysis time of 3 minutes per sample makes the method attractive for high-throughput settings and clinical studies.

Compared with other methods and the traditional immuno-assays for the detection of MTX in plasma, our method has improved LLOQ and a larger dynamic range. As with our erythrocyte MTX-PG method, improvements in LLOQ can be made with the addition of more extensive sample clean-up, such as solid-phase extraction, but this would also increase the cost and preparation time of the method.

When comparing with the standard immunoassay-based analysis, good agreement was found. This was somewhat unexpected due to reports about the poor specificity of the immunoassay. [18-20] The developed method will be very valuable for pharmacokinetics studies due to the high specificity and low LLOQ. Also, in certain situations, such as in the case of carboxylase-G2 (glucarpidase) rescue of toxic plasma levels of MTX, immunoassays may be severely hampered by interference of MTX metabolites such as DAMPA. [18-21] In such cases, a more selective method for the determination of MTX in plasma is very important. We showed that during glucarpidase rescue of toxic MTX levels in ALL patients,

the immunoassay experienced strong positive interference from DAMPA while our LC-MS/MS assay was free from interference and was able to correctly measure the disappearance of the plasma MTX induced by glucarpidase. [22] In conclusion, we developed an LC–MS/MS–based method to measure MTX in plasma with minimal sample pre-treatment, relative short analysis time, and good performance that can be applied for routine therapeutic drug monitoring.

The methods described in *chapter 3* and *chapter 4* were both tested thoroughly for the presence of matrix effects. For both methods, the recovery and matrix effect experiments emphasize the need for a proper internal standard when working with complex and highly variable matrices such as biological samples in an LC–MS/MS setting.

We were able to compare the plasma method to the routine and 'gold standard' method for measuring MTX in plasma. This demonstrated that we measure comparable and ensured that the developed assay does not differ from other reported values, enabling comparison between methods and studies. For the erythrocyte MTX-PG method there is no standard method. Although multiple methods have been reported for the measurement of erythrocyte MTX-PG, none of these is used routinely. [23-26] Although validation of our method was good, this was done using erythrocyte samples with extra-cellular added MTX-PG. The extend of extraction is impossible to determine and could differ widely per method, leading to very different results which could in turn lead to differences in the perceived relation between erythrocyte MTX-PG and response to treatment. Cross-calibration between methods would be very important to adequately compare and standardize results. In order to do this a proficiency testing scheme would need to be set up similar to the testing that is done regularly for assays such as vitamin B6, using well defined samples that are measured by each method. Such a comparison would greatly enhance the clinical usefulness of erythrocyte MTX-PG measurement.

Although there is no golden standard for the measurement of erythrocyte MTX-PG, the company Exagen offers a commercial measurement of erythrocyte MTX-PG. [23] Although they only measure and report MTX-PG3, it enabled us to compare results when measuring the same sample set. The comparison of 60 samples showed good agreement between methods (LC-MS/MS=-0.89 [95% CI: -1.59 to 0.42] × FPIA – 0.87 nM [95% CI: 0.84 to 0.90], Figure 1). Considering the difference in detection method and sample preparation this is good and shows that results obtained using our method can be compared to results obtained from methods based on the method used by Exagen.

Due to the increasing evidence of the relation between MTX-PG and response in RA and JIA it would be beneficial to have a quick, easy-to-use assay for the measurement of the total MTX-PG content in erythrocytes that utilizes an existing, robust platform like the FLx immuno-analyser in order to enhance the clinical availability of this measurement.
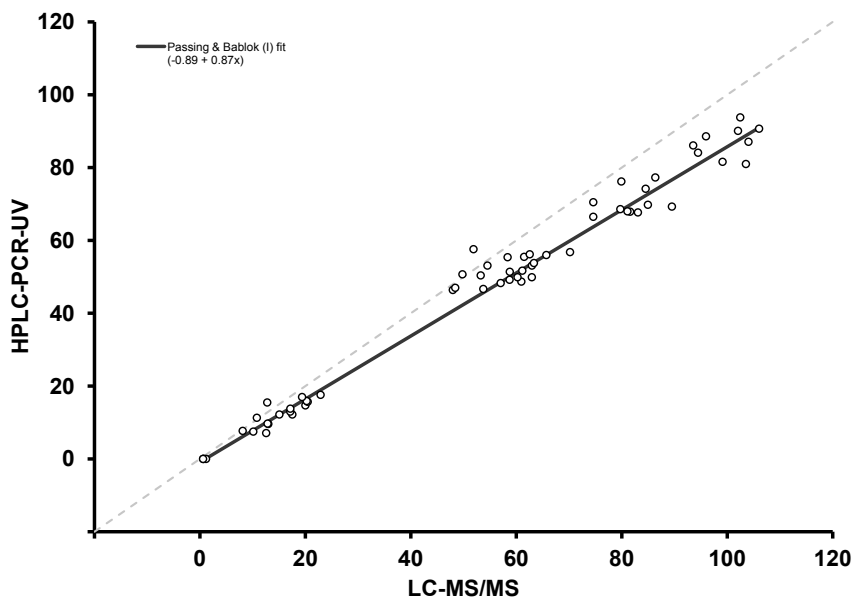
Figure 1. Preliminary results of the comparison of 60 samples between HPLC/UV and our LC/MS-MS method.

In *chapter 5* we describe our attempt to utilize an adapted FLx-based MTX assay for the quantitation of total erythrocyte MTX-PG content. This method utilizes the immunoassay's high cross-reactivity between MTX-PG species in order to measure total erythrocyte MTX-PG. Although the method performed well during validation using spiked erythrocyte samples, and Passing and Bablok method comparison between the FLx-based assay with our in-house validated LC-MS/MS assay showed no significant bias, a clear difference between both methods was visible. FLx measured samples generally yielded higher concentrations of total MTX-PG than the same samples measured on LC-MS/MS. Furthermore, the correlation between FPIA and LC-MS/MS in patient samples was much poorer than in calibration samples (R^2 = 0.42 vs 0.999), which generated large differences in total erythrocyte MTX-PG levels in individual patients between methods.

Although we demonstrate that an adapted FLx-based assay can be used to measure erythrocyte total MTX-PG levels, the method in general shows a positive bias, low specificity and high imprecision. This might be partially due to pre-analytical variability as the turbidity and color were not consistent between different samples which might influence the measurement or be a reflection of extraction efficiency. Interference from cross-reacting MTX metabolites such as DAMPA and 7-OH-MTX might also yield these differences. [19, 21, 27] While the FLx-based assay seems promising, sample preparation needs to be further optimized before it can be utilised in a clinical setting. As the method cannot be translated directly from the original article [28], this study shows the importance of cross-validating newly developed or implemented methods with a (reference) method on a different platform using real patient material.

Chapter

9

Based on our studies we advise the following concerning the measurement of plasma or erythrocyte MTX:

•   When using mass spectrometry the use of stable isotopes for each individual MTX-PG species is mandatory for quantification;

•   LC-MS/MS should be the preferred method for routine clinical measurement of erythrocyte MTX-PG levels in low-dose MTX therapy;

•   MALDI-QqQ-MS/MS could be a useful method in large-scale epidemiological studies, especially coupled with stable isotope internal standards;

•   LC-MS/MS is the preferred method to monitor plasma MTX in patients on glucarpidase therapy;

•   Methods that use immunoassays to measure intracellular MTX should not be used in patients because of the high imprecision;

•   A proficiency testing scheme should be developed for erythrocyte MTX-PG levels in order to standardize the different methods;

•   More research should indicate whether other tissues such as WBC are also/more useful to monitor MTX response in patients;

•   More research will need to be done to determine which MTX-PG species correlates best to response in order to simplify measurement.

Aim 2: determinants of erythrocyte MTX-PG in low-dose MTX treatment

In order to be able to individualize treatment with MTX, it is important to understand the factors that influence the intracellular levels of MTX-PGs. Therefore, in *chapter 6* we investigated which biological, genetic and behavioral factors influence the intracellular levels of MTX-PGs using two independent prospective cohorts.

Our results show that in both cohorts age, MTX dosage, erythrocyte folate content and the FPGS rs4451422 SNP were major determinants of intracellular MTX-PG levels at three months of treatment. We chose the MTX-PG levels at three months of treatment as in our study this was the first clinical decision point for the rheumatologist, after which medication would be adapted if necessary. When tailoring treatment to the individual patient it is important to know which factors determine the MTX-PG levels at the earliest time-point relevant to treatment in order to optimize the use of these factors.

There have been multiple studies focusing on the determinants of MTX-PG levels during treatment and previous research found dose of MTX, route of administration, age and renal function to be strongly associated with MTX-PG levels. [5, 29, 30] In concordance with this data, we found MTX dose and age to be strong determinants. However, renal function was not significantly associated with MTX-PG levels in our cohorts.

The discrepancy between results from previous studies and our study can be partially explained by the cross-sectional nature of the cohorts that were used in previous studies. In our study, patients were prospectively followed while other studies used patients that had been treated with MTX for up to 18 years. The MTX-PG accumulation over such a

long period of time would be very different, as patients will have received multiple dosage adjustments. Also, long-term MTX-PG levels might be controlled by different factors.

Despite strong correlations, the determinants found in this study only explained up to 21% of the variability in the derivation cohort, and even less in the validation cohort (up to 10%). This was also seen in the combined cohort where only up to 16% of variability was explained by the model which included dose of treatment. This is lower than found previously in a cross-sectional study of RA patients [30], where up to 30% of the intracellular variability in MTX-PG levels was explained by dose of treatment, route of administration age and renal function. This indicates that other, as of yet undiscovered, factors influence the MTX-PG status besides the factors found in our study.

One such factor could be the alternative splicing of FPGS [31], which has been shown to influence response to MTX in leukemia cell lines. Alternative splicing leads to loss of function of FPGS, resulting in a different polyglutamylation status and loss of MTX retention in the cell. Another possibility could be differences in methylation status [unpublished data] [32], which could lead to differences in expression of the folate pathway genes, thereby leading to variation in MTX uptake, or metabolism.

Finally, compliance is a very important factor as it is directly connected to exposure and known to be poor in chronic diseases. [33-35] We did not include any measure for the above mentioned factors in our study and these should be explored in future studies. Measurement of erythrocyte MTX-PG levels could also be very useful to monitor compliance in patients on low-dose MTX.

In our cohorts, the MTX-PG levels were similar to those previously reported in cross-sectional studies. [2, 23, 29, 30, 36] This was unexpected because in these cross-sectional studies patients were treated with MTX anywhere between 3 months and 18 years. While other studies used lower dosages of MTX, the longer time of treatment combined with the previously reported time to steady state of the erythrocyte MTX-PGs [37] suggested that more time was needed to reach similar levels and as we looked at the prospective accumulation at three months of treatment, we expected the MTX-PG levels to be (much) lower. This shows that the bulk of the MTX-PG accumulation occurs during the first three months.

Aim 3: To examine the prospective accumulation of MTX-PGs in erythrocytes during low-dose pulse MTX treatment and its association with response

Although the MTX-PGs are potential targets for a TDM based approach to RA treatment, not much is known about the speed of accumulation and distribution of intracellular MTX-PGs or what effect different treatment dosages have. In *chapter 7*, we focused on the accumulation of MTX-PG in erythrocytes over the first 9 months of treatment in two separate prospectively collected cohorts that have been treated with two different dosage regimes. We found that for both cohorts, the largest part of the accumulation of the short- and medium-chain MTX-PGs occurred during the first 3 months of treatment with only a

Chapter

9

marginal increase in the months thereafter. Steady state levels for all MTX-PGs were reached around 6 months of treatment in both cohorts. However, for the long-chain MTX-PGs the low-dose (15 mg/week) treated cohort took significantly longer to achieve similar levels than the cohort treated with a higher dose (25 mg/week). After 9 months of treatment the low-dose treated patients had achieved similar levels of long-chain MTX-PGs, but these constituted a significantly smaller part of the total erythrocyte MTX-PG content.

During the early phase of treatment, low-dose treated patients had significantly higher levels of the short-chain MTX-PGs but lower levels of long-chain MTX-PGs. Patients treated with low-dose MTX also had a selective redistribution of MTX-PGs towards short-chain MX-PGs whereas in high-dose treated patients the distribution favored the long-chain MTX-PGs. This difference in MTX-PG distribution persevered throughout the 9 months that patients were followed, even though at 9 months of treatment the total MTX-PG levels were not different between cohorts.

Interestingly, in the few patients that received parenteral MTX, there was no difference in distribution or concentration of the MTX-PG levels between cohorts, despite a difference in treatment dose. But parenteral MTX did lead to similar effects as the high dose treatment, i.e., lower levels of short chain MTX-PGs and higher levels of long chain MTX-PGs.

Long chain MTX-PGs are considered to be the more active MTX-species. As higher treatment dose of MTX led to a faster accumulation and higher concentrations of long-chain MTX-PGs as well as resulting in a selective distribution towards long-chain MTX-PGs, we propose that more intensive dosage regimes may be more efficient in the treatment of RA. This is supported by a shift towards higher treatment doses and extended co-medication over time in the low-dose treated patients. In fact, half of the patients that started at low-dose MTX, were increased to 20-25 mg/week MTX after 6 months of treatment, whereas in the high-dose treated patients medication stayed more or less stable.

Because in our cohorts the main bulk of the accumulation of MTX-PGs happens during the first three months of treatment, we propose that the optimal point of MTX prediction will be during this time, when accumulation is more likely to be in a linear phase, enhancing the differences between patients.

There have been multiple studies that show a relation between MTX-PGs and clinical response. However, no large prospective studies with a validation cohort have been performed. In *chapter 8* we investigate the relation of if the erythrocyte MTX-PG concentrations in RA patients are associated with disease activity or adverse events over time in two independent prospective RA cohorts. Although we did not find a relation between the erythrocyte MTX-PGs and adverse events, we found that in both cohorts an increase in erythrocyte MTX-PG concentrations at three months of treatment was associated with a decrease in disease activity during the first 9 months of treatment as measured by the DAS28. Erythrocyte MTX-PG levels are therefore a potential tool for therapeutic drug monitoring of MTX in RA.

In this study a cut-off value of 74 nmol/L erythrocytes of total MTX-PG was determined at three months of treatment, which can be used for the identification of moderate/good-responders to MTX. Patients below this cut-off might benefit from a dose increase to achieve lower disease activity. Maybe more importantly, this cutoff will be able to identify patients that have high MTX-PG concentrations (above the cut-off) but show no decrease in disease activity. These patients will have no benefit from increased MTX and will need additional medication to achieve remission of disease activity.

Based on this study, MTX-PG2 and MTX-PG3 would be the most suited candidates for TDM for the prediction of clinical response. MTX-PG2 was slightly superior to MTX-PG3 in terms of the effect size. On the other hand, MTX-PG3 is more abundant and therefore, can be detected more easily and with more (analytical) precision.

Although the established cut-off enables discrimination of moderate/good-responders to MTX at three months of treatment, from a clinical point of view, it would be preferable to predict response much earlier than at 3 months of treatment to adapt treatment to the individual patient as earlier and reach a decrease in disease activity as quickly as possible. Because of this, MTX-PG2 might be better candidate because of its much shorter accumulation half-time compared to MTX-PG3.

We strongly recommend future studies to investigate the predictive power of erythrocyte MTX-PGs in the first three months of treatment, preferably as soon as possible after the start of MTX treatment.

Having longitudinal data of MTX-PGs for 3 visits in first 9 months of MTX treatment in two different cohorts is unique. Even though the cohorts used in this study have different treatment regimens and differ in disease activity and baseline characteristics, the relation between erythrocyte MTX-PG and response is shown in both cohorts, supporting the conclusion that erythrocyte-MTX-PG levels are related to clinical response.

Future perspectives

In this thesis we have developed a method for the quantitation of MTX-PG in erythrocytes. Using this method we have investigated the erythrocyte MTX-PG content of multiple MTX treated RA patients in two independent prospective cohorts. We have determined some of the factors that influence the erythrocyte MTX-PG levels. By comparing two different treatment regimens we have shown that dosage drives the speed of accumulation and distribution of the MTX-PGs, and we have determined the relation between MTX-PG levels and response. However there is still much room for improvement and research.

Validation

We have determined a cut-off concentration which can be used for the identification of moderate/good-responders to MTX. Further validation will need to be done in order to determine the clinical use of the cut-off value and an erythrocyte MTX-PG based intervention study should be performed. Ideally future research will also determine cut-off value for non-

Chapter

9

response and non-compliance. Though these cut-off values will be of great use, the clinical use will be greatly enhanced if the cut-off values are translatable between methods and treatment strategies. In order to make the cut-off values useful on multiple methods, it is important that different methods are compared through standardization or harmonization of the MTX-PG assays.

Dried blood spots
While our LC-MS/MS based method is easily applicable and well suited for study and routine use, it would be more patient-friendly if patients do not need to have blood samples drawn at the clinic. Dried blood spots would be a possible solution as patients can spot these themselves from the comfort of their home and send them to the laboratory by regular post. This would help those patients that have low mobility due to the severity of their disease, as well as preventing high amounts of sick-leave and days off from work because patients need to attend the clinic to have blood drawn. This may be of special interest for application in remote areas and less developed countries where analytical laboratories are more difficult to reach.

White blood cells
We have used the erythrocyte MTX-PG levels in this project, but the physiological action of methotrexate (MTX) is most likely exerted by the white blood cells (WBCs). Because of this, the WBC MTX-PG levels might be a better predictor of response than the erythrocyte MTX-PG levels. A previous study found a relation between WBC MTX-PGs and response but in the same study it was found that the effect of erythrocyte MTX-PG was more pronounced and more significant.[8]

    Currently there is no clinically validated method for the measurement of MTX in WBCs in patient samples. The LC-MS/MS based method we developed could be adapted for the measurement of WBC MTX-PGs, however, a WBC based method may be analytically less robust because of the lower numbers of WBCs and the more complicated pre-analytical phase.

    Because erythrocytes lack an active MTX metabolism, the erythrocyte MTX-PG content is likely to be stable throughout sample handling and for at least two days at room temperature *(unpublished data)*. WBCs on the other hand contain an active MTX metabolism and will continue to process MTX-PGs during transport and sample preparation, possibly leading to irreproducible results. This may be averted by strict standardization of sample drawing and collection, but this will limit its use in a clinical setting, making the collection of a trustworthy sample population difficult.

Compliance
The determinants found in our study only explain a small part of the total variability of the erythrocyte MTX-PG levels, more research is needed to find out what other factors influence

this variability. An obvious and important target is compliance. Because compliance has been shown to be low in chronic patients, it is important to have a measure for compliance to be able to correct for levels of compliance. MTX-PG levels themselves might be an indicator of compliance as the extend of polyglutamylation is dose-(and therefore exposure-) driven. The use of MTX-PG levels as a measure for compliance needs to be verified first by comparison to an independent measure of compliance.

(epi-)genetics
In our study we have investigated the effect for different genetic polymorphisms in the folate metabolism. While this is an important part of determining the genetic factors that influence MTX levels. a great start, there are many more ways that genetics can influence the metabolism. Methylation, for example, might down regulate the expression of certain genes. It has been shown that certain disease types, and certain drugs influence methylation, and methylation might prove to be a factor in the variation of the inter-patient MTX-PG levels and response. Differences in gene splicing might also explain part of the variability of MTX-PG levels, and there are more ways that expression can be altered. This suggests that we should look not only into genetic polymorphisms, but also at the mRNA levels and eventual protein levels to build a complete picture of the genetic influences.

Interaction between DMARDS
Because of the strictly protocolized nature of the validation cohort, and the low number of patients in the derivation cohort we have not been able to study the possible interaction between the co-medication and MTX. As some DMARDS may interact, this is worth researching in order to optimise treatment.

Earlier prediction point
While we can, with reasonable certainty, discriminate non-responders from moderate/ good-responders, the timepoint at which we are currently able to do this is not optimal, as it coincides with the earliest clinical decision point of the rheumatologist. Ideally we would be able to predict in advance (preferably at the start of treatment) how, and if, a patients will respond to treatment. Using the MTX levels as a guide, this is of course not possible. But considering the fast build-up of MTX-PGs in the first three months of treatment, it is reasonable to assume that a better prediction point can be found in an earlier phase of treatment.

Interaction between MTX and folic acid
Patients treated with MTX also receive folic acid 'rescue' therapy, one day after taking MTX. This is hypothesized to reduce adverse events though it there is no empirical evidence for the treatment scheme. The patients in our cohorts had a significant increase in folate levels in both erythrocytes and serum over the course of treatment. Because MTX and folate use the

Chapter

9

same mechanism, increasing the folate levels this drastically could increase the competition for enzymes in the folate pathway, possibly reducing the working of MTX. In addition, it is not know which dosage of folic acid, or at what frequency folic acid should be given to reduce adverse events. As the erythrocyte and serum levels increase during treatment in our patient group, we consider the current folic acid dosage too high. More research should be done on the effect of folic acid supplementation and what would be the optimal strategy. Our study was not designed to determine this and we have not been able to correct for folic acid dose, frequency or time of intake in relation to MTX intake.

Chapter

9

References

1   Stamp LK, Roberts RL. Effect of genetic polymorphisms in the folate pathway on methotrexate therapy in rheumatic diseases. Pharmacogenomics. 2011 Oct;12(10):1449-63.

2   Dervieux T, Furst D, Lein DO, Capps R, Smith K, Caldwell J, et al. Pharmacogenetic and metabolite measurements are associated with clinical status in patients with rheumatoid arthritis treated with methotrexate: results of a multicentred cross sectional observational study. Ann Rheum Dis. 2005 Aug;64(8):1180-5.

3   Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum. 2006 Oct;54(10):3095-103.

4   Bulatovic Calasan M, den Boer E, de Rotte MC, Vastert SJ, Kamphuis S, de Jonge R, et al. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in juvenile idiopathic arthritis patients. Ann Rheum Dis. 2013 Nov 28.

5   Becker ML, van Haandel L, Gaedigk R, Lasky A, Hoeltzel M, Stobaugh J, et al. Analysis of intracellular methotrexate polyglutamates in patients with juvenile idiopathic arthritis: effect of route of administration on variability in intracellular methotrexate polyglutamate concentrations. Arthritis Rheum. 2010 Jun;62(6):1803-12.

6   Hobl EL, Jilma B, Erlacher L, Duhm B, Mustak M, Broll H, et al. A short-chain methotrexate polyglutamate as outcome parameter in rheumatoid arthritis patients receiving methotrexate. Clin Exp Rheumatol. 2012 Mar-Apr;30(2):156-63.

7   Hornung N, Ellingsen T, Attermann J, Stengaard-Pedersen K, Poulsen JH. Patients with rheumatoid arthritis treated with methotrexate (MTX): concentrations of steady-state erythrocyte MTX correlate to plasma concentrations and clinical efficacy. J Rheumatol. 2008 Sep;35(9):1709-15.

8   Angelis-Stoforidis P, Vajda FJ, Christophidis N. Methotrexate polyglutamate levels in circulating erythrocytes and polymorphs correlate with clinical efficacy in rheumatoid arthritis. Clin Exp Rheumatol. 1999 May-Jun;17(3):313-20.

9   Volmer DA, Sleno L, Bateman K, Sturino C, Oballa R, Mauriala T, et al. Comparison of MALDI to ESI on a triple quadrupole platform for pharmacokinetic analyses. Anal Chem. 2007 Dec 1;79(23):9000-6.

10 Meesters RJ, Cornelissen R, van Klaveren RJ, de Jonge R, den Boer E, Lindemans J, et al. A new ultrafast and high-throughput mass spectrometric approach for the therapeutic drug monitoring of the multi-targeted anti-folate pemetrexed in plasma from lung cancer patients. Anal Bioanal Chem. 2010 Dec;398(7-8):2943-8.

11 Meesters RJ, van Kampen JJ, Scheuer RD, van der Ende ME, Gruters RA, Luider TM. Determination of the antiretroviral drug tenofovir in plasma from HIV-infected adults by ultrafast isotope dilution MALDI-triple quadrupole tandem mass spectrometry. J Mass Spectrom. 2011 Mar;46(3):282-9.

12 van Kampen JJ, Burgers PC, Gruters RA, Osterhaus AD, de Groot R, Luider TM, et al. Quantitative analysis of antiretroviral drugs in lysates of peripheral blood mononuclear cells using MALDI-triple quadrupole mass spectrometry. Anal Chem. 2008 Jul 1;80(13):4969-75.

13 van Kampen JJ, Reedijk ML, Burgers PC, Dekker LJ, Hartwig NG, van der Ende IE, et al. Ultra-fast analysis of plasma and intracellular levels of HIV protease inhibitors in children: a clinical application of MALDI mass spectrometry. PLoS One. 2010;5(7):e11409.

14 van Kampen JJ, Burgers PC, de Groot R, Gruters RA, Luider TM. Biomedical application of MALDI mass spectrometry for small-molecule analysis. Mass Spectrom Rev. 2011 Jan-Feb;30(1):101-20.

15 Wagner M, Varesio E, Hopfgartner G. Ultra-fast quantitation of saquinavir in human plasma by matrix-assisted laser desorption/ionization and selected reaction monitoring mode detection. J Chromatogr B Analyt Technol Biomed Life Sci. 2008 Sep 1;872(1-2):68-76.

16 Hatsis P, Brombacher S, Corr J, Kovarik P, Volmer DA. Quantitative analysis of small pharmaceutical drugs using a high repetition rate laser matrix-assisted laser/desorption ionization source. Rapid Commun Mass Spectrom. 2003;17(20):2303-9.

17   FDA. Guidance for Industry - Bioanalytical Method Validation. http://wwwfdagov/downloads/Drugs/Guidances/ucm070107pdf. 2001.

18   Kumar VS, Law T, Kellogg M. Liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for monitoring methotrexate in the setting of carboxypeptidase-G2 therapy. Methods Mol Biol. 2010;603:359-63.

19   Albertioni F, Rask C, Eksborg S, Poulsen JH, Pettersson B, Beck O, et al. Evaluation of clinical assays for measuring high-dose methotrexate in plasma. Clin Chem. 1996 Jan;42(1):39-44.

20   Eksborg S, Albertioni F, Rask C, Beck O, Palm C, Schroeder H, et al. Methotrexate plasma pharmacokinetics: importance of assay method. Cancer Lett. 1996 Nov 29;108(2):163-9.

21   Al-Turkmani MR, Law T, Narla A, Kellogg MD. Difficulty measuring methotrexate in a patient with high-dose methotrexate-induced nephrotoxicity. Clin Chem. 2010 Dec;56(12):1792-4.

22   Zandvliet ML, Jacobs A, Den Boer E, meesters RJW, de Goede AI, Engels FK, et al. Monitoring of methotrexate levels following glucarpidase rescue treatment requires detection by mass spectrometry since immunoassay is not applicable. Eur J Hosp Pharm. 2012;19(2).

23   Dervieux T, Orentas Lein D, Marcelletti J, Pischel K, Smith K, Walsh M, et al. HPLC determination of erythrocyte methotrexate polyglutamates after low-dose methotrexate therapy in patients with rheumatoid arthritis. Clin Chem. 2003 Oct;49(10):1632-41.

24   van Haandel L, Becker ML, Williams TD, Leeder JS, Stobaugh JF. Measurement of methotrexate polyglutamates in human erythrocytes by ion-pair UPLC-MS/MS. Bioanalysis. 2011 Dec;3(24):2783-96.

25   Mo X, Wen Y, Ren B, Chen J, Xu C, Wang X, et al. Determination of erythrocyte methotrexate polyglutamates by liquid chromatography/tandem mass spectrometry after low-dose methotrexate therapy in Chinese patients with rheumatoid arthritis. J Chromatogr B Analyt Technol Biomed Life Sci. 2012 Oct 15;907:41-8.

26   Hawwa AF, Albawab A, Rooney M, Wedderburn LR, Beresford MW, McElnay JC. A Novel Dried Blood Spot-LCMS Method for the Quantification of Methotrexate Polyglutamates as a Potential Marker for Methotrexate Use in Children. PLoS One. 2014;9(2):e89908.

27   den Boer E, Heil SG, van Zelst BD, Meesters RJ, Koch BC, Te Winkel ML, et al. A U-HPLC-ESI-MS/MS-based stable isotope dilution method for the detection and quantitation of methotrexate in plasma. Ther Drug Monit. 2012 Aug;34(4):432-9.

28   Hayashi H, Fujimaki C, Tsuboi S, Matsuyama T, Daimon T, Itoh K. Application of fluorescence polarization immunoassay for determination of methotrexate-polyglutamates in rheumatoid arthritis patients. Tohoku J Exp Med. 2008 May;215(1):95-101.

29   Dervieux T, Zablocki R, Kremer J. Red blood cell methotrexate polyglutamates emerge as a function of dosage intensity and route of administration during pulse methotrexate therapy in rheumatoid arthritis. Rheumatology (Oxford). 2010 Dec;49(12):2337-45.

30   Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Frampton C, James J, et al. Determinants of red blood cell methotrexate polyglutamate concentrations in rheumatoid arthritis patients receiving long-term methotrexate treatment. Arthritis Rheum. 2009 Aug;60(8):2248-56.

31   Stark M, Wichman C, Avivi I, Assaraf YG. Aberrant splicing of folylpolyglutamate synthetase as a novel mechanism of antifolate resistance in leukemia. Blood. 2009 Apr 30;113(18):4362-9.

32   Liu Y, Aryee MJ, Padyukov L, Fallin MD, Hesselberg E, Runarsson A, et al. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. Nat Biotechnol. 2013 Feb;31(2):142-7.

33   Viller F, Guillemin F, Briancon S, Moum T, Suurmeijer T, van den Heuvel W. Compliance to drug treatment of patients with rheumatoid arthritis: a 3 year longitudinal study. J Rheumatol. 1999 Oct;26(10):2114-22.

34   de Klerk E, van der Heijde D, Landewe R, van der Tempel H, van der Linden S. The compliance-questionnaire-rheumatology compared with electronic medication event monitoring: a validation study. J Rheumatol. 2003 Nov;30(11):2469-75.

Chapter

9

35   de Klerk E, van der Heijde D, Landewe R, van der Tempel H, Urquhart J, van der Linden S. Patient compliance in rheumatoid arthritis, polymyalgia rheumatica, and gout. J Rheumatol. 2003 Jan;30(1):44-54.

36   Stamp LK, O'Donnell JL, Chapman PT, Zhang M, James J, Frampton C, et al. Methotrexate polyglutamate concentrations are not associated with disease control in rheumatoid arthritis patients receiving long-term methotrexate therapy. Arthritis Rheum. 2010 Feb;62(2):359-68.

37   Dalrymple JM, Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Barclay ML. Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. Arthritis Rheum. 2008 Nov;58(11):3299-308.

Chapter

9

Addendum

Summary
Dutch summary/Nederlandse samenvatting
List of abbreviations
Publications
PhD portfolio
Acknowledgements/Dankwoord
About the Author

Summary

Methotrexate (MTX) is the 'anchor drug' in the treatment of Rheumatoid Arthritis (RA) due to its high efficacy, low cost and good safety profile. However, its use is hampered by a highly variable inter-patient response and 20-40% of patients do not respond adequately to treatment. Also, about 30% of patients suffer from adverse events leading to lower adherence and possible premature switching of medication by the treating rheumatologist.

The intracellular levels of MTX have been correlated with the response to treatment and similarly to the clinical response to MTX, the (time to reach) steady-state erythrocyte MTX levels are highly variable between patients. This link between inter-patient MTX response variability and the large inter-individual variation in the rate and extent of erythrocyte MTX accumulation suggests that erythrocyte MTX measurement may be a valuable tool for the clinician to individualize MTX treatment in an early phase in order to achieve faster disease remission and less erosive damage.

While MTX plasma levels can be measured easily, low-dose MTX is rapidly cleared from plasma. The therapeutic effects of MTX are thought to be mediated by its intracellular levels, which are more difficult to measure. MTX is transported into the cell primarily by the reduced folate carrier. Once in the cell, MTX-PG1 is rapidly converted to MTX polyglutamates (MTX-PGs). Polyglutamylation retains MTX in the cell because the MTX-PGs are a poor substrate for the MTX efflux proteins.

The objectives of this thesis were: 1) to develop a new, clinically applicable method for the measurement of MTX-PGs in erythrocytes, 2) the application of this method to determine the factors that influence the erythrocyte MTX-PG levels, and 3) examining the prospective accumulation of erythrocyte MTX-PGs and the association between erythrocyte MTX-PGs and response to treatment.

In *Chapter 2* we describe the development of a new bioanalytical assay to measure methotrexate polyglutamates in erythrocytes using a novel high-throughput rapid mass-spectrometric technology. This technology merges a high-repetition matrix-assisted laser desorption/ionization (MALDI) source with a triple quadruple mass analyzer platform. The great power of this system lies in the speed at which it can measure, it is at least twenty-five times faster than using the fasted LC-MS/MS based system currently available. Although at the time stable isotopes of the MTX-PGs were not yet available, acceptable precision, LOQ and linearity were achieved. This method will be very valuable when large cohort studies are being performed, especially with the addition of stable isotope internal standards.

Although the MALDI-MS/MS method is a promising method for the high-throughput analysis of MTX-PGs, for the routine clinical lab a more accessible technique is preferred that does not need such specialized and expensive equipment. In order to provide routine labs with a clinically applicable method of measuring MTX-PGs we developed an LC-MS/MS method that uses a standard UPLC-MS/MS system similar to what many clinical

labs have available. In **Chapter 3** the validation of this method is outlined. This method uses stable isotope-labelled internal standards making it more robust and therefore ideal for the use of clinical samples. The method was fully validated according to clinical chemistry and FDA guidelines and has high specificity and sensitivity. Because no complicated or expensive sample preparation is used it is ideally suited for the routine clinical lab.

In **Chapter 4** this method is adapted and validated for the measurement of MTX in plasma in order to complement our erythrocyte method. Compared with the in-house immuno-assay the LC-MS/MS based method performed at least similarly, while having a better resolution and larger linear range.

As not every lab has an LC-MS/MS system available but might be interested in measuring intracellular MTX-PG levels, an easy, accessible method for the determination of total MTX-PGs in erythrocytes is described in **Chapter 5**. Although the validation of this method was good, with reasonable accuracy and precision, it was carried out using spiked erythrocyte samples. When in the final phase of method validation the method was compared to our previously developed LC-MS/MS assay using patient samples, the immuno-assay based method displayed high proportional and constant bias as well as a very low precision (i.e. high spread).

We established the LC/MS-MS method for the determination of MTX-PG of erythrocyte samples from multiple prospective RA cohorts. In **Chapter 6** we investigate which factors influence the erythrocyte MTX-PG levels during the first three months of treatment. We measured various potential socio-demographic, clinical and biochemical determinants at the start of treatment and correlated them to MTX-PG levels in erythrocytes. We found that higher age, higher erythrocyte folate levels and the FPGS rs4451422 wildtype were all associated with higher medium and long-chain MTX-PG levels in both cohorts. Because both cohorts had too little variation in dose of treatment at baseline, the cohorts were combined to look at the difference of dosage. In the combined cohort, higher MTX dose had a strong association with higher levels of erythrocyte MTX-PG levels. These factors together explained up to 21% of the total variability of MTX-PGs, suggesting stronger associations are still to be found.

Because there is little information available about the effect of different MTX dosing schemes on the concentration, distribution and speed of accumulation of the intracellular MTX-PGs we investigated in **Chapter 7** how these are influenced by two different dosing schemes. We found that the main bulk of accumulation occurred during the first three months of treatment, with only marginal increase in the months thereafter. Patients treated with a lower dosage of MTX needed a longer time to reach the same levels of MT-XPGs as patients treated with a higher dose of MTX. Also, patient that were treated with a lower dose had a selective distribution towards the short-chain MTX-PGs, whereas high dosage treatment led to a larger percentage of long-chain MTX-PGs.

Finally, **Chapter 8** describes the relation between MTX-PGs and response in two prospective RA cohorts. We found that higher levels of MTX-PGs were associated with

a decrease in disease activity during the first 9 months of treatment. A cut-off value of 74 nmol/L erythrocytes was determined which can be used for the identification of moderate/ good-responders to MTX. We did not find an association between MTX-PGs and adverse events and there was no difference in frequency of adverse events between cohorts.

The data we present in this thesis show that therapeutic drug monitoring in rheumatoid arthritis is feasible and that erythrocyte MTX-PG levels can be used to this end. Furthermore, our results suggest that more intensive treatment (high-dose or parenteral administration) is better in reducing disease activity as higher levels of erythrocyte MTX-PGs are reached earlier in treatment, while adverse events do not differ from patients treated with low-dose MTX. Because the bulk of the accumulation of MTX-PGs happens during the first three months of treatment, we think that monitoring of MTX-PG levels will be the most valuable during this time. Additionally, apart from prediction of clinical (non-)response, measurement of erythrocyte MTX-PG may also be used to assess drug adherence.

Nederlandse samenvatting

Ruim 160.000 patiënten in Nederland lijden aan de gewrichtsziekte reumatoïde artritis (hierna: reuma). Elk jaar komen er ongeveer 7200 nieuwe patiënten bij. Reuma komt voor onder alle leeftijden, maar openbaart zich in de meeste gevallen tussen het 40e en 60e levensjaar. Vrouwen worden vaker getroffen door deze ziekte dan mannen. Reuma is een chronische ziekte, die (pijnlijke) ontstekingen veroorzaakt in de gewrichten. Indien reuma in een vroeg stadium wordt herkend, is de aandoening goed te behandelen. Echter, als de ziekte niet (tijdig) wordt behandeld heeft dit grote gevolgen: permanente gewrichtsschade, beperkingen in de mobiliteit en een sterke vermindering van de kwaliteit van leven. Bovendien kan het niet behandelen van de reuma leiden tot ontstekingen aan hart, longen en bloedvaten.

Methotrexaat (MTX) is één van de belangrijkste geneesmiddelen om reuma te behandelen. MTX wordt standaard als eerste geneesmiddel ingezet omdat het middel effectief, veilig en goedkoop is. Patiënten krijgen in eerste instantie een standaarddosering voorgeschreven. Als dit onvoldoende effect heeft, wordt de dosering aangepast. Ondanks het succes van dit geneesmiddel blijkt 20 tot 40% van de patiënten onvoldoende te reageren op MTX. Daarnaast ervaart 30% van de patiënten bijwerkingen, zoals mucositis, misselijkheid en algehele malaise, waardoor zij soms voortijdig stoppen met het innemen van MTX.

Op dit moment is er nog geen methode om de dosering van het medicijn op de individuele patiënt af te stemmen. Daarmee kan nog niet goed worden voorspeld hoe patiënten reageren op het gebruik van MTX. Als gevolg hiervan wordt iedere patiënt blootgesteld aan een onvoorspelbare zoektocht naar de juiste dosering totdat de gewenste dosering op basis van 'trial and error' kan worden vastgesteld. Dit leidt tot onnodige overlast voor patiënten en hogere kosten voor het bestrijden van reuma.

Uit eerder onderzoek blijkt dat de concentraties van MTX in de bloedcellen een mogelijk aanknopingspunt vormen om tot een voorspelbare, op de individuele patiënt afgestemde dosering van het medicijn te kunnen komen. De concentraties van MTX in de cel blijken namelijk sterk samen te hangen met de wijze waarop de patient reageert op de behandeling met MTX.

Bij behandeling met MTX is er niet alleen veel variatie in de mate waarin het medicijn effectief is, maar ook de tijd die nodig is om te komen tot stabiele MTX concentraties in de bloedcellen èn de uiteindelijke hoogte van die concentraties lopen erg uiteen. Dit suggereert dat het meten van MTX mogelijk een manier is om te komen tot een op de individuele patiënt toegesneden MTX dosering. Hierdoor kan reuma eerder en met minder overlast voor de patiënt worden bestreden.

Het therapeutisch effect van MTX wordt waarschijnlijk gemedieerd door de intracellulaire spiegels van MTX. Wanneer MTX wordt opgenomen in de cel door de reduced folate carrier, worden er stapsgewijs meerdere glutamaat groepen aan toegevoegd. Hierdoor worden de MTX poly-glutamaten (MTX-PG's) gevormd. Deze MTX-PG's zorgen ervoor dat de

intracellulaire MTX in de cel wordt behouden omdat MTX-PG's geen goed substraat zijn voor de export enzymen.

Dit proefschrift is het verslag van de zoektocht naar: 1) een nieuwe, klinisch toepasbare methode voor het meten van de intracellulaire MTX-PG spiegels, 2) het toepassen van deze methode voor het bepalen van de factoren die de hoogte van de intracellulaire MTX-PG spiegels beïnvloeden en 3) het onderzoeken van de opbouwsnelheid van MTX-PG's in de rode bloedcel en de relatie tussen MTX-PG's in de rode bloedcel en de afname van ziekteactiviteit.

Deze methode moet het voor de reumatoloog mogelijk maken om eerder en efficiënter in te grijpen waardoor de overlast voor patiënten wordt beperkt, de kosten voor de gezondheidszorg worden beperkt en patiëntentrouw bij het innemen van medicatie bevordert.

In *hoofdstuk 2* wordt de ontwikkeling van een nieuwe methode beschreven voor het meten van de intracellulaire MTX-PG spiegels in rode bloedcellen door middel van een nieuwe, zeer snelle massa-spectrometrische methode. Deze technologie maakt gebruik van een MALDI ionisatiebron gekoppeld aan een tandem massaspectrometer (MS/MS). De kracht van het systeem ligt in de snelheid waarmee er gemeten kan worden, per monster is het minimaal 24 keer sneller dan met de snelste huidige reguliere LC-MS/MS gebaseerde methode. Tijdens de ontwikkeling van deze methode waren er jammer genoeg nog geen stabiele isotopen beschikbaar. Desondanks werden acceptabele precisie, detectie limiet en lineariteit bereikt. Deze methode zal bijzonder waardevol blijken bij grote cohort studies en de toevoeging van een stabiele isotoop als interne standaard zal de metingen een stuk betrouwbaarder maken.

De MALDI-MS/MS methode is een veelbelovende methode voor de analyse van grote aantallen monsters. Echter, voor de meer routinematig ingestelde klinische laboratoria zou een meer toegankelijke techniek beter toepasbaar zijn. Om aan die behoefte te voldoen hebben wij een vloeistof chromatografische (LC) gebaseerde analyse methode ontwikkeld die gebruikt maakt van een standaard LC-MS/MS systeem waarover vele klinische laboratoria al beschikken.

In *hoofdstuk 3* wordt de validatie van deze methode beschreven. Tijdens het ontwikkelen van deze methode waren wel stabiele isotoop gelabelde standaarden beschikbaar, hierdoor is de methode extra robuust en daardoor ideaal voor het gebruik met klinische monsters. De methode is volledig gevalideerd op basis van klinisch-chemische en FDA richtlijnen en heeft hoge specificiteit en gevoeligheid. Omdat er geen ingewikkelde of kostbare monster-voorbehandeling word gebruikt is de methode ideaal voor het gebruik in het klinische laboratorium.

In *hoofdstuk 4* wordt deze methode aangepast en gevalideerd voor het meten van MTX in plasma. Vergeleken met de huidige 'gouden standaard' methode voor het meten van MTX in plasma heeft de in dit hoofdstuk ontwikkelde massaspectrometrische methode

een betere specificiteit en gevoeligheid voor MTX .

Aangezien niet elk laboratorium de beschikking heeft over een LC-MS/MS, maar wellicht wel de intracellulaire MTX-PG spiegels wilt meten, zou een snelle, makkelijke en toegankelijke methode uitkomst bieden. In **hoofdstuk 5** beschrijven wij zo een methode. Ondanks redelijke resultaten tijdens de validatiestap, was de uiteindelijke vergelijking van patiëntenmonsters tussen deze methode en de eerder ontwikkelde LC-MS/MS methode niet goed. Tussen methoden was er een hoge mate van spreiding en de beschreven methode had een lage precisie. Hierdoor is deze methode niet geschikt voor het betrouwbaar meten van patiëntenmonsters.

De methode ontwikkeld in **hoofdstuk 4** is toegepast op monsters van reumapatiënten uit meerdere prospectieve cohorten. In **hoofdstuk 6** onderzoeken we welke factoren de MTX-PG spiegels in de rode bloedcel beïnvloeden gedurende de eerste drie maanden van therapie. Hiervoor hebben we meerdere potentiele socio-demografische, klinische en biochemische determinanten gemeten bij aanvang van de behandeling. Deze hebben we vervolgens gecorreleerd aan de MTX-PG spiegels in de rode bloedcellen. Hieruit bleek dat in beide cohorten een hogere leeftijd, hogere folaat spiegels in de bloedcel en de wildtype variant van een polymorfisme in het gen dat MTX metaboliseerd (FPGS rs4451422) geassocieerd waren met hogere rode bloedcel MTX-PG spiegels. Omdat er in beide cohorten te weinig variatie in behandeldosis was om deze apart te toetsen, werden de cohorten gecombineerd om te kijken naar de invloed van het verschil in behandeldosis op de rode bloedcel MTX-PG spiegels. In het gecombineerde cohort bleek een hogere behandeldosis sterk geassocieerd was met de rode bloedcel MTX-PG spiegels. Samengenomen voorspellen de gevonden factoren tot 21% van de gevonden MTX-PG variatie. Dit suggereert dat er andere, nog sterkere associaties zijn die niet gevonden werden in dit onderzoek.

Omdat er weinig bekend is over het effect van verschillende doseringen op de concentratie, distributie en accumulatie snelheid van de intracellulaire MTX-PG's hebben wij in **hoofdstuk 7** gekeken hoe deze beïnvloed worden door twee verschillende doseringsschema's. Hier vonden wij dat het grootste deel van de opbouw van MTX-PG's in rode bloedcellen plaatsvindt tijdens de eerste drie maanden van behandeling, met slechts een kleine stijging in de maanden erna. Patiënten die behandeld werden met een lagere dosis MTX hadden meer tijd nodig om vergelijkbare spiegels te bereiken als patiënten die en hogere dosis MTX kregen. Tevens werden er bij lage MTX dosering minder additionele glutamaatgroepen toegevoegd aan de MTX-PG's terwijl er bij een hoge MTX dosering juist meer glutamaatgroepen toegevoegd.

Tenslotte beschrijven we in **hoofdstuk 8** de relatie tussen MTX-PG's en de reactie op behandeling in twee prospectieve studies. Hieruit bleek dat hogere rode bloedcel MTX spiegels geassocieerd waren met een sterkere afname in ziekteactiviteit tijdens de eerste negen maanden van therapie. We hebben een afkappunt kunnen bepalen waarmee er een onderscheid kan worden gemaakt tussen 'moderate/good responders' en 'non-responders.' Er werd geen associatie gevonden tussen MTX-PG's en bijwerkingen.

De data die in dit proefschrift zijn gepubliceerd laten zien dat therapeutic drug monitoring in reuma haalbaar is en dat de rode bloedcel MTX-PG spiegels hiervoor kunnen worden gebruikt. Verder suggereren de resultaten van dit onderzoek dat een hogere behandeldosis van MTX een effectievere methode is om reuma te bestrijden omdat er dan sneller hogere MTX spiegels worden bereikt waarvan wij hebben aangetoond dat deze gerelateerd zijn aan de afname van ziekteactiviteit, terwijl bijwerkingen niet afwijken van patiënten die met een lagere dosis worden behandeld. Omdat het grootste deel van de opbouw van de MTX-PG spiegels plaatsvind tijdens de eerste drie maanden van de behandeling verwachten we dat het monitoren van de MTX-PG spiegels het meest waardevol zal blijken in deze periode. De MTX-PG meting zou mogelijk ook kunnen worden ingezet voor het controleren van de therapietrouw van patienten.

List of abbreviations

7-OH-MTX	7-hydroxy methotrexate
ABC	ATP-binding cassette
ACR	American College of Rheumatology
ALAT	Alanine-aminotransferas
ALL	Acute lymphoblastic leukemia
Anti-CCP	Anti-cyclic citrullinated peptide antibody
CRP	C-reactive protein
CV	Coefficient of variation
DAMPA	4-amino-4-deoxy-N-methylpteroic acid
DAS	Disease Activity Score
DMARD	Disease modifying anti-rheumatic drug
eGFR-MDRD	estimated Glomerular filtration rate using Modification of Diet in Renal Disease formula
EMIT	Enzyme multiplied immunoassay technique
ESI	electrospray ionization
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FDA	United States Food and Drug Administration
FPGS	folylpolyglutamate synthase
FPIA	fluorescence polarization immunoassay
GGH	γ-glutamyl hydrolase
IQR	Interquartile range
IS	Internal Standard
JIA	Juvenile Idiopathic Arthritis
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLOQ	Lower Limit of Quantitation
LOD	Lower Limit of Detection
MALDI	Matrix-Assisted Laser Desorption/Ionization
MTX	Methotrexate
MTXd3	Methotrexate deuterium labeled internal standard
MTX-PG	Methotrexate polyglutamate
MTX-PG(M+6)	Methotrexate polyglutamate (13C5, 15N)- stable isotope labeled internal standard
MTX-R	Methotrexate in Rotterdam, Netherlands cohort
NSAID	Non-steroidal anti-inflammatory drugs (NSAID)
RA	Rheumatoid Arthritis
TDM	Therapeutic drug monitoring
tREACH	treatment in Rotterdam Early Arthritis CoHort

SD	standard deviation
SNP	Single nucleotide polymorphisms
S/N	signal-to-noise ratio
SPE	Solid phase extraction
ULOQ	Upper limit of quantitation
Var	Variant genotype
Wt	Wildtype
WBC	White Blood Cell
QC	Quality control

Publications

First author

1   den Boer E, Heil SG, van Zelst BD, Meesters RJW, Koch BCP, Te Winkel ML, van den Heuvel-Eibrink MM, Luider TM, de Jonge R. A U-HPLC-ESI-MS/MS-based stable isotope dilution method for the detection and quantitation of methotrexate in plasma. Ther Drug Monit. 2012 Aug;34(4):432-9.

2   den Boer E, Meesters RJW, van Zelst BD, Luider TM, Hazes JMW, Heil SG, de Jonge R. Measuring methotrexate polyglutamates in red blood cells: a new LC-MS/MS-based method. Anal Bioanal Chem. 2013 Feb;405(5):1673-81.

3   den Boer E, Koch BCP, Huisman R, de jonge R. Using Fluorescence Polarization Immunoassay for Determination of Erythrocyte Methotrexate-Polyglutamates, a quick and easy test? Ther Drug Monit. *(accepted)*

4   den Boer E, de Rotte MCFJ, Pluijm SMF, Heil SG, Hazes JMW, de Jonge R. Determinants of erythrocyte methotrexate polyglutamate levels in rheumatoid arthritis. J Rheumatol. *(provisionally accepted)*

5   den Boer E, de Rotte MCFJ, Koch BCP, Hazes JMW, de Jonge R. Effect of two MTX dosing regimens on intracellular MTX accumulation in rheumatoid arthritis patients. *(in preparation)*

Second Author

6   Meesters RJW, den Boer E, Mathot RAA, de Jonge R, van Klaveren RJ, Lindemans J, Luider TM. Ultrafast selective quantification of methotrexate in human plasma by high-throughput MALDI-isotope dilution mass spectrometry. Bioanalysis. 2011 Jun;3(12):1369-78.

7   Meesters RJW, den Boer E, de Jonge R, Lindemans J, Luider TM. Assessment of intracellular methotrexate and methotrexate-polyglutamate metabolite concentrations in erythrocytes by ultrafast matrix-assisted laser desorption/ionization triple quadrupole tandem mass spectrometry. Rapid Commun Mass Spectrom. 2011 Oct 30;25(20):3063-70.

8   Bulatovic Calasan M, den Boer E, de Rotte MCFJ, Vastert SJ, Kamphuis S, de Jonge R, Wulffraat NM. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in juvenile idiopathic arthritis patients. Ann Rheum Dis. *(accepted)*

9   de Rotte MCFJ, den Boer E, de Jong PH, Pluijm SMF, Bulatovic Calasan M, Weel AE, Huisman AM, Gerards AH, van Schaeybroeck B, Wulffraat NM, Lindemans J, Hazes JM, de Jonge R. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in patients with rheumatoid arthritis. Ann Rheum Dis. *(accepted)*

Other

10   Meesters RJW, Cornelissen R, van Klaveren RJ, de Jonge R, den Boer E, Lindemans J, Luider TM. A new ultrafast and high-throughput mass spectrometric approach for the therapeutic drug monitoring of the multi-targeted anti-folate pemetrexed in plasma from lung cancer patients. Anal Bioanal Chem. 2010 Dec;398(7-8):2943-8.

11   Effect of methotrexate use and erythrocyte-methotrexate polyglutamate on glycosylated hemoglobin in rheumatoid arthritis compared with other therapies. de Rotte MCFJ, de Jong PHP, den Boer E, Pluijm SMF, Ozcan B, Weel AE, Lindemans J, Hazes JMW, de Jonge R. *(Accepted)*

PhD portfolio

Name PhD student:	Ethan den Boer
Erasmus MC Department:	Clinical Chemistry
Research School:	Erasmus Postgraduate school Molecular Medicine
PhD period:	2009-2014
Promotor:	Prof.dr. J. Lindemans
Co-promotors:	dr. R. de Jonge
	dr. T.M. Luider

Courses

LC-MS introductiecursus, Avans+	2010
Researchmanagement for PhD students and Postdocs, the Erasmus Postgraduate School Molecular Medicine (MolMed)	2010
Principles of research in medicine, Netherlands institute for health science (NIHES)	2011
Introduction to data-analysis, NIHES	2011
English biomedical writing and communication, Erasmus Medical Centre	2011
Development and Validation of quantitative LC-MS/MS assays for use in clinical diagnosis, American Association for Clinical Chemistry	2012
Workshop inDesign CS5 for PhD students and other researchers, MolMed	2012
Photoshop and Illustrator CS5, MolMed	2012
Presenting skills for scientists, MolMed	2012
Integrity in research, Erasmus Medical Centre	2014

(inter)national scientific meetings

6th International Conference of the Metabolomics Society, Amsterdam (poster)	2010
Molecular Medicine Day (MOLMED) 14th annual meeting, Rotterdam (poster)	2010
NVKC annual scientific meeting, amersfoort (poster)	2011
Molecular Medicine Day (MOLMED) 15th annual meeting, Rotterdam (poster)	2011
Mass spectrometry: applications to the clinical laboratory 4th annual conference, San Diego, USA (oral presentation + poster)	2012
NVKC annual scientific meeting, amersfoort (oral presentation +poster)	2012

Molecular Medicine Day (MOLMED) 16th annual meeting, Rotterdam (poster)	2012
Annual European congress of rheumatology (EULAR) 2013, Madrid, Spain (poster)	2013
Annual European congress of rheumatology (EULAR) 2013, Paris, France (poster)	2014

Teaching

Supervising research internship of HLO student 7 months	2011-2012
Supervising research internship of HLO student 5 months	2012
Supervising research internship of University Minor student 5 months	2013

Other activities

PhD-student committee member of the MolMed Postgraduate School, ErasmusMC	2010-2014
Organising committee of the ´Get out of your lab days´, a scientific and cultural retreat for PhD-student of the MolMed Postgraduate School, ErasmusMC	2012
Organising committee of the yearly scientific gathering of the MolMed Postgraduate School, ErasmusMC	2012
Abstract committee MolMed day	2013
Organising and maintaining the blood sample logistics of several clinical studies conducted at the department of Clinical Chemistry of the EMC	2010-2013

Grants and awards

Reumafonds conference grant for The European League Against Rheumatism (EULAR 2013)	2013
Mass Spectrometry Applied to the Clinical Lab (MSACL2012) young investigator travel award	2012
Erasmus MC pilot grant (overall coordinator) for the project: Using intracellular methotrexate levels to monitor patient adherence in rheumatoid arthritis (50.000 euro)	2012

Dankwoord

Het is af, klaar, over; mijn proefschrift is gearriveerd. Hoewel ik er ruim vier en een half jaar over gedaan heb, lijkt het allemaal te snel te zijn gegaan en met veel te weinig resultaat; ik heb zeker nog voor drie jaar werk liggen aan ideeën en data.

Uiteraard zijn er vele mensen die op de een of andere manier, op meer of mindere wijze hebben bijgedragen aan dit boekje. Bij deze wil ik allen bedanken voor de hulp en steun die jullie mij gegeven hebben.

Hoewel ik niet zo'n fan ben van uitgebreide dankwoorden wil ik toch een paar mensen specifiek bedanken.

Mijn promotor, Jan Lindemans. Beste Jan, ik ben je zeer dankbaar voor het mogelijk maken van mijn promotie door het aannemen van iemand die al bijna vier jaar afgestudeerd was en eigenlijk niet ideaal aansloot bij de functie, ik dus. Vijf jaar geleden had ik eigenlijk de hoop op een AIO-plaats al opgegeven dus toen ik in juni 2009 jouw bericht kreeg aangenomen te zijn als UD op de afdeling Klinische Chemie was ik zeker zeer verrast. Ook toen bleek dat uiteindelijk ging om die AIO-plaats waar ik op had gesolliciteerd was ik nog steeds blij verrast, ik heb dan ook geen moment getwijfeld en het aanbod aangenomen. Hoewel je tijdens het project wat op de achtergrond bent gebleven, stond je altijd klaar om vragen te beantwoorden of te helpen.

Mijn co-promotor, Robert de Jonge. Beste Robert, jouw losse stijl van leiding geven sloot perfect aan bij wat ik prettig werken vond. Ik heb in ieder geval het gevoel gekregen dat ik alles zelf deed en bedacht, met slechts wat sturing van jouw kant. Hoe dit verhoudt tot de werkelijkheid is maar de vraag. Vooral ook bedankt voor het geduld om eindeloos de slordigheden in mijn stukken aan te stippen en verbeteren, ik vrees dat ik daar nog wel even aan moet werken. Ook je hulp met het zoeken naar geldbronnen om het project iets te verlengen of te vervolgen ben ik je zeer dankbaar voor. Ik hoop ten zeerste dat de aanvraag bij ZONMW doorgaat zodat we onze samenwerking kunnen voortzetten.

Bertrand, bedankt voor alle praktische en theoretische hulp bij het opzetten van de MS methoden en het regelmatige trouble-shooten, ook op afstand en buiten werktijd. Zonder jou was de MS methode waarschijnlijk niet zo snel opgezet of zo degelijk gevalideerd. Ook de (vaak bijzondere) gesprekken heb ik altijd zeer gewaardeerd, het is zelden dat ik een gelijke vind in directheid, interesses en hoeveelheid genuanceerde opmerkingen. Ik ben dan ook heel blij dat je mijn paranimf wilt zijn.

Mijn vader, die soms meer gemotiveerd leek dan ik en die ondanks het niet altijd begrijpen van mijn onderzoek nooit afliet het in ieder geval te proberen. Bedankt voor je steun, de gesprekken, het op korte termijn nakijken van lekensamenvattingen en het (ad hoc) opvangen van de kinders. Ik vind het fijn dat je mij ook bij deze belangrijke gebeurtenis wilt steunen door als paranimf aan mijn zijde te staan.

Professor Hazes, Mieke, bedankt voor de nuchtere klinische blik op de artikelen waar jij co-auteur was, en ook voor de fijne samenwerking in het schrijven van nieuwe (jammer genoeg nog niet succesvolle) projectaanvragen. Ik hoop dat we in de toekomst nog eens samen kunnen werken.

Sandra Heil, bedankt voor de begeleiding en goede raad omtrent het AIO-traject en ook voor de leuke gesprekken van de afgelopen jaren.

Saskia Pluijm, heel erg bedankt voor de hulp met de statistiek waar ik altijd zo'n hekel aan had en nu (mede dankzij jou) graag meer in zou willen doen. Ook was het geweldig iemand tegen te komen met een gelijke liefde voor koffie.

Maurits de Rotte, het was zeer prettig met jou samenwerken, vooral het sparren over resultaten en onderzoekslijnen was bijzonder waardevol. Ik wens je veel geluk met je nu nog prille huwelijk, en ook met je aankomende promotie waarna de stand tussen je Florentien en jij weer gelijk is (wil je een nieuw naambordje?).

Monique de Waart, bedankt voor het eindeloze (on)geduld waarmee je altijd weer klaarstond om antwoord te geven op (soms de meest idiote) vragen, te helpen met de logistiek van de bloedmonsters en dat je ondanks de problemen met sommige monsters altijd bereid was om te blijven helpen.

Verder natuurlijk het lab, Samira voor de gezelligheid en het soms meeslepen naar koffie of lunch, Pamela for the movie (nerd) talk and sambal (did you ever get that recipe?), Pieter voor de hulp bij alles DNA en het helpen begeleiden van mijn studenten. Evert voor het MS overleg en de soms bijzondere gesprekken, Martin voor de droge opmerkingen en Marjan voor Marjan.

And my fellow PhD-students, Cherina, the undisputed 'queen-of-body-fluids', your enthusiasm and interest was at times enough to make my day, especially when the homefront was in turmoil, your never ending appetite for my wife's food will not be forgotten soon.

Maja, it was fun being your roommate, the help with all things pharmacological is still very much appreciated. I wish you much luck and love with your upcoming marriage.

Pooja, thank you for the help with R, I could not have made my splines without you. Also, thank you so much for the Indian food, just don't forget to bring more thingies.

And all of you for the nice conversations and discussions, despite not being overly present at lunch or dinners I really appreciated your company. I wish you all much luck with your projects.

De mensen van de stofwisseling en dan speciaal Wilma, Wim en Peggy, jullie hadden een bijna eindeloos geduld ondanks dat ik constant de planning overhoop gooide door mijn tijden verkeerd in te schatten, monsters tussendoor wilde draaien, weer eens niets kon

vinden of gewoon in de weg liep.

Rob en Marga, voor het helpen met de Sysmex helemaal aan het begin van mijn project, en het altijd helpen als ik bloedmonsters nodig had of hulp met de analyes/microscopie.

Ook mijn studenten, Sander, Trung en Anniek, voor de hulp en de chaos die zij verzorgden, het was leuk (en leerzaam) jullie te begeleiden.

Mijn vrienden, en dan specifiek Michael, Bob (Dennis) en Dennis voor jullie begrip voor afgezegde afspraken, verjaardagen, trainingen, game-nights etc. Michael, nog reuze bedankt voor het maken van de mooie splines plaatjes, jammer dat ze hier niet meer in zijn gekomen, maar ze zullen zeker gebruikt worden.

Mijn schoon moeder, Doris, die altijd klaar stond op de kinderen te passen zodat ik mij kon richten op mijn project of naar een congres kon.

Mijn twee zoons, Devin en Aedyn, voor het vermaak en de ontspanning, voor het nooit ech begrijpen wat ik nou deed en waarom ik het nou leuk vond (Devin) en het vertellen op school dat ik heel de dag met bloed speel (Aedyn).

En als laatste en belangrijkste, mijn vrouw (mijn allesie ^_^), Melanie, bedankt voor het mogelijk maken van de afgelopen jaren, zonder jou was dit proefschrift er überhaupt niet gekomen. Zelfs in de tijden dat het minder ging en je moeder in het ziekenhuis lag bleef je me helpen en steunen, zij het soms met wat gemor.
En ondanks het feit dat ik zelden op de afgesproken tijd thuis was, soms op onmogelijke tijden nog wilde werken, altijd te laat was voor het eten, je nooit precies snapte wat ik nou uitvoerde op het Erasmus en dat je van tijd tot tijd 'part-time single mom' was, heb je me altijd geprobeerd te motiveren en ook aan het werk geschopt waar nodig.

About the Author

Ethan den Boer was born on April 20th 1982 in Rotterdam, the Netherlands. After finishing secondary school in 1999 at the Rotterdam Montessori Lyceum he started a Bachelor of Applied Science in Biochemistry at the Rotterdam University of Applied Sciences and graduated in 2003. In that same year he started his Masters studies in Cellular and Molecular Biology at Leiden University which he finished in 2005.

From 2006 he worked as a research technician at DSM anti-infectives, from which he left in 2008 to work as a research fellow at the LACDR (Leiden-Amsterdam Centre for Drug Research).

In September 2009 he started to work on the research project described in this thesis at the department of Clinical Chemistry of the Erasmus MC, Rotterdam under the supervision of prof.dr. J. Lindemans and dr. R. de Jonge. During this project Ethan collaborated closely with the department of Rheumatology of the Erasmus MC, Rotterdam headed by prof.dr. J.M.W. Hazes.

In this period he was also active as member of the PhD-committee of the MOLMED (Molecular Medicine) post-graduate school.