A Toolbox for Personalized Medicine of Methotrexate Therapy in Arthritis

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A Toolbox for Personalized Medicine of Methotrexate Therapy in Arthritis

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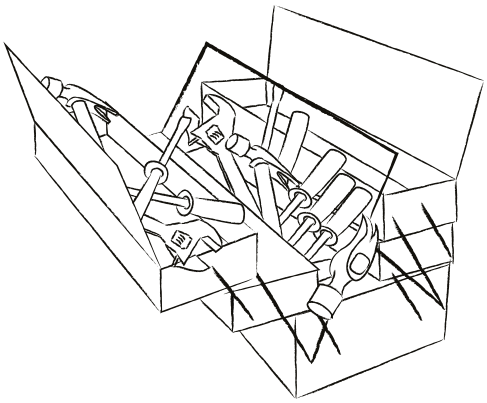
Aan mijn ouders en Florentien

Every man builds his world in his own image. He has the power to choose, but no power to escape the necessity of choice. *(Ayn Rand, Atlas Shrugged, 1957)*

CONTENTS



CHAPTER 1

General Introduction

Partly based on: M.C.F.J. de Rotte, E. den Boer, M. Bulatović Ćalasan, M.W. Heijstek, M.L. te Winkel, S.G. Heil, J. Lindemans, G. Jansen, G.J. Peters, S.S.M. Kamphuis, R. Pieters, W.J.E. Tissing, M.M. van den Heuvel-Eibrink, J.M.W. Hazes, N.M. Wulffraat, R. de Jonge. Personalized medicine of methotrexate therapy. *Nederlands Tijdschrift voor Klinische Chemie en Laboratoriumgeneeskunde (2012) 37 (1): 50-53*.

Arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease. It is characterized by swelling and pain of the joints, uncontrolled proliferation of synovial tissue and multisystem co-morbidities like cardiovascular disease and thyroid disease. RA mainly affects the joints of the extremities like hands, feet, knees, wrist and elbows. Joint damage can occur early in the disease course when the disease is not treated effectively. More than 21% of United states adults (46.4 million persons) were found to have self-reported physician diagnosed arthritis. The specific diagnose of RA has a prevalence of 1%.[1] The prevalence of RA among women is approximately double that in men. There is still no cure for RA, despite the fact that treatment strategy has changed considerably over the years. Early initiation of therapy is effective in prevention of joint damage and results in milder medication regimens while maintaining disease remission.[2-5] Early in the disease, 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. For RA, a prediction model has been developed that divides patients into 3 groups according to their likelihood of progressing to persistent arthritis.[6]

Juvenile idiopathic arthritis (JIA) is one of the most common autoimmune diseases in childhood with a reported prevalence between 16 and 150 per 100.000.[7] JIA is a heterogeneous disease, characterized by chronic inflammation of one or more joints, which begins before age of 16, persists for more than 6 weeks and is of unknown origin.[7,8] It encompasses various subtypes, defined by the International League of Associations for Rheumatology (ILAR) criteria, whose severity and clinical course differ.[8,9] Although heterogeneous, the common denominator in JIA is chronic arthritis, which can lead to joint destruction and long-term disabilities.[7,8,10] This puts a heavy toll on children, their parents and society.[11,12]

Such serious consequences in both RA and JIA put the aim of attaining tight disease control in these arthritic diseases to the forefront.

Methotrexate therapy in arthritis

High-dose methotrexate (MTX) originally was developed in the 1940s as a chemotherapeutic drug in the treatment of neoplastic diseases such as (pediatric) Acute Lymphoblastic Leukemia (ALL) and other proliferative diseases.[13] In the 1970s and 1980s, it appeared that low-dose MTX also was effective for the treatment of RA and JIA and in today's practise is the cornerstone disease-modifying anti-rheumatic drug (DMARD) in treating these diseases. Although MTX is an effective drug, there is large inter-individual variation in the efficacy and toxicity of MTX limiting its use.[14-16] In RA and JIA, efficacy varies between 30-70% depending on the treatment regime and outcome measure. In RA, 10-30% of patients discontinue MTX because of toxicity.[17] In children with JIA, hepatotoxicity and gastrointestinal intolerance are major problems and are the main reasons for MTX withdrawal.[18,19] In current practice, MTX is administered based on historical precedent rather than on scientific knowledge and it is seldom individually

tailored, implicating a wide range in MTX levels and variability in adverse events.[15] Patients who do not respond to MTX or develop severe adverse events within 3 months after MTX start are frequently given biologicals, alone or in combination with MTX.[2]

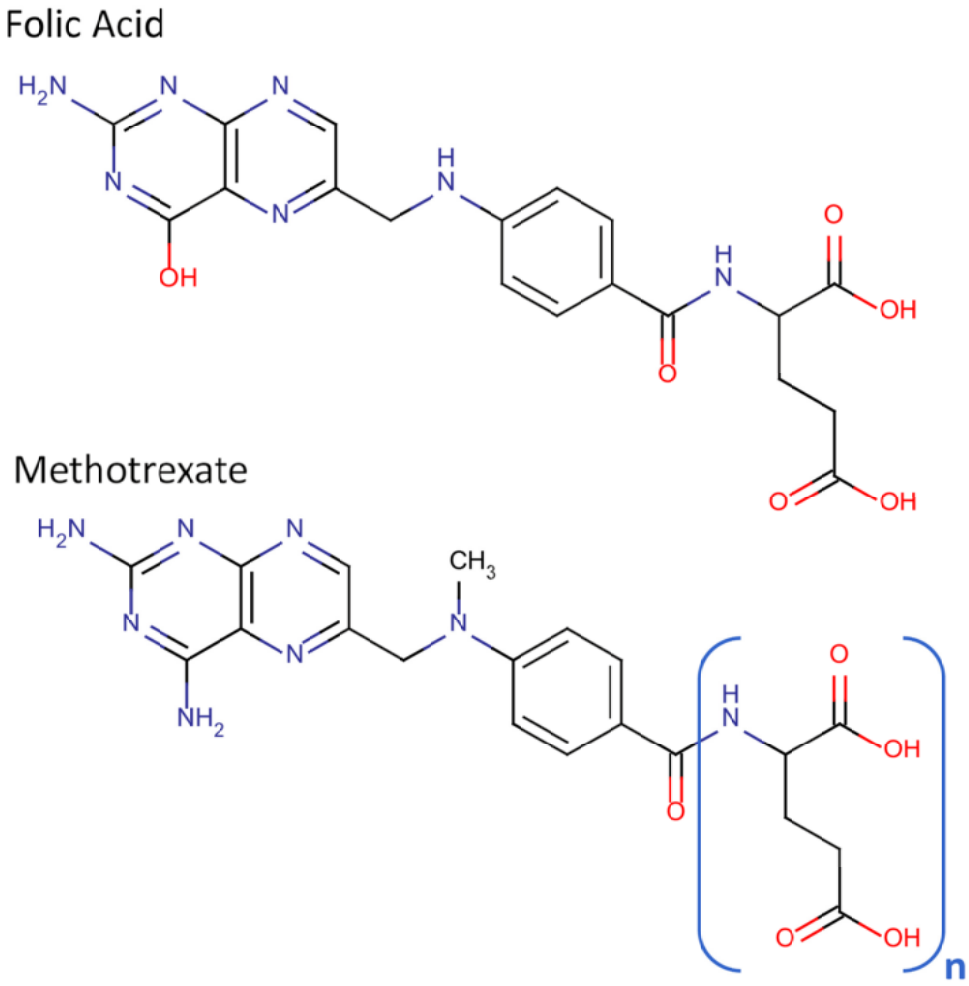


Figure 1 Molecular structures of folic acid and methotrexate. The glutamate group of methotrexate is indicated between brackets, up to 4 additional (n=5) glutamate groups are added during polyglutamation in the cell. *Courtesy of dr. E. den Boer.*

Prediction of MTX non-response and MTX-induced adverse events before MTX start is paramount since the first months upon diagnosis represent a window of opportunity during which outcomes can be more effectively modulated by therapy.[20] To ensure that only patients unresponsive to MTX receive early additional treatment with biologicals

and those responsive to MTX are spared costly biologicals, it is necessary to identify non-responders and patients prone to experience adverse events at baseline. In order to identify these patients, prediction models for MTX non-response and adverse events have to be developed. These prediction models could function as toolbox for personalized medicine of methotrexate therapy. Physicians could predict whether their patients will be unresponsive to MTX or develop adverse events before starting treatment and can adapt MTX dose or change to biologicals at forehand. To fill this toolbox, first association studies have to be done, so that possible determinants of MTX non-response and adverse events can be identified. To set the field for these association studies, we hypothesized that individual differences and derangements in the patient's metabolism of MTX modifies the response to MTX treatment. In order to understand the metabolism of MTX, the folate pathway has to be studied further, since MTX is a folate antagonist.

MTX, uptake, efflux and polyglutamylation

The molecular structures of folic acid and MTX are almost the same (figure 1). Folic acid is a synthetically produced form of folate and used in fortified foods and supplements. Folate is the naturally occurring form, found in food. MTX is a folate antagonist that uses the same transport mechanisms as folate.[21] MTX as well as food-derived folate or supplemented folic acid are incorporated into the folate pathway (one-carbon metabolism). In one-carbon metabolism, all forms of folate are taken up by the intestine and circulate in plasma as methyl-tetrahydrofolate (THF). Circulating MTX and methyl-THF are taken up into cells via the solute carrier 19A1 (*SLC19A1*), formerly known as reduced folate transporter (*RFC*), and are additionally transported into the cell via the solute carrier 46A1 (*SLC46A1*), formerly known as protein coupled folate transporter (*PCFT*), and folate receptors (*FOLR*) 1 and 2 (Figure 2).[22] In the intestine, most transport goes via *SLC46A1*. Members of the adenosine triphosphate (ATP) binding cassette (*ABC*) transporters including *ABCB1*, *ABCC* as well as *ABCG2* function as ATP-dependent MTX efflux transporters.[22] Intracellularly, MTX and methyl-THF are polyglutamylated (MTX-PG) by folylpolyglutamate synthetase (*FPGS*) to a variability of chain-lengths (PG2-5) competing with γ-glutamyl hydrolase (*GGH*) that deconjugates glutamate residues (Figure 2).[23] Polyglutamylation retains MTX intracellularly because it is no substrate for the MTX efflux proteins and a higher degree of MTX polyglutamylation leads to stronger inhibition of the target enzymes in one-carbon metabolism and purine the novo synthesis. In low dose MTX treatment, the pentaglutamate (PG5) is the highest order of glutamylation detected while the triglutamate form (PG3) of MTX predominates.[24,25] The polyglutamated forms of MTX inhibit enzymes in the one-carbon metabolism (figure 3). Single nucleotide polymorphisms (SNPs) in genes involved in MTX transport and polyglutamylation affect intracellular MTX accumulation.[26] Therefore, they could be a potential candidate for association studies, searching for determinants of MTX non-response and adverse events.

In RA and JIA, low dose MTX (15-25 mg/week in RA and 10-15 mg/m^2 in JIA) is given orally in a fixed dose that may be increased when response is insufficient; folic acid is used to prevent adverse events.

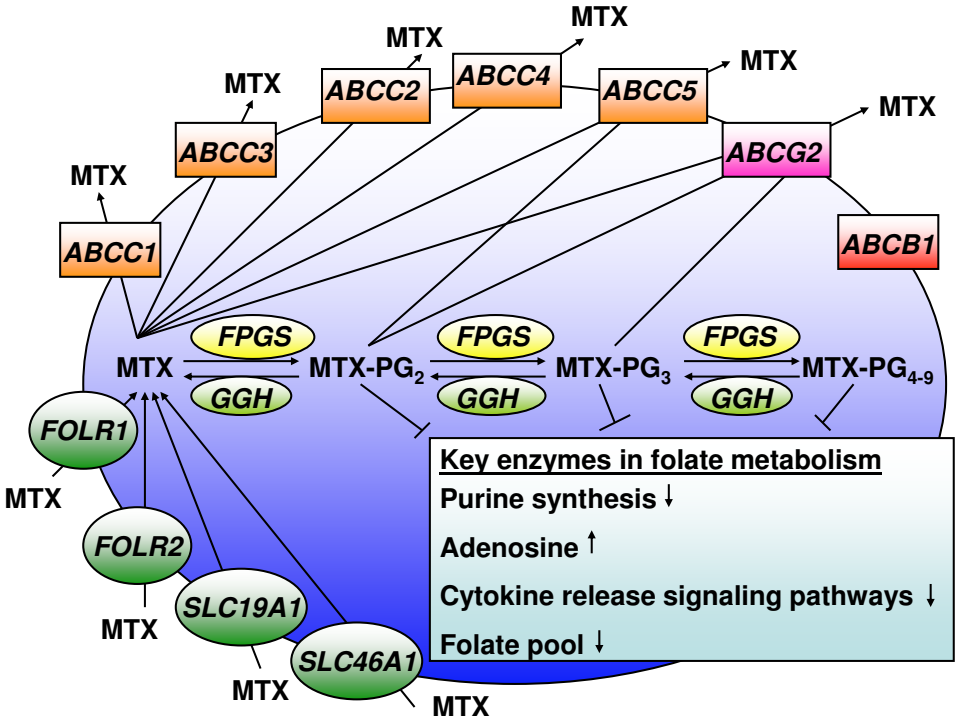


Figure 2 Cellular MTX transport routes for MTX influx and efflux in relation to polyglutamylation and mechanisms for arthritis suppression. MTX-PGs can inhibit several key enzymes in folate metabolism and therefore may cause a decreased de novo purine biosynthesis, increased adenosine release, direct or indirect effects on cytokine release signaling pathways and folate depletion, which all may lead to arthritis suppression. MTX, methotrexate; MTX-pg, methotrexate polyglutamates; *ABCB1*, adenosine triphosphate-binding cassette transporter B1; *FPGS*, folylpolyglutamate synthetase; *FOLR1*, folate receptor 1; *GGH*, γ-glutamyl hydrolase; *SLC19A1*, solute carrier 19A1. *De Rotte et al. Journal of Rheumatology (2012) 39 (10): 2032-2040.*

Subcutaneous or intramuscular MTX injections are given when response is insufficient, or when patients do not tolerate oral tablets. Oral MTX is actively absorbed in a capacity-limited process by the proximal jejunum. Because of the short half-live (6-15 hours), intermittent low dose MTX administration once a week does not lead to accumulation of MTX in plasma and hence, therapeutic drug monitoring with plasma MTX concentrations is not possible in low dose MTX treatment. Plasma MTX is mainly eliminated by the kidneys; 65-80% is eliminated within 12 hours after administration.

Inhibitory actions of MTX on one-carbon metabolism

Inside cells, MTX-PGs inhibit key-enzymes in one-carbon metabolism which is responsible for its therapeutic effects as well as its adverse-event profile. In one-carbon metabolism, methyl-THF is demethylated intracellularly to THF (figure 3). The formed THF is converted to methylene-THF by the vitamin B6 dependent serine-hydroxymethyltransferase (*SHMT*). Methylene-THF is on the cross-road for methylation or DNA synthesis. Methylene-THF is converted to methyl-THF by 5,10-methylenetetrahydrofolate reductase (*MTHFR*) which is the substrate for the vitamin B12 dependent methionine synthase reductase (*MTRR*). *MTRR* remethylates homocysteine into methionine. The formed methionine is transferred to S-adenosyl methionine (SAM). SAM is turned into S-adenosyl homocysteine (SAH) and hereby finally provides methylation for DNA, RNA, proteins and more molecules by losing a one-carbon molecule (figure 3). DNA-methylation is essential in signalling DNA transcription.

DNA synthesis is served by the one-carbon metabolism by making pyrimidines and purines. The enzyme thymidylate synthase (*TS*) uses methylene-THF to form pyrimidines and produces dehydrofolate (DHF). DHF is converted back to THF by dihydrofolate reductase (*DHFR*). Methylene-THF can also be formed into formyl-THF by methylenetetrahydrofolate-dehydrogenase (*MTHFD*). Formyl-THF can be used by 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (*ATIC*) to transform its substrate 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) finally into purines. Pyrimidines and purines are building blocks for DNA and are needed for cell proliferation of for instance leukocytes.

Intracellular MTX prevents cell proliferation and DNA-methylation by displacing the preferred substrates of the folate-dependent enzymes *DHFR*, *TS* and *ATIC*.[27] Levels of AICAR are elevated in the urine of RA patients treated with MTX.[28] The effect of the inhibition of *ATIC*, besides a reduced purine production is the accumulation of AICAR and its metabolites, which are inhibitors of adenosine deaminase (*ADA*) and adenosine monophosphate deaminase (*AMPD*). This inhibition results in increasing adenosine levels. The released adenosine induces a variety of anti-inflammatory effects.[28]

Important phenotypic markers of derangements in one-carbon metabolism, such as plasma-homocysteine, serum-vitamin-B12, serum-folate, erythrocyte-vitamin B6 and erythrocyte-folate may determine the extent of MTX non-response and MTX-related adverse events. Nevertheless, they have hardly been studied as risk factors of MTX outcome.[29,30]

Prediction of MTX efficacy and adverse events

Numerous association studies have been done that investigated the association between potential predictors and MTX response. Recently, the following clinical determinants of response to MTX and other DMARDs were summarized in an editorial review: male gender, non-smoking, early RA, DMARD naïve and disease activity.[31]

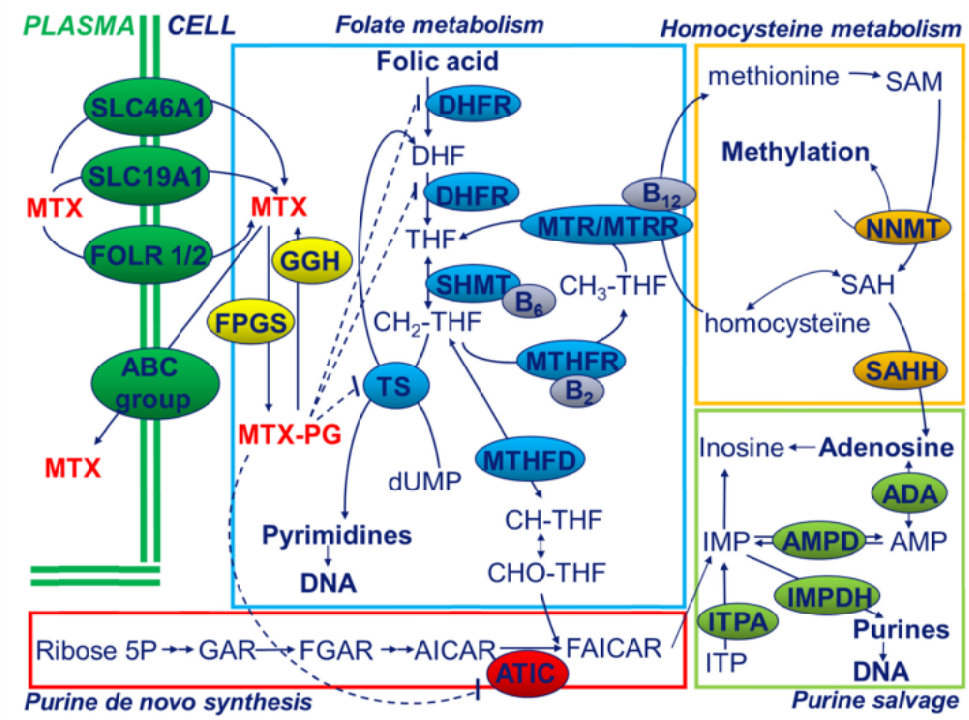


Figure 3 One-carbon metabolism and inhibitory effects of MTX. *SLC46A1*, solute carrier 46A1; *SLC19A1*, solute carrier 19A1; *FOLR1*, folate receptor; ABC group, Adenosine triphosphate binding cassette transporters; MTX, methotrexate; *GGH*, γ-glutamyl hydrolase; *FPGS*, folylpolyglutamate synthetase; *DHFR*, dihydrofolate reductase; *SHMT*, serine hydroxymethyltransferase; DHF, dihydrofolate; THF, tetrahydrofolate; *MTHFD*, methylenetetrahydrofolate-dehydrogenase; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase, *MTRR*, methionine synthase reductase; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; *NNMT*, nicotinamide N-methyltransferase; *SAHH*, S-adenosyl homocysteine hydrolase; *ADA*, adenosine deaminase; *AMPD*, adenosine monophosphate deaminase; AMP, adenosine monophosphate; IMP, inosine monophosphate; *ITPA*, inosine triphosphatase; *IMPDH*, inosine-5'-monophosphate dehydrogenase; *TS*, thymidylate synthetase; dUMP, deoxyuride monophosphate; *ATIC*, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; GAR; glicinamide ribonucleotide; FGAR, formil glicinamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, 5-formamidoimidazole-4-carboxamide ribonucleotide.

In a systematic review,[32] the following variables were found to be independent predictors of RA remission: male gender, young age, late-onset RA, short disease duration, non-smoker, low baseline disease activity, mild functional impairment, low baseline radiographic damage, absence of rheumatoid factor and anti-citrullinated peptide, low serum level of acute-phase reactant, interleukin-2, and RANKL at baseline, *MTHFR* 677T alleles and *MTHFR* 1298C alleles in the methotrexate treated patients.

Also, glucocorticoid response at 2 weeks is a useful tool for recognizing those patients who will probably have active disease after 3 months of DMARD treatment.[33]

In low dose MTX treatment such as in RA and JIA, various association studies for genetic determinants and MTX (non)response have been done.[27] SNPs in *ABCB1*, *ABCC2*, *AMPD1*, *MTHFR, MTRR, SLC19A1, TS* and *GGH* were associated with (non)response in RA.[27] In JIA, SNPS in *ATIC* were found to be associated with (non)response. For adverse events associations with SNPs in *ABCB1, ABCC2, AMPD1, ATIC, MTHFR, MTR, MTRR, SHMT, SLC19A1* and *TS* have been found in RA.[27] In JIA, *MTHFR* was associated with adverse events.[27] In contrast to the many association studies for genetic determinants, associations studies for metabolic determinants and MTX (non)response or adverse events have not been performed yet.

Earlier, prediction models for MTX non-response have been developed for juvenile idiopathic arthritis (JIA)[34] and for RA.[31,35-37] However, these models did not use metabolic predictors,[35-37] were not validated[36] or the model was developed in patients on MTX monotherapy[35-37] rather than in therapy with a combination of DMARDs.

The first prediction model for MTX response was successfully constructed in 205 RA patients.[35] The model for MTX response consisted of sex, rheumatoid factor and smoking status, the DAS, and 4 polymorphisms in the *AMPD*1, *ATIC*, *ITPA*, and *MTHFD*1 genes. This prediction model was transformed into a scoring system ranging from 0 to 11.5. Scores of ≤3.5 had a true positive response rate of 95%. Scores of ≥6 had a true negative response rate of 86%. The area under the curve (AUC) for the receiver operating characteristics (ROC) curve was 85% (95%CI: 80-91%).

Also for JIA a prediction model has been developed. In a derivation cohort of 183 patients, a prediction model to identify JIA patients not responding to MTX has been developed. The prediction model included: erythrocyte sedimentation rate and SNPs in genes coding for *MTRR, ABCB1, ABCC1*, and *SLC46A1*. The AUC of the ROC was 73% (95%CI: 64-81%). The prediction model was transformed into a total risk score (range 0 to 11). At a cut-off score of ≥3, sensitivity was 78%, specificity 49%, positive predictive value was 83% and negative predictive value 41%. In the validation cohort (n=104), the AUC was 65% (95%CI: 54-77%).[34]

A prediction model for adverse events has not been published yet.

Intracellular MTX-PG measurement and treatment response

In those RA patients who are non-responsive to MTX, 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.[30] 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, or other DMARDs is instituted.[2] 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 levels can be easily measured but low dose MTX is rapidly cleared from plasma and hence, plasma MTX levels do not correlate with MTX non-response and are therefore not routinely measured.[24,38,39] Higher MTX-dose leads to higher intracellular erythrocyte-MTX-polyglutamate (MTX-PG) concentrations.[40,41] Measurement of MTX-PG in erythrocytes or white blood cells (WBC) may be a strong predictor of response[26,29,42-45] but is generally not measured by clinical laboratories. This is mainly because there is no rapid and specific method to measure MTX-PG in routine laboratories. Therefore, our group developed earlier fast and high-throughput MALDI-MS/MS methods to measure MTX in erythrocytes and plasma.[46,47] Although this method is very fast, the machinery is not standard for routine laboratories. Using stable isotope dilution LC-ESI-MS/MS, it is now possible to measure erythrocyte MTX-PGs in a fast and precise way.[48] Sample pre-treatment is simple and consists of a lysing and a deproteinization step.

Erythrocyte-MTX-PGs have been related to (non)response in several studies in adult RA.[29,38,43,44,49] In addition, we showed in an accompanying paper that in JIA long-chain erythrocyte-MTX-PGs were associated with lower disease activity at 3 months and during one year of MTX treatment.[50] In 113 JIA patients, higher concentrations of MTX-PG3 (β=-0.005, p=0.028), MTX-PG4 (β=-0.014, p=0.014), MTX-PG5 (β=-0.049, p=0.023) and MTX-PG3-5 (β=-0.004, p=0.018) were associated with lower disease activity over 1 year. However, there have been reports with contrasting results in RA and in JIA.[30,51,52] Most of these studies used cross-sectional analyses[29,30,44,51,52] in which patients were in different stages of MTX-treatment varying from 3 months to >10 years. Longitudinal validated studies that follow patients disease activity and erythrocyte-MTX-PG concentrations from baseline until remission are needed to investigate MTX-PGs as tool for TDM. 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.

Aims of this thesis

In this thesis, the central hypothesis is that derangements in cellular MTX pathway metabolism influences MTX non-response and adverse events, through direct effects on the mechanism of MTX action or indirectly mediated via changes in intracellular MTX-PG accumulation (Figure 4).

The primary aim was to identify determinants for non-response and adverse events of MTX therapy in arthritis, so we could finally develop a prediction model. Physicians could use this model for more personalized medicine for their patients. A simple blood test and filling out some questions, before prescription, would provide the physician with an advice which therapy to start. A second aim of this thesis was to better understand the mechanism between determinants and MTX non-response or co-effects. Co-effects can be adverse events, but also positive co-effects on comorbidities

such as prevention of cardiovascular diseases and diabetes. We have investigated these mechanisms of non-response or co-effects by looking at MTX-PG concentrations as an intermediate that caused the effects on non-response and adverse events. A third aim of this thesis was to find out whether cellular erythrocyte-MTX-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. Besides adverse events, MTX may has positive co-effects. The fourth aim was to assess metabolic co-effects of MTX therapy. The fifth aim, was to combine all found determinants for MTX non-response or adverse events and develop a prediction model for 3 months MTX non-response and adverse events.

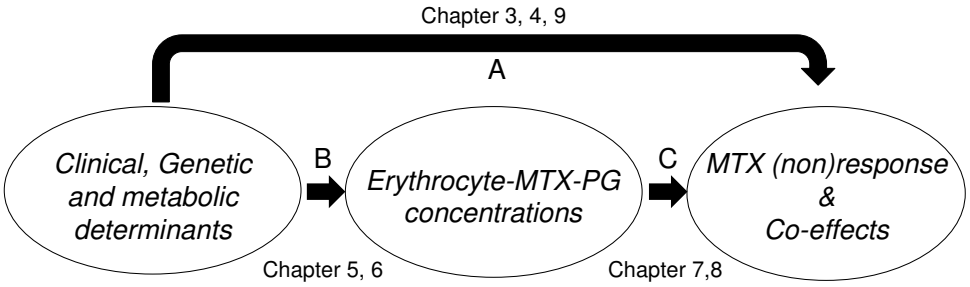


Figure 4 Relations that will be investigated in this thesis. MTX, methotrexate; MTX-PG, methotrexate-polyglutamate.

Before, all aims were assessed first the advantages and disadvantages of cross-sectional versus longitudinal study designs were investigated.

Figure 4 shows the relations that were investigated in this thesis. Relation A represents the associations between determinants and MTX (non)response / co-effects. Relation B represents the associations between determinants and intracellular MTX-PG concentrations. Relation C represents the associations between MTX-PGs and MTX (non)response and co-effects.

Aims:

1) To identify clinical, genetic and metabolic determinants of non-response and adverse events of methotrexate therapy in arthritis (relation A).
2) To better understand the mechanisms of non-response and co-effects of methotrexate therapy in arthritis (relation B and C).
3) To investigate if intracellular erythrocyte-MTX-PG concentrations are related to non-response or adverse events in RA patients on MTX and thus if MTX-PGs could be a tool for therapeutic drug monitoring (relation C).
4) To assess metabolic co-effects of MTX therapy in arthritis (relation C).
5) To develop a prediction model for non-response and adverse events of methotrexate therapy in arthritis (relation A).

Outline of this thesis

This thesis gives an overview of direct and indirect, via erythrocyte MTX-PG, determinants of MTX non-response and adverse events in arthritis. In addition, this thesis reveals the mechanism of action of some determinants for methotrexate non-response and shows the influence of MTX on non-response and glycosylated hemoglobin (HbA_{1c}). Finally this thesis presents a prediction model for MTX non-response in RA. **Chapter 1** gives an introduction on the topics described in this thesis. In **chapter 2,** we show the advantages of longitudinal analysis compared to snapshot analysis in pharmacogenetic studies in JIA patients. Therefore, a longitudinal JIA cohort was used in **chapter 3A** to assess associations of SNPs in genes involved in cellular MTX transport and polyglutamylation in relation to MTX response. In addition we also assessed in **chapter 3B** the associations with MTX adverse events of SNPs in genes involved in cellular MTX transport and polyglutamylation in JIA and RA cohorts. Metabolic determinants of MTX non-response and adverse events in arthritis have not yet been investigated. In **chapter 4**, the associations of one-carbon metabolism biomarkers with MTX non-response and adverse events were investigated in two prospective RA cohorts. The association described in **chapter 4** between low baseline erythrocyte-folate and MTX non-response in RA was more extensively investigated in **chapter 5**, were we assessed the association between baseline erythrocyte folate-PG distributions and 3 months erythrocyte MTX-PG distributions in MTX treated RA patients. Other clinical, metabolic and genetic determinants of 3 months erythrocyte MTX-PG concentrations in RA were identified in **chapter 6**. In **chapter 7,** the association of erythrocyte MTX-PG concentrations and MTX non-response and adverse events was investigated. The metabolic co-effects of MTX therapy and MTX-PG concentrations on HbA_{1c} concentrations were further investigated in **chapter 8**. Finally, in **chapter 9** all known associations of MTX non-response and adverse events were assessed for their use in prediction models for MTX non-response and adverse events in RA. In **chapter 10** the results and implications of our studies were discussed.

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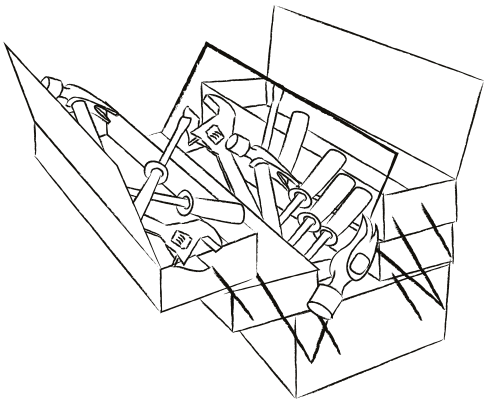

CHAPTER 2

Do Snapshot Statistics Fool us in Methotrexate Pharmacogenetic Studies in Arthritis Research?

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Rheumatology (2010) 49 (6): 1200-1201.

An interesting discussion was published in Rheumatology (Oxford) on discrepant literature results concerning methotrexate pharmacogenetics in rheumatoid arthritis (RA).[1-3] This discussion was triggered by the paper of Lee et al.[2] introducing the concept of false-positive report probability (FPRP) in the field of arthritis research. The discussion focused on the discrepant results observed for single nucleotide polymorphisms (SNP) in the *ATIC* gene (rs4673993 and rs2372536, both in linkage disequilibrium): the 347 C-allele[4,5] and the G-allele[2,6] were both associated with increased MTX efficacy. Similar discrepancies for SNPs in the methylenetetrahydrofolate reductase (MTHFR) gene were reported in a meta-analysis earlier this year.[7] In trying to explain the discrepancy, Dervieux[1] pointed out the challenges and difficulties researchers face when validating associations between low-penetrance genetic polymorphisms and complex phenotypes such as drug response. The discussion focused on differences between studies in the FPRP, differences in sample size or power, demographic dissimilarities among cohorts, environmental factors such as folate status, duration of disease, and treatment duration.

We would like to argue that one of the most important reasons for discrepant studies is because of cross-sectional analysis, also called the snapshot approach. Most pharmacogenetic studies examine only one time point during (MTX) treatment. For instance, MTX response was assessed at 6 months in the European studies[4,5] and after >50 months in the US cohorts.[2,6] The snapshot approach suffers from several methodological flaws. First, the snapshot approach may not reflect the true response characteristics over the whole treatment phase. To illustrate this, we have plotted the typical treatment response patterns of patients with juvenile idiopathic arthritis (JIA; Figure 1). From the Figure it becomes clear that treatment response can be roughly divided into three profiles: A) patients who will respond to treatment at any time-point between start of treatment and one-year of follow-up and will stay in remission (47%); B) patients who shift back and forth from responder to non-responder (31%) and C) patients who do not show any response during the first year of treatment (22%).

This study was performed in the University Medical Centre Utrecht (UMCU), Wilhelmina Children's Hospital, The Netherlands. Patients with a confirmed JIA diagnosis according to the ILAR criteria were included. All included patients had started MTX therapy between 1990 and 2006. All patients gave their informed consent. The study was approved by the Medical Ethics Committee of the UMCU. Patients had been systematically followed every 3 months using a standardized report form on disease activity.

Similar profiles were observed in adult RA patients. From a clinical point of view, prediction of treatment response at only one time-point (e.g. 6 months) is less informative because, at the next hospital visit, a substantial number of patients may become non-responders and vice-versa. Second, the snapshot approach only evaluates patients that are still available at the analyzed time-point and hence, ignoring dropouts or missing data. Often missing data is not missing completely at random (MCAR) and

could be related to the primary outcome, i.e. toxicity or intolerance. As a consequence, the estimators will be biased for the investigated SNP on treatment response.

Assessing the FPRP in snapshot approach pharmacogenetic studies may be helpful in detecting spurious findings. However, future pharmacogenetic studies in arthritis research should preferably evaluate the treatment response in a longitudinal way. Longitudinal analysis will allow us I) to better characterize the different response profiles of patients (see Figure 1) and II) to perform sophisticated repeated measurement statistics that are not affected by the disadvantages of snapshot statistics. This method allows estimating the occurrence of response for a group as a whole over a certain period of time. This approach will generate clinically more relevant information because it will predict the long-term response characteristics of patients better and will reduce the risk of false-positive and –negative findings.

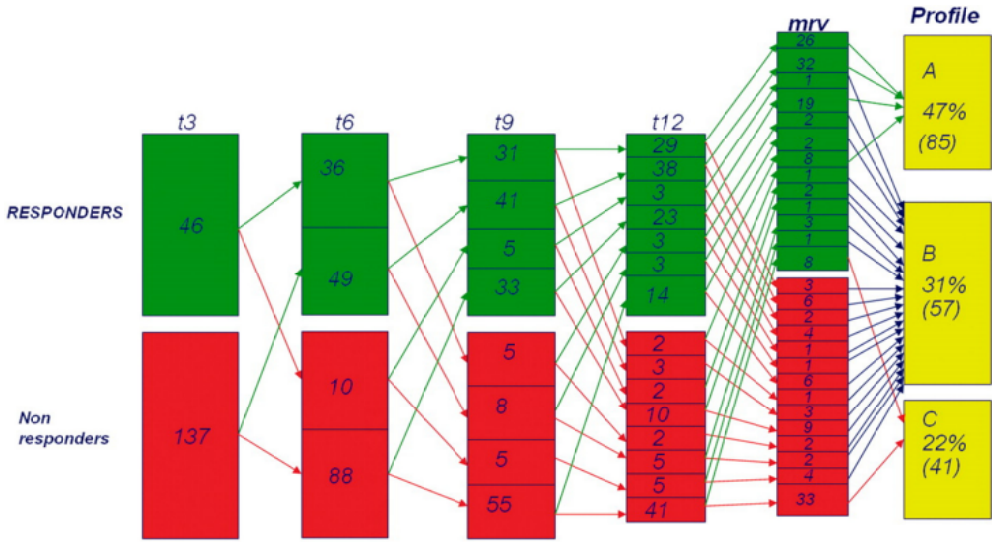

Figure 1 Responders and non-responders in 183 JIA patients following Pediatric American College of Rheumatology 30% (ACRped30) criteria in 3 months intervals up to the most recent visit after start of treatment with methotrexate. Response is divided into three profiles: A) patients who will respond towards treatment at any time-point between start of treatment and 1-year of follow-up and will stay in remission (47%); B) patients who shift back and forth form responder to non-responder (31%); and C) patients who do not show any response during first year of treatment (22%). t3,6,9,12, time-point 3,6,9 and 12 months, respectively, after start of methotrexate treatment; mrv, most recent visit.

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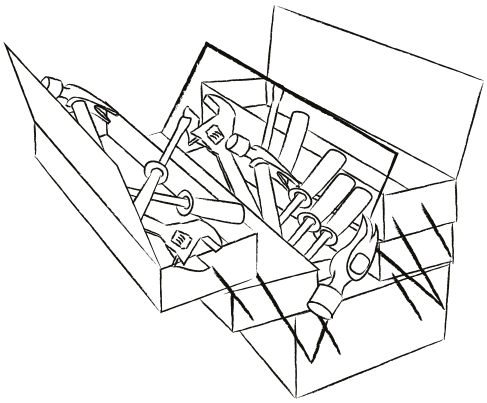

CHAPTER 3A

ABCB1 and *ABCC3* gene polymorphisms are associated with first-year response to methotrexate in juvenile idiopathic arthritis

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ABSTRACT

Objective

Although methotrexate (MTX) is the most widely prescribed drug in juvenile idiopathic arthritis (JIA), 30% of patients fail to respond to it. To individualize treatment strategies, the genetic determinants of response to MTX should be identified.

Methods

A cohort of 287 patients with JIA treated with MTX was studied longitudinally over the first year of treatment. MTX response was defined as the American College of Rheumatology pediatric 70 criteria (ACRped70). We genotyped 21 single nucleotide polymorphisms in 13 genes related to MTX polyglutamylation and to cellular MTX uptake and efflux. Potential associations between ACRped70 and genotypes were analyzed in a multivariate model and corrected for these 3 covariates: disease duration prior to MTX treatment, physician's global assessment of disease activity at baseline, and MTX dose at all study visits.

Results

MTX response was more often achieved by patients variant for the adenosine triphosphate-binding cassette transporter B1 (*ABCB1*) gene polymorphism rs1045642 (OR 3.80, 95% CI 1.70-8.47, p=0.001) and patients variant for the *ABCC3* gene polymorphism rs4793665 (OR 3.10, 95% CI 1.49-6.41, p=0.002) than by patients with other genotypes. Patients variant for the solute carrier 19A1 (*SLC19A1*) gene polymorphism rs1051266 were less likely to respond to MTX (OR 0.25, 95% CI 0.09-0.72, p=0.011).

Conclusion

ABCB1 rs1045642, *ABCC3* rs4793665 and *SLC19A1* rs1051266 polymorphisms were associated with response to MTX in 287 patients with JIA studied longitudinally. Upon validation of our results in other JIA cohorts, these genetic determinants may help to individualize treatment strategies by predicting clinical response to MTX.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is the most frequent rheumatic disease in infants, affecting 1 in 1000 children, and is an important cause of disability.[1] Methotrexate (MTX) is the most widely used disease modifying-antirheumatic drug (DMARD) in JIA.[2] Although patients can go into prolonged remission, 30% of the patients treated with MTX do not respond to the drug.[2] The delay in identifying the optimal treatment at an early stage of the disease can lead to joint damage. Therefore, there is a need to identify determinants of response to MTX that can be used to individualize treatment strategies.

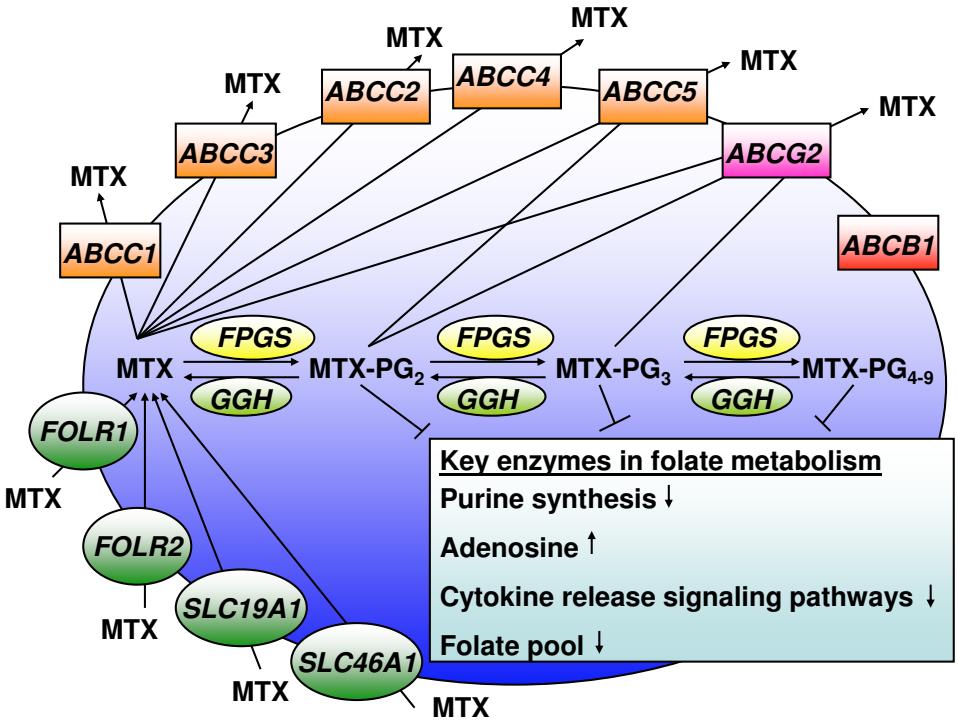

Figure 1 Cellular MTX transport routes for MTX influx and efflux in relation to polyglutamylation and mechanisms for arthritis suppression. MTX-PGs can inhibit several key enzymes in folate metabolism and therefore may cause a decreased de novo purine biosynthesis, increased adenosine release, direct or indirect effects on cytokine release signaling pathways and folate depletion, which all may lead to arthritis suppression. MTX, methotrexate; MTX-PG, methotrexate polyglutamates; *ABCB1*, adenosine triphosphate-binding cassette transporter B1; *FPGS*, folylpolyglutamate synthetase; *FOLR1*, folate receptor 1; *GGH*, γ-glutamyl hydrolase; *SLC19A1*, solute carrier 19A1.

In weekly low-dose MTX treatment, MTX polyglutamates accumulate intracellularly and as such inhibit several key enzymes in the folate metabolism and *de novo* purine

synthesis (figure 1).[3,4] MTX polyglutamates correlate with MTX efficacy in adult rheumatoid arthritis (RA).[5-8] Non-responders accumulate fewer MTX polyglutamates in red blood cells compared to responders in an early phase of treatment.[6] Single nucleotide polymorphisms (SNP) in genes involved in MTX transport and polyglutamylation affect intracellular MTX accumulation.[9] MTX enters mammalian cells mainly through the solute carrier 19A1 / reduced folate carrier (*SLC19A1/RFC*) and is additionally transported into the cell via the solute carrier 46A1 / proton coupled folate transporter (*SLC46A1/PCFT*) and the folate receptors (*FOLR*) 1 and 2.[4] Members of the adenosine triphosphate (ATP) binding cassette (*ABC*) transporters, including *ABCB1* / P-glycoprotein (P-gp), multidrug resistance proteins (*MRP*/*ABCC*), and breast cancer resistance protein (*BCRP*/*ABCG2*), function as ATP-dependent MTX efflux transporters.[4] Cellular retention of MTX is mediated by the dynamic interplay between formation of MTX-polyglutamates via folylpolyglutamate synthetase (*FPGS*) and MTX-polyglutamate breakdown via γ-glutamyl hydrolase (*GGH*).[3]

In contrast to RA,[10] studies in JIA examining associations of SNPs in genes involved in MTX transport (uptake/efflux) and polyglutamylation are scarce.[11-17] Moreover, they report inconsistent findings and the majority has a cross-sectional design. Therefore, the aim of our study was to perform a comprehensive analysis of SNPs in genes involved in cellular MTX transport and polyglutamylation in relation to MTX response in a longitudinal JIA cohort. We hypothesize that SNPs in genes involved in MTX transport and polyglutamylation affect response to MTX in JIA.

MATERIALS AND METHODS

Patients and study design

We used a cohort study performed at the University Medical Center Utrecht (UMCU), Wilhelmina Children's Hospital, The Netherlands was used for this study. The cohort included 295 patients who started MTX therapy between 1990 and 2010. Patients with a confirmed JIA diagnosis according to the International League of Associations for Rheumatology criteria were included.[18] Patients were excluded if full clinical data or blood for DNA analysis were not available. All patients gave informed consent. The study was approved by the Medical Ethics Committee of the UMCU and was in compliance with the declaration of Helsinki. Patients had been systematically followed at 0, 3, 6 and 12 months after initiation of MTX therapy using a standardized report form on disease activity. Information was collected from the patients' medical files at every visit until 1 year after the start of MTX therapy. The data were disease activity, MTX usage and route of administration, MTX dose, reasons for ending MTX treatment, concomitant therapy and laboratory measurements.

Definition of response

The international validated core set criteria for the assessment of patients with JIA was used to define disease activity: 1) Physician global assessment of disease activity on a 10 cm visual analog scale (PGA), 2) Parent/patient assessment of overall wellbeing

using the Childhood Health Assessment Questionnaire (CHAQ wellbeing), 3) Functional ability, measured using the Childhood Health Assessment Questionnaire (CHAQ disability) on a 0-3 scale, 4) Number of joints with active arthritis, defined by the presence of swelling and/or limitation of movement accompanied by pain and/or tenderness, 5) Number of joints with limited range of motion, defined as a loss of at least 5 degrees in any articular movement from the normal amplitude, 6) Erythrocyte sedimentation rate in mm/first hour. MTX response was defined by the American College of Rheumatology 70 pediatric criteria (ACRped70):[19] Patients with >70% improvement in at least 3 of the 6 criteria, without >30% worsening in 1 of the remaining variables, were defined as good clinical responders. Use of anti-tumor necrosis factor (TNF)-alpha was used as a criterion for non-response.

SNP selection

SNPs 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) database[20,21] or a proven functionality in relation to MTX, JIA, RA or folate metabolism.[22-30] If no information was known for a particular gene, we selected tagging SNPs by Hapmap database and haploview (version 4.2, 29 April 2008).[20] We chose an MAF >0.10 instead of the commonly chosen >0.05. Because our sample size was relatively small, we expect that SNPs with an MAF <0.10 would not have sufficient data distribution for statistical analysis. Preferably, 2 SNPs were selected per gene, which were located in different haplotype blocks. The following 21 SNPs in 13 genes were chosen: *ABCB1* rs1128503, rs2032582, rs1045642; *ABCC1* rs35592, rs3784862; *ABCC2* rs4148396, rs717620; *ABCC3* rs4793665, rs3785911; *ABCC4* rs868853; rs*2274407*, *ABCC5* rs2139560, *ABCG2* rs13120400, rs2231142; *FPGS* rs4451422, *FOLR1* rs11235462, *FOLR2* rs514933, *GGH* rs10106587; rs3758149, *SLC46A1* rs2239907 and *SLC19A1* rs1051266. Subsequently, we calculated the gene coverage[31] in order to assess the percentage of genetic variation that was covered by the investigated SNPs of all the genetic variation possible within each gene.

We standardized our SNP nomenclature based on the VIC and FAM-labeled probes for which the Taqman assays were designed for allele detection. The major allele was analyzed as wild type allele and the minor allele as variant allele (supplemental table 1).

A haplotype is a combination of alleles at adjacent locations on the chromosome that are transmitted together. We included haplotype analysis in this study to test whether the effect of the haplotypes on MTX response was larger than that of the corresponding SNPs alone. Lewontin's D prime (D') and correlation coefficient (R^2) were calculated by haploview to assess linkage disequilibrium of SNPs within each gene. SNPs that were in linkage disequilibrium (D' ≠ 0) with a correlation coefficient <0.80 were selected for haplotype reconstruction by the PHASE method.[32]

Genotyping

Genomic DNA was isolated from 0.2 mL EDTA whole blood with a Total Nucleic Acid Extraction kit on a MagNA Pure LC, (Roche Molecular Biochemical's, Almere, Netherlands). Genotyping was performed using Taqman allelic discrimination assays on the Prism 7000 sequence detection system, (Life Technologies, Applied Biosystems, Bleiswijk, Netherlands). Each assay consisted of 2 allele-specific minor groove binding probes, labeled with the fluorescent dyes VIC and FAM. The primer and probe sequences were ordered from stock by Applied Biosystems and otherwise by their Assay-by-Design service (*ABCB1* rs1128503, rs2032582, rs1045642 and *SLC19A1* rs1051266). Samples were the Taqman did not perform an automatic calling were rejected. Of these samples, duplicate samples were genotyped. When the Taqman could not perform an analysis the second time, the result was included as missing in the database. For every new genotyping test in our laboratory, 50 random blood samples were analyzed. From these results a wild type, heterozygous and homozygous variant control sample was chosen. In each run with patient samples, control samples for each genotype were included. A run was rejected when the results for the control samples changed from the original results. For 5% of the patients, duplicate samples were run for each SNP on random patients. All allele frequencies were compared with Hapmap and NCBI databases[20,21] and if discrepancies existed samples were sequenced to confirm genotypes. Therefore, we designed primers for these SNPs. The quality control samples were sequenced with the obtained primers. Deviation from Hardy-Weinberg equilibrium (HWE) was tested.

Statistical analysis

Before analysis we plotted the percentage responders within each genotype group, and the inheritance of all SNPs followed the recessive mode of inheritance. We therefore chose for a recessive inheritance model to increase the statistical power. Consequently, genotypes and haplotypes were dichotomized accordingly: Genotypes into: wild-type/heterozygous = 0 and homozygous variants = 1; haplotypes into: heterozygous and all other homozygous haplotypes = 0 and homozygous for the specific haplotype = 1. For example, for the *ABCB1* haplotype GCA, the patients with the genotypes rs1128503 GG, rs2032582 CC and rs1045642 AA = 1 and all other patients = 0. Statistical analyses were done with SPSS PASW 17.02 for windows (SPSS inc. Chicago, IL, USA) unless stated otherwise. P-values <0.05 were considered significant.

SNPs or haplotypes with sufficient distribution of data for statistical analysis (at least 1 responder and 1 non responder for each genotype on every visit) were further analyzed for associations with MTX response. The associations between genotype, or haplotype and response were analyzed with a generalized linear mixed model to account for the correlations between the repeated measurements and to obtain an overall odds ratio (OR) and confidence intervals (CI) over the whole treatment period.[33] Generalized linear mixed models were fitted using proc glimmix in the Statistical Analysis Software (SAS) version 9.2 (SAS Institute Inc. Cary, NC, USA). A random

intercept logistic model was used. This model considers random variation within individuals and random variation between individuals. We used empirical (sandwich) estimators to make analysis robust against misspecification of the covariance structure and to adjust for small-sample bias. The estimation is based on integral approximation by adaptive quadrature.

Univariate relations between genotype or haplotype and ACRped70 with a significance of $p<0.2$ were further investigated in a multivariate analysis. This analysis combined potential univariate associations ($p<0.2$) with clinical covariates, namely disease duration prior to start of MTX treatment, PGA at baseline and MTX dose, which were previously reported to be significantly associated with MTX response in JIA.[12]

To test whether our results suffered from multiple testing problems we tested the significant SNPs from the multivariate analysis also in relation with ACRped50 as criterion for MTX response. We also used an alternative outcome (responders as patients with an ACRped70 at 2 or more consecutive visits) to obtain an ordinary logistic regression analysis to test our significant results. Finally we used a Bonferroni correction to assess our significant results.

RESULTS

Patient characteristics

Blood for DNA isolation was available for 295 patients. Five patients were excluded because longitudinal clinical data could not be retrieved and 3 patients were excluded since they received biologicals (Anakinra) at start of MTX. That left, 287 patients for further analyses. Baseline characteristics are shown in table 1. Of the 287 patients, 29 (10.1%) patients were ACRped70 responders after 3 months, 83 (28.9%) after 6 months and 132 (46.0%) after 12 months of MTX therapy.

After 3 months, 1 patient received anti-TNF-α therapy; after 6 months, 3 patients; and after 12 months, 17 patients because of insufficient response to MTX. Those patients were considered non-responders on those visits. Patients taking sulfasalazine were not considered non-responders. Despite the heterogeneity of the study population, we did observe equal MTX response rates among different JIA subtypes.

SNP analysis

Only the *ABCC2* rs717620 SNP deviated from HWE (p-value = 0.038). However, this SNP had a low number of homozygous variants (5 patients). This could have contributed to the HWE p-value <0.05. We decided to keep this SNP in the analysis. Failure for genotyping was between 0 and 6% per SNP. Allele frequencies for *ABCC3* rs4793665, *ABCC3* rs3785911, *ABCC4* rs868853 and *ABCC4* rs2274407 were not confirmed in the Hapmap/NCBI database and therefore a sequencing analysis was performed. For all 4 SNPs investigated, the sequencing analysis confirmed the expected SNPs. There were <5% discrepancies between duplicate runs.

Of the 21 genotyped SNPs, statistical analyses, for the univariate association between genotype and MTX response in JIA, could be performed on 17 SNPs (table 2).

Table 1 Characteristics of patients with JIA at time of starting MTX treatment.

Characteristics	(n=287)
Polyarticular JIA, n (%)	107 (37.3)
Systemic-onset JIA, n (%)	47 (16.4)
Oligoarticular persistent JIA, n (%)	63 (22.0)
Oligoarticular extended JIA, n (%)	48 (16.7)
Enthesitis-related JIA, n (%)	11 (3.8)
Psoriatic JIA, n (%)	11 (3.8)
Male sex, n (%)	104 (36.2)
Age, years, median (range)	9.0 (1.4-18.8)
Disease duration at MTX start, years, median (range)	1.4 (0.0-15.6)
PGA, median (range)	3.4 (0.0-10.0)
Joints with limited motion, median (range)	2 (0-26)
Joints with active arthritis, median (range)	3 (0-30)
CHAQ disability, mean (SD)*	1.1 (0.7)
CHAQ well-being, cm, mean (SD)*	4.3 (2.7)
ESR, mm/h, median (range)	24 (1-140)
RF seropositivity, n (%)**	23 (8.0)
MTX dose at start (mg/m^2/week), median (range)	9.6 (2.8-25.0)
NSAIDs, n (%)	250 (87.1)
Sulfasalazine, n (%)	8 (2.8)
Oral steroids, n (%)	43 (15.0)
Intra-articular steroids, n (%)	41 (14.3)

*CHAQ was assessed for 280 patients, included after 1994, when the CHAQ was introduced in our clinic. **RF was assessed for 234 patients. JIA, juvenile idiopathic arthritis; MTX, methotrexate; PGA, physician global assessment of disease activity; CHAQ, child health assessment questionnaire; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; NSAID, non-steroidal anti-inflammatory drug.

For the other 4 SNPs investigated, there was insufficient distribution of data for statistical analysis (Not at least 1 responder and 1 non responder for each genotype on every visit). A p-value <0.2 for ACRped70 after univariate analysis was observed for the following 6 SNPs: *ABCB1* rs1045642 (p=0.002), *ABCC1* rs35592 (p=0.045), *ABCC3* rs4793665 (p=0.005), *ABCG2* rs13120400 (p=0.036), *FPGS* rs4451422 (p=0.087) and *SLC19A1* rs1051266 (p=0.054).

These SNPs were entered together in a multivariate model and were corrected for the clinical covariates disease duration prior to the start of MTX treatment, PGA at baseline, and dose of MTX (figure 2). Three of these 6 investigated SNPs remained significant (p<0.05) in this multivariate analysis. *ABCB1* rs1045642 showed a 3.80 higher OR (95% CI 1.70-8.47, p=0.001), and *ABCC3* rs4793665 a 3.10 higher OR (95% CI 1.49-6.41, p=0.002) to achieve an ACRped70 response in the first year after start of MTX therapy, whereas *SLC19A1* rs1051266 showed a 0.25 lower OR (95% CI 0.09-0.72, p=0.011) to achieve the ACRped70 response.

To address the issue of subtype heterogeneity we investigated whether the effect sizes of the significant SNPs remained the same in the oligoarticular and polyarticular JIA subtypes only. We found similar effects sizes as reported for MTX response in the

entire JIA cohort, namely *ABCB1* rs1045642 OR 4.07 (95% CI 1.40-11.90, p=0.010), *ABCC3* rs4793665 OR 2.78 (95% CI 1.07-7.19, p=0.036) and *SLC19A1* rs1051266 OR 0.09 (95% CI 0.01-0.65, p=0.017). There were no differences in the frequency of ACRped70 responses in patients on oral MTX and patients on parental MTX. We checked also the prevalence of SNPs between routes of administration. The MAFs for the patients on oral MTX at baseline (n=270) were comparable with the MAFs for the patients on parental MTX at baseline (n=17).

Table 3 shows the reconstructed haplotypes. None of the haplotypes remained significant after multivariate analysis.

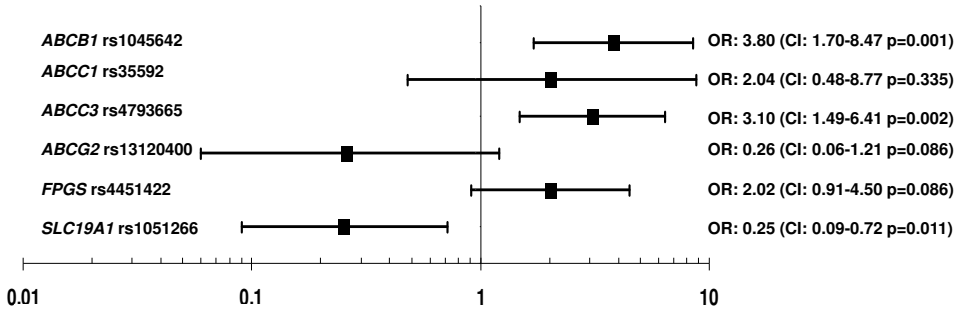

Figure 2 Multivariate analysis of relation between *ABCB1* rs1045642, *ABCC1* rs35592, *ABCC3* rs4793665, *ABCG2* rs13120400, *FPGS* rs4451422 and *SLC19A1* rs1051266 and ACRped70 with OR, 95% CI and p-values. Covariates in multivariate analysis: Disease duration prior to start of MTX treatment, PGA at baseline, and MTX dose. *ABCB1*, adenosine triphosphate-binding cassette transporter B1; *FPGS*, folylpolyglutamate synthetase; *SLC19A1*, solute carrier 19A1; JIA, juvenile idiopathic arthritis; MTX, methotrexate; ACRped70, American College of Rheumatology 70% pediatric criteria; PGA, physicians global assessment of disease activity.

Table 2 Genes and 21 SNPs within cellular MTX transport routes and polyglutamylation in relation to response (ACRped70) over the first year of MTX therapy in 287 patients with JIA.

SNP	GC (%)	HWE p	WT/het/var	MAF	Genotype Frequency (WT/het/var)	OR (95%CI) univariate	p	Study
ABCB1 rs1128503	7.5	0.560	GG/GA/AA	0.40	0.38/0.46/0.16	1.85 (0.64-5.35)	0.254	22
ABCB1 rs2032582	7.5	0.682	CC/CA/CT/TA/AA	0.38/0.02	0.36/0.47/0.01/0.02/0.13	1.86 (0.63-5.52)	0.263	22
ABCB1 rs1045642	7.5	0.060	GG/GA/AA	0.48	0.29/0.45/0.26	3.72 (1.62-8.55)	0.002	23
ABCC1 rs35592	2.8	0.175	TT/TC/CC	0.23	0.61/0.32/0.07	4.93 (1.04-23.26)	0.045	24
ABCC1 rs3784862	2.8	0.260	AA/AG/GG	0.28	0.53/0.38/0.09	1.49 (0.49-4.50)	0.482	24
ABCC2 rs4148396	6.3	0.329	CC/CT/TT	0.36	0.39/0.49/0.12	1.02 (0.34-3.03)	0.973	24
ABCC2 rs717620	6.3	0.038*	CC/CT/TT	0.19	0.63/0.35/0.02	2.87 (0.14-58.82)	0.493	24
ABCC3 rs4793665	3.8	0.347	CC/CT/TT	0.57	0.17/0.52/0.31	2.99 (1.39-6.41)	0.005	25
ABCC3 rs3785911	3.8	0.298	AA/AC/CC	0.32	0.48/0.41/0.11	1.09 (0.35-3.40)	0.879	†
ABCC4 rs868853	0.5	0.638	TT/TC/CC	0.07	0.86/0.14/0.00	**		26
ABCC4 rs2274407	0.5	0.243	CC/CA/AA	0.06	0.87/0.13/0.00	**		26
ABCC5 rs2139560	17.5	0.092	GG/GA/AA	0.40	0.34/0.53/0.13	0.51 (0.18-1.46)	0.208	‡
ABCG2 rs13120400	9.1	0.622	TT/TC/CC	0.27	0.53/0.39/0.08	0.17 (0.03-0.89)	0.036	24
ABCG2 rs2231142	9.1	0.351	GG/GT/TT	0.11	0.80/0.18/0.02	**		27
FPGS rs4451422	16.2	0.568	AA/AC/CC	0.40	0.37/0.46/0.17	2.14 (0.90-5.13)	0.087	‡
FOLR1 rs11235462	5.6	0.925	TT/TA/AA	0.16	0.71/0.27/0.02	**		†
FOLR2 rs514933	7.1	0.514	TT/TC/CC	0.35	0.44/0.43/0.13	0.59 (0.20-1.73)	0.338	28
GGH rs10106587	14.9	0.992	AA/AC/CC	0.29	0.50/0.42/0.08	2.28 (0.54-9.62)	0.260	‡
GGH rs3758149	14.9	0.921	GG/GA/AA	0.30	0.49/0.42/0.09	0.69 (0.19-2.47)	0.563	24
SLC46A1 rs2239907	48.4	0.643	CC/CT/TT	0.44	0.32/0.48/0.20	1.05 (0.42-2.63)	0.914	29
SLC19A1 rs1051266	57.3	0.839	CC/CT/TT	0.37	0.39/0.47/0.14	0.34 (0.11-1.02)	0.054	30

Analyses were performed according to a recessive inheritance model. **ABCC2* rs717620 had a low number of homozygous variants (5 patients). As this could have contributed to the HWE p-value <0.05, we kept this SNP in the analysis. **Insufficient distribution of data for statistical analysis (Not at least 1 responder and 1 non responder for each genotype on every visit). †No tagging SNPs were available and an SNP with MAF >0.10 was chosen. ‡Tagging SNPs were selected by Hapmap database and Haploview. SNP, single nucleotide polymorphism; MTX, methotrexate; ACRped70, American College of Rheumatology 70% pediatric criteria; JIA, juvenile idiopathic arthritis; GC, gene coverage; HWE, Hardy-Weinberg equilibrium; WT, wild-type; het, heterozygous; var, variant; MAF, minor allele frequency; *ABCB1*, adenosine triphosphate-binding cassette transporter B1; *FPGS*, folylpolyglutamate synthetase; *FOLR1*, folate receptor 1; *GGH*, γ-glutamyl hydrolase; *SLC19A1*, solute carrier 19A1.

Table 3 Haplotypes of SNPs in genes within cellular MTX transport routes and polyglutamylation in relation to response (ACRped70) over the first year of MTX therapy in JIA.

Gene	rs numbers	Haplotypes	Frequency	OR (95%CI) Univariate	p-value
ABCB1	rs1128503/rs2032582/rs1045642	GCA	0.10	*	
ABCB1	rs1128503/rs2032582/rs1045642	AAA	0.37	2.44 (0.80-7.46)	0.117
ABCB1	rs1128503/rs2032582/rs1045642	GCG	0.46	0.37 (0.15-0.89)	0.026
ABCC2	rs4148396/rs717620	TC	0.17	1.23 (0.24-6.29)	0.806
ABCC2	rs4148396/rs717620	TT	0.19	2.87 (0.14-58.82)	0.493
ABCC2	rs13120400/rs2231142	CC	0.63	1.08 (0.51-2.30)	0.837
ABCG2	rs13120400/rs2231142	TT	0.11	*	
ABCG2	rs13120400/rs2231142	CG	0.27	0.17 (0.03-0.89)	0.036
ABCG2	rs13120400/rs2231142	TG	0.62	2.10 (1.00-4.39)	0.049
GGH	rs10106587/rs3758149	AA	0.29	1.11 (0.31-3.91)	0.875
GGH	rs10106587/rs3758149	CG	0.30	1.29 (0.36-4.57)	0.692
GGH	rs10106587/rs3758149	AG	0.41	0.74 (0.29-1.92)	0.541
Gene	**rs numbers**	**Haplotypes**	**Frequency**	**OR (95%CI) Multivariate**	**p-value**
ABCB1	rs1128503/rs2032582/rs1045642	AAA	0.37	3.01 (0.72-5.65)	0.184
ABCB1	rs1128503/rs2032582/rs1045642	GCG	0.46	0.48 (0.21-1.10)	0.081
ABCG2	rs13120400/rs2231142	CG	0.27	0.26 (0.05-1.42)	0.120
ABCG2	rs13120400/rs2231142	TG	0.62	1.69 (0.83-3.43)	0.149

Haplotype analysis was performed according to a recessive inheritance model and therefore only homozygous haplotypes were analyzed. *Insufficient distribution of data for statistical analysis (not at least 1 responder and 1 non responder for each haplotype on every visit). MTX, methotrexate; SNP, single nucleotide polymorphism; ACRped70, American College of Rheumatology 70% pediatric criteria; *ABCB1*, adenosine triphosphate-binding cassette transporter B1; *GGH*, γ-glutamyl hydrolase.

DISCUSSION

In the present longitudinal study, we identified two SNPs that were potentially associated with a positive MTX response and 1 SNP associated with a negative MTX response in patients with JIA. The presence of *ABCB1* rs1045642 or *ABCC3* rs4793665 variant genotypes increased the likelihood of becoming an MTX responder 2-3 fold. For *SLC19A1* rs1051266 the likelihood decreased 2-3 fold. For children who failed to respond to MTX, the delay in finding the appropriate treatment may be crucial for their disease outcome, with the risk of joint damage and potential permanent disability.[34] Therefore, identifying determinants of MTX response would be a major development in JIA therapy.

The SNPs in the *ABCC1, ABCC2, ABCC5, ABCG2, FPGS, FOLR1, FOLR2, GGH and SLC46A1* genes were not associated with response to MTX in our study. In a recent study,[13] a total of 14 genes in the MTX pathway in relation to MTX response were investigated in a cross-sectional JIA cohort and replication cohort. Similarly to the present study, the authors did not find a significant association for SNPs in the genes *FPGS* and *GGH* with response to MTX. Another recent cross-sectional study in 92

Japanese patients with JIA also showed no evidence for a relation between SNPs in *FPGS* and *GGH* and response to MTX.[14]

To our knowledge, the present longitudinal study is the first to evaluate *ABCB1* and *ABCC3* gene polymorphisms with response to MTX in patients with JIA. Previous studies in adult patients with RA reported a positive association,[35,36] a negative association[37] and no statistical significant association[38-40] between *ABCB1* polymorphisms and response to MTX.

ABCB1 belongs to the efflux transporters of the *ABC* super family, subfamily B, and was formerly referred to as multidrug resistance 1 gene. The product of the *ABCB1* gene is P-gp.[4] Although the *ABCB1* rs1045642 polymorphism is synonymous (i.e., not leading to amino acid exchange), it is associated with altered P-gp expression and reduced P-gp function.[41] Early in vitro experiments in cell lines with high levels of MTX resistance suggested that P-gp could transport MTX.[42,43] From this perspective, the *ABCB1* rs1045642 polymorphism may result in impaired cellular efflux of MTX in heterozygous and homozygous variants, with concomitant increased intracellular MTX levels and increased MTX efficacy. However, recent research showed that MTX is unlikely to be a substrate of P-gp.[44,45] P-gp is expressed as a cell membrane-associated protein in natural killer cells, CD4 and CD8 lymphocytes and bone marrow progenitor cells[46] and plays a role in the transport of some inflammatory mediators, in particular bioactive lipids.[47] This could explain why *ABCB1* gene polymorphisms have been associated with increased response to MTX in adult RA[35,36] and in JIA in the present study; if the *ABCB1* rs1045642 polymorphism is associated with a diminished extrusion of inflammatory mediators, it could facilitate a better therapeutic effect of MTX. Changes in the physiological function of P-gp could provide an alternative explanation for the association between the *ABCB1* rs1045642 polymorphism and MTX response.

ABCC3 is involved in the efflux of MTX.[4,48] The rs4793665 SNP is located in the 5'-promoter region of the *ABCC3* gene and was associated with significantly lower *ABCC3* transcript levels, and a trend towards lower protein expression in human liver, and it could affect the binding of nuclear proteins to the *ABCC3* promoter.[49] Less expression of *ABCC3* transporter could have a positive effect on the cellular retention of MTX, leading to higher intracellular levels (figure 1). This could explain our finding that the rs4793665 SNP was associated with response to MTX. However, others have shown that this polymorphism determined neither the expression of the *ABCC3* gene nor the response to MTX therapy in acute leukemia.[50] Nevertheless, the treatment dosage is much lower in the JIA context, and thus these studies are not comparable. We expect that SNPs in efflux transporters have a greater influence on low-dose MTX therapy.

The membrane transporter *SLC19A1* is involved in the influx of MTX. Previously, we associated *SLC19A1* rs1051266 with an increased risk of pediatric acute lymphoblastic leukemia and elucidated the effects of this carrier on MTX metabolism.[30] SNPs in *SLC19A1* have been associated with response to MTX in RA[8] but not in JIA.[13] The association between *SLC19A1* rs1051266 ($p=0.011$) and MTX response was not

significant after Bonferroni adjustments (significant p-value = 0.05/17 SNPs tested = 0.003), and hence this finding should be judged with some skepticism. Therefore, the *SLC19A1* rs1051266 needs to be replicated in larger JIA cohort studies.

Haplotype analysis revealed no associations between haplotypes and MTX response in JIA. Therefore, our results suggest that testing of the 3 *ABCB1* SNPs has no additional value, and that determination of the rs1045642 SNP alone may suffice.

Some limitations of this study should be considered. Because of the large number of SNPs tested, the observed positive associations may be spurious. However, when we analyzed all SNPs in relation to ACRped50, similar results were obtained. Multivariate analysis yielded ORs of 3.18 (95% CI 1.41-7.19, p=0.006), 3.47 (95% CI 1.66-7.25, p=0.001) and 0.34 (95% CI 0.12-0.95, p=0.040) to be an ACRped50 responder for *ABCB1* rs1045642, *ABCC3* rs4793665 and *SLC19A1* rs1051266, respectively. In addition, we alternatively defined MTX responders as patients with an ACRped70 at 2 or more consecutive visits. Ordinary logistic regression analysis on this alternative outcome measure for MTX response yielded results comparable to those of the repeated measures analysis using generalized linear mixed modeling: *ABCB1* rs1045642 OR 2.46 (95% CI 1.39-4.34, p=0.002), *ABCC3* rs3785911 OR 1.86 (95% CI 1.07-3.22, p=0.003) and the *SLC19A1* rs1051266 OR 0.38 (95% CI 0.14-1.01, p=0.053). Further, if Bonferroni adjustments for multiple comparisons were applied (significant p-value = 0.05/17 SNPs tested = 0.003), *ABCB1* rs1045642 (p=0.001) and the *ABCC3* rs4793665 (p=0.002) SNP remained significant with MTX response.

Our findings can only be interpreted as associations, because the selected SNPs may be in linkage disequilibrium with the true causal variant. For the other genes investigated in this study, gene coverage (table 2) was not high enough (0.5-57.3%) to conclude that there is no association between these genes and response to MTX, because not all the genetic variation within these genes was covered with our analysis. We are aware of the relatively small sample size (n=287) of our cohort. This may have caused overestimation of OR.[51] Therefore, this study should be replicated in a cohort with a larger sample size. Finally, our study lacks an independent validation cohort and therefore our results should be replicated. For that, multicenter studies with large patient numbers are needed, which for rare diseases as JIA can be difficult. Therefore, an international collaboration is warranted to pool clinical data for analysis of gene associations and to validate the observed associations.

Unlike other studies that examined the associations of SNPs within genes in the MTX metabolic pathway with MTX response in JIA,[11-13,15] we analyzed our data longitudinally. A study in RA patients revealed that multiple measurements per patient with the same number of patients reduces the between-subject variability and will increase power.[52] In addition, we showed earlier that response to MTX in JIA can fluctuate over time and thus should be analyzed in a longitudinal way.[53] For this reason we did not apply a multifactor dimensionality reduction (MDR) analysis on our data. Recently other authors[15,54] have introduced MDR into the field of predicting MTX response in arthritis. This is an elegant method to reveal interactions between

covariates on an outcome in a cohort. However, for MDR analysis, our longitudinal MTX response data has to be transformed into 1 binary variable, missing cases have to be removed and continuous data have to be stratified. This would mean a loss of most of the benefits of longitudinal analysis.[52,53] Instead we chose to analyze our data with a generalized linear mixed model to make use of the longitudinal character of our data. Nonetheless, MDR identified identical SNPs significantly associated with MTX response as the general linear mixed model.

The present longitudinal study is the first to our knowledge to associate *ABCB1* and *ABCC3* gene polymorphisms with response to MX in patients with JIA. *ABCB1* rs1045642, *ABCC3* rs4793665 and *SLC19A1* rs1051266 are possibly associated with MTX outcome according to ACRped70 criteria. These polymorphisms may be used to optimize the treatment of patients with JIA.

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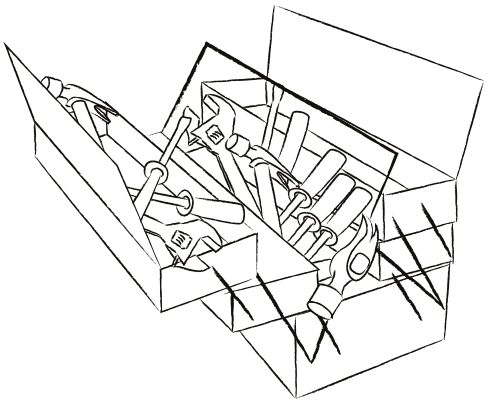
Supplemental table 1 SNP nomenclature.

Gene	rs number	HGVS c.code	VIC Allele	FAM Allele	Minor Allele	Code
ABCB1/MDR1	rs1128503	c.1236T>C	G	A	A	G>A
	rs2032582	c.2677T>G	C	A	A	C>A
		c.2677T>A	C	T	T	C>T
	rs1045642	c.3435T>C	G	A	A	G>A
ABCC1/MRP1	rs35592	c.1219-176T>C	C	T	C	T>C
	rs3784862	c.615+413G>A	A	G	G	A>G
ABCC2/MRP2	rs4148396	c.3258+56T>C	C	T	T	C>T
	rs717620	c.-24C>T	C	T	T	C>T
ABCC3/MRP3	rs4793665		C	T	C	C>T
	rs3785911	c.4476-1022A>C	A	C	C	A>C
ABCC4/MRP4	rs868853		C	T	C	T>C
	rs2274407	c.912G>C	A	C	A	C>A
ABCC5/MRP5	rs2139560	c.3854+1820T>C	A	G	A	G>A
ABCG2/BCRP	rs13120400	c.1194+928A>G	C	T	C	T>C
	rs2231142	c.421C>A	G	T	T	G>T
FPGS	rs4451422		A	C	C	A>C
FOLR1	rs11235462		A	T	A	T>A
FOLR2	rs514933	c.150+429T>C	C	T	C	T>C
GGH	rs10106587		A	C	C	A>C
	rs3758149		A	G	A	G>A
SLC46A1/PCFT	rs2239907*		C	T	T	C>T
SLC19A1/RFC	rs1051266	c.80A>G	C	T	T	C>T

SNP nomenclature used in our study based upon the combination of Taqman VIC and FAM labeled probes for allele detection and MAF. Gene names are according to HUGO gene nomenclature. *rs2239907 is in the Hapmap/NCBI database located to the *SARM1* gene which has an overlap with the *SLC46A1* gene. SNP, single nucleotide polymorphism; HGVS c.code, Human genome variation society coding DNA reference sequence; *MDR1*, multidrug resistance 1; *MRP1*, multidrug resistance protein 1; *BCRP*, breast cancer resistance protein; *ABCB1*, adenosine triphosphate-binding cassette transporter B1; *FPGS*, folylpolyglutamate synthetase; *FOLR1*, folate receptor 1; *GGH*, γ-glutamyl hydrolase; *SLC19A1*, solute carrier 19A1; *PCFT*, proton coupled folate transporter; *RFC*, reduced folate carrier; MAF, minor allele frequency; HUGO, human genome organization; NCBI, national center for biotechnology information; *SARM1*, sterile alpha and TIR motif containing 1.



CHAPTER 3B

Transporter Gene Polymorphisms and Methotrexate Adverse Events in Arthritis

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In our recently published prediction model for methotrexate (MTX) non-response in juvenile idiopathic arthritis (JIA),[1] *ABCB1* rs1045642 was described, indicating the relative importance of this polymorphism to predict non-response to MTX in JIA. Also, we were able to reproduce this finding in a prospective cohort of 387 adult patients with rheumatoid arthritis (RA) receiving MTX: the *ABCB1* rs1045642 polymorphism showed an association with improved clinical response (lower disease activity score 28: β=-0.16, p=0.001). We agree that finding genetic predictors for MTX-induced toxicity and gastrointestinal (GI) adverse events is equally as important as response, because toxicity limits the considerations of a dose increase or continuation of MTX and GI adverse events could result in overt refusal by children with JIA to take MTX, making an alternative therapy more appropriate.

In JIA, effects of toxicity such as bone marrow suppression and elevated liver enzymes occurs rarely, leading to a lack of power to perform pharmacogenetic testing. In contrast, GI adverse events occur frequently.[2] We recently developed and validated a questionnaire for GI adverse events in patients with JIA.[2] Using this questionnaire, we documented GI adverse events, such as abdominal pain, nausea and vomiting as well as fatigue in a prospective cohort of 140 patients with JIA. We then tested associations of GI adverse events with polymorphisms in MTX transporter genes,[3] in particular 3 months after start of MTX because GI adverse events shortly after starting MTX have the most influence on limiting dose increases or continuation. Associations were tested with a univariate logistic regression analysis.

Within 3 months of starting MTX, 46% of the patients reported abdominal pain, 43% nausea, 11% vomiting and 49% fatigue. There was a trend towards association of *ABCC2* rs4148396 (OR=0.52, 95%CI=0.26-1.05, p=0.070) and *ABCC3* rs4793665 (OR=0.49, 95%CI=0.24-1.01, p=0.052) polymorphisms with nausea. The *SLC19A1* rs1051266 polymorphism showed a trend to association with abdominal pain (OR=2.76, 95%CI=0.88-8.62, p=0.081). The *ABCC3* rs4793665 (OR=0.33, 95%CI= 0.12-0.91, p=0.031) and *SLC19A1* rs1051266 (OR=2.94, 95%CI=1.37-6.31, p=0.006) polymorphisms were associated with fatigue. For these findings to be useful in clinical practice, multivariate analyses, meta-analyses and validation studies are needed, eventually leading to construction of prediction models. We are currently constructing a prediction model for GI intolerance as we did for MTX non-response in JIA.[1]

As dr. Ranganathan stated,[4] it would be interesting to know whether MTX pharmacogenetic associations are comparable between children with JIA and adults with RA. Therefore, we also have investigated transporter gene polymorphisms in a cohort of 387 adult patients with RA. The following adverse events were analysed after 3 months of therapy: GI adverse events (diarrhoea, vomiting, and sickness or abdominal pain) and malaise (fatigue, dizziness, headache, sleeplessness or feeling not well). GI adverse events were observed in 43% of the patients and malaise in 45%. In a univariate logistic regression analysis, we found only 1 significant association: The *ABCB1* rs1045642 polymorphism was associated with malaise (OR 2.57, 95% CI 1.59-4.15, p<0.001).

Hence, in our RA cohort, we did not reproduce the associations between polymorphisms and adverse events observed in our JIA cohort. These findings could indicate that the genetics/MTX-outcome relations may differ between children and adults. Although we did observe effects of transporter gene polymorphisms on GI adverse events in JIA and RA, we were not able to replicate the findings of Ranganathan et al.[5] This underscores the need for meta-analysis and collaborations between centres to build prediction models for MTX outcomes of MTX therapy in paediatric and adult rheumatic diseases.

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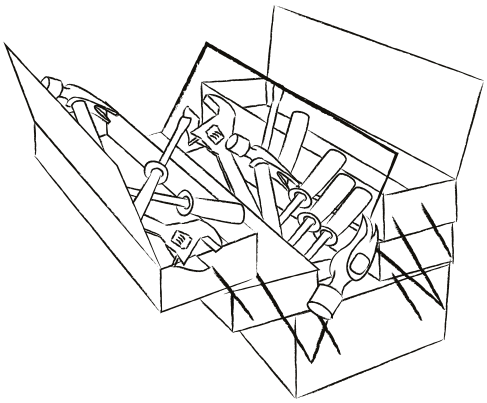

CHAPTER 4

Association of Low Baseline Levels of Erythrocyte-Folate with Treatment Non-Response at 3 Months in Rheumatoid Arthritis Patients Receiving Methotrexate

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ABSTRACT

Objective

To investigate whether baseline concentrations of one-carbon metabolism biomarkers were associated with non-response and adverse events in rheumatoid arthritis (RA) patients on methotrexate (MTX).

Methods

A prospective derivation cohort (n=285) and validation cohort (n=102) of RA patients receiving MTX were studied. Concentrations of plasma-homocysteine, serum-vitamin B12, serum-folate, erythrocyte-vitamin B6 and erythrocyte-folate were determined at baseline and after 3 months of treatment. Non-response after 3 months was assessed using the disease activity score in 28 joints (DAS28) and the European League Against Rheumatism (EULAR) response criteria. Adverse events at 3 months were assessed using biochemical parameters and health status questionnaires. Analyses were corrected for baseline DAS28, age, gender, MTX dose, co-medication and presence of the methylenetetrahydrofolate reductase 677TT genotype.

Results

In the derivation cohort, the mean DAS28 at baseline and 3 months were 4.94 and 3.12, respectively, and 78% of patient's experienced adverse events. This was similar between the cohorts, despite a lower MTX dose in the validation cohort. Patients with lower levels of erythrocyte-folate at baseline had a higher DAS28 at 3 months in both the derivation cohort (ß=-0.15, p=0.037) and the validation cohort (β=-0.20, p=0.048). In line with these results, lower baseline erythrocyte-folate levels were linearly associated with a 3 months DAS28 >3.2 in both cohorts (derivation cohort, p=0.049; validation cohort, p=0.021), and with non-response according to the EULAR criteria (derivation cohort, p=0.066; validation cohort, p=0.027). none of the other biomarkers (levels at baseline or changes over 3 months) were associated with the DAS28 or treatment non-response. Baseline levels of the biomarkers and changes in levels after 3 months were not associated with incidence of adverse events.

Conclusion

A low baseline concentration of erythrocyte-folate is associated with high disease activity and non-response at 3 months after the start of MTX treatment and could be used in prediction models for MTX outcome. None of the investigated one-carbon metabolism biomarkers were associated with incidence of adverse events at 3 months.

INTRODUCTION

Methotrexate (MTX) is the cornerstone disease-modifying anti-rheumatic drug (DMARD) in the treatment of rheumatoid arthritis (RA). In significant numbers of patients, MTX fails to achieve adequate suppression of disease activity and induces adverse events, which impacts the ability to increase or even continue the therapeutic dose.[1] Patients who do not respond to MTX or develop severe adverse events within 3 months after the start of MTX treatment are frequently treated with biologicals, alone or in combination with MTX.[2] The ability to predict MTX non-response and MTX-induced adverse events before the initiation of this DMARD treatment is paramount, since the first months following diagnosis represent a window of opportunity during which outcomes can be more effectively modulated by therapy.[3] To ensure that only patients who are nonresponsive to MTX receive early additional treatment with biologicals, and those who are responsive to MTX are spared from treatment with costly biologicals, it is necessary to identify non-responders and patients prone to experience adverse events at baseline. In order to predict MTX non-response and occurrence of adverse events, risk factors of these outcomes should be identified.[4,5]

Earlier studies have examined clinical and genetic risk factors for MTX non-response in RA patients.[1,6] Besides clinical and genetic determinants, phenotypic markers (metabolites/proteins) could also be potential predictors of MTX non-response. MTX is a folate antagonist that uses the same transport mechanisms as folate.[7] MTX as well as food-derived folate or supplemented folates, are taken up intracellularly via solute carrier 19A1 and incorporated into the folate pathway (one-carbon metabolism). Inside cells, MTX inhibits key-enzymes involved in one-carbon metabolism, and this mechanism is responsible for the therapeutic effects of MTX, as well as its adverse event profile. Important phenotypic markers of one-carbon metabolism, such as concentrations of plasma-homocysteine, serum-vitamin B12, serum-folate, erythrocyte-vitamin B6, and erythrocyte-folate, may determine the extent of MTX non-response and MTX-related adverse events. Nevertheless, these biomarkers have rarely been studied as risk factors of MTX outcome.[8,9]

We therefore investigated whether these one-carbon metabolism biomarkers, measured at baseline, could be associated with MTX non-response and incidence of adverse events over 3 months of follow-up in a prospective cohort study of RA patients receiving MTX. We also validated our findings in an independent validation cohort of RA patients.

PATIENTS AND METHODS

Patients

Data from 2 prospective cohorts of RA patients, all of whom were white, were collected. The derivation cohort consisted of patients who were enrolled in the treatment in Rotterdam Early Arthritis Cohort (tREACH) study, which is a multicentre, stratified single-blind clinical trial (ISRCTN26791028) of patients with early RA, as previously described.[10] The validation cohort consisted of patients with RA from the methotrexate

in Rotterdam, Netherlands (MTX-R) cohort. These latter patients were started on MTX treatment between January 2006 and March 2011 in the department of rheumatology of Erasmus University Medical Center (Erasmus MC), Rotterdam, the Netherlands. The medical ethics committee of Erasmus MC approved both studies, and patients gave their written informed consent before inclusion.

The derivation cohort included patients receiving MTX who fulfilled the American College of Rheumatology/European League Against Rheumatism (EULAR) 2010 criteria for RA.[11] Patients in the validation cohort were included when diagnosed as having RA by the physician. Patients from the derivation cohort were started on an MTX dosage of 25 mg/week. The patients in this cohort were randomized to receive either MTX alone or co-treatment with sulfasalazine, hydroxychloroquine, and glucocorticosteroids,[10] whereas in the validation cohort, the dosage of MTX and co-medications were chosen by the physician. In both cohorts, patients received folic acid (10 mg/week) during MTX treatment. All patients were assessed at baseline and after 3 months of treatment.

Biomarkers

Three research tubes of blood samples were obtained during every study visit, in addition to routine blood samples for determination of the erythrocyte sedimentation rate (ESR) and levels of C-reactive protein (CRP), alanine-aminotransferase (ALAT), leukocytes, and thrombocytes. One serum tube was centrifuged for 10 minutes at 1,700 g at a temperature of 4 ^{o}C, and the serum was divided into aliquots and stored at -80 ^{o}C. One EDTA tube was immediately put on ice after collection, and centrifuged for 10 minutes at 1,700 g at a temperature of 4 ^{o}C, and plasma and aliquots of cell pellets were stored at -80 ^{o}C. One EDTA tube was kept at room temperature, and the whole blood was divided into aliquots and stored at -80 ^{o}C.

The concentration of homocysteine was determined in EDTA-plasma using isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS; waters Acquity UPLC Quattro Premier XE), by an adapted method.[12] For chromatographic separation, a Waters Symmetry C_8 column (2.1x100mm) with a pre-column (Waters, Etten-Leur, Netherlands) was used. Concentrations of vitamin B12 and folate in the serum were measured using an electrochemiluminescence immunoassay (Modular E170, Roche, Almere, Netherlands). The concentration of vitamin B6 was measured in whole blood with an isotope-dilution LC-MS/MS assay, as described previously.[13] For the erythrocyte-folate assay, 100 µl whole blood was diluted with 1,600 µl of a 10 g/L ascorbic acid solution (pH 4) and incubated for 3 hours at room temperature. Tubes were centrifuged at 2,000 g and analysed with an electrochemiluminescence immunoassay for folate (Modular E170, Roche). The concentration of erythrocyte-folate was measured in whole blood from the EDTA tube at room temperature within 24 hours after sample collection. The sustained stability of erythrocyte-folate at room temperature for up to 24 hours has been proven in a previous study.[14] The Erythrocyte-folate levels were corrected for those of serum-folate and haematocrit.

Routine haematology parameters were measured using a Sysmex XE-2100 instrument (Sysmex, Etten-Leur, Netherlands), and the ESR was measured using a Sysmex InteRRliner.. Routine chemistry parameters were measured on a Roche Modular P analyser. Isolation of DNA and genotyping for the methylenetetrahydrofolate reductase (*MTHFR*) 677T-allele was done using a previously described method.[5]

Assessment of treatment non-response and adverse events

The primary outcome assessed was the disease activity score in 28-joints (DAS28) with ESR,[15] which was assessed at baseline and after 3 months of follow-up. In rheumatology practice, physicians assess disease activity levels using a DAS28 cutoff value of >3.2 to define active disease, and assess treatment response according to whether changes in disease activity meet the EULAR response criteria for RA;[16] both of these are used as the decision points for continuing or stopping medication. Therefore, MTX non-response was defined as a DAS28 score of >3.2 and failure to meet the EULAR response criteria.

Specifically, the EULAR response criteria are based on an attained level of improvement in the DAS28 as well as extent of change in the DAS28 over a defined follow-up period. Patients are classified as either non-responders, moderate-responders or good-responders. In this study, we dichotomised the EULAR criteria into non-response versus moderate/good-response. Of note, the EULAR response criteria allow assessment of only those patients whose DAS28 at baseline is ≥3.3.

Adverse events were assessed with biochemical and self-reported measures. Gastrointestinal symptoms, malaise, psychological disorders, MTX-related hepatotoxicity, MTX-related depression of bone marrow, and “other” adverse events were counted as an adverse event. Dichotomized categories of ≥1 adverse event (versus non) and ≥3 adverse events (versus ≤2) were also analysed as outcome variables. The adverse event categories of gastrointestinal symptoms, malaise, psychological disorders, and “other” adverse events were all accumulations of different symptoms that were reported on the patient health status questionnaires. Gastrointestinal symptoms comprised diarrhoea, vomiting, sickness, and abdominal pain. Malaise comprised fatigue, dizziness, headache, sleeplessness, and not feeling well. Psychological disorders involved depression and personality changes. “Other” adverse events comprised dyspnoea, alopecia, infection, mucositis, epistaxis, and skin-related disorders. If a patient reported experiencing none of these symptoms over the follow-up and also did not meet the criteria for hepatotoxicity or bone marrow depression, the patient was scored as having no adverse event. Hepatotoxicity was defined as ALAT >3 times the upper limit of normal. Bone marrow depression was defined as a leucocyte count $<3.0 \times 10^9$/L or thrombocyte count $<100 \times 10^9$/L.

We did not determine the percentage of patients with adverse events at baseline. The category of ≥3 adverse events was assigned to patients who experienced more than 1 symptom over the follow-up. For example, if a patient reported having the

symptoms of diarrhoea, vomiting, and sickness, he or she was scored as having gastro intestinal symptoms and as having ≥3 adverse events.

Statistical analysis

Statistical comparisons were made using Student's *t*-test, chi-square test, or Mann-Witney U-test, as appropriate. Multivariate regression analysis was used to examine the associations between biomarker levels at baseline and the change in biomarker levels and the DAS28 at 3 months. Results are expressed as the standardised β coefficient. Logistic regression analysis was used for dichotomous outcome measures of DAS28 >3.2 (versus DAS28 ≤3.2), non-response (versus moderate/good-response) according to the EULAR criteria, and presence (versus absence) of adverse events. Results are expressed as the odds ratio (OR) with 95% confidence interval (95% CI).

If necessary, biomarker levels were normalised by transformation to their natural logarithm, to improve plots of the residual analyses. To examine whether there was a linear or non-linear association, biomarkers were analysed continuously, and concentrations were analysed into quintiles according to ranges of values (from lowest levels in the first quintile to highest levels in the fifth quintile). To test whether there was a significant ($p < 0.05$) effect modification, interaction terms, defined as, for example, biomarker x co-medication, were included in all multivariate models. If an interaction terms was significant, analyses were stratified. Analyses were corrected for confounders, including age, gender, baseline DAS28, MTX dose, presence of *MTHFR* 677TT genotype, use of other DMARDs, and use of glucocorticoids.[6,17]

In addition, we investigated whether the results based on the DAS28-ESR, the routine outcome measure used in our studies, would be comparable to results assessed with the DAS28-CRP in the pooled cohort. We also examined the potential effect modification by *MTHFR*-677 T allele genotype, by stratifying the significant biomarker-outcome associations by genotype in the pooled cohort. The potential effect modification was explored by defining the interaction term as significant biomarker x presence of *MTHFR* 677 T allele variants.

All statistical analyses were performed using the SPSS statistical package, version 20.0.0.1 (SPSS Inc. Chicago, USA).

RESULTS

Characteristics of the patients

Flow charts for the derivation cohort and validation cohort, with numbers of patients assessed for eligibility and reasons for dropping out, are given in figure 1. The 3 month follow-up data from the derivation cohort were reported in an earlier study.[18] For the present study, 285 patients from the derivation cohort were included at baseline, and 270 were still participating after 3 months. For the validation cohort, 102 patients were included at baseline, and 84 were still participating after 3 months. Table 1 shows the baseline characteristics of both cohorts. The mean MTX dosage was higher in the derivation cohort compared to the validation cohort. In the validation cohort, the DAS28

was lower, more patients were taking non-steroidal anti-inflammatory drugs, fewer patients were taking glucocorticoids, and more patients had received MTX via subcutaneous injection when compared to patients in the derivation cohort.

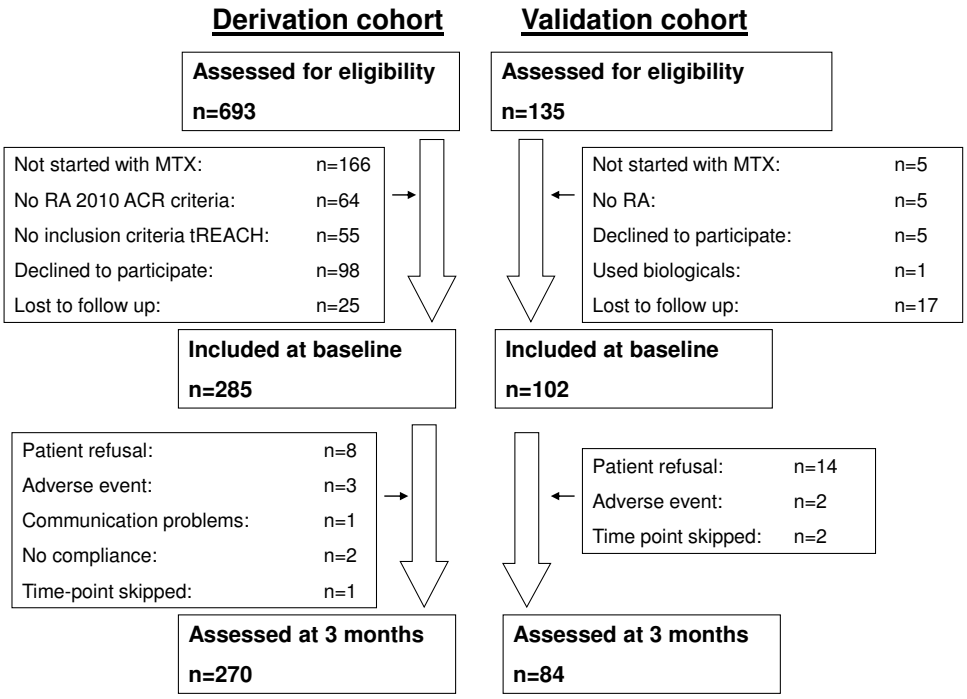

Figure 1 Flow chart of the distribution of rheumatoid arthritis patients in the derivation and validation cohorts at baseline and follow up. MTX, methotrexate; RA, rheumatoid arthritis; ACR, American College of Rheumatology; tREACH, treatment in Rotterdam Early Arthritis Cohort.

Biomarker concentrations

Baseline levels of the one-carbon metabolism biomarkers were comparable in both cohorts, with the exception of the baseline erythrocyte-folate level, which was lower in the derivation cohort (median 844 nmol/L versus 1,079 nmol/L, $p<0.001$) (Table 1). Figure 2 shows all biomarker concentrations at baseline and at 3 months in both cohorts. The majority of one-carbon metabolism biomarker concentrations remained stable over time, apart from serum-folate, whose concentration increased in both cohorts (derivation cohort, median 17 nmol/L at baseline versus 31 nmol/L at 3 months; validation cohort, median 17 nmol/L at baseline versus 26 nmol/L at 3 months) (each $p<0.001$). The erythrocyte-folate level increased only in the derivation cohort (median 844 nmol/L at baseline versus 940 nmol/L at 3 months, $p=0.023$) (figure 2).

Table 1 Baseline characteristics of the cohort

Laboratory parameters	**Derivation cohort (n=285)**	**Validation cohort (n=102)**	**p**
Plasma-homocysteine, µmol/l, median (IR)	11 (10-14)	12 (10-16)	0.264
Serum-vitamin B12, pmol/l, median (IR)	290 (231-404)	286 (230-376)	0.588
Serum-folate, nmol/l, median (IR)	17 (13-24)	17 (13-23)	0.742
Erythrocyte-vitamin B6, nmol/l, median (IR)	80 (64-97)	74 (64-102)	0.485
Erythrocyte-folate, nmol/l, median (IR)	844 (662-1165)	1079 (868-1326)	<0.001
Rheumatoid factor positive, %	66	41	<0.001
Anti-cyclic citrullinated peptide antibody positive, %	70	41	<0.001
Erythrocyte sedimentation rate, mm/h, median (IR)	23 (13-40)	19 (9-33)	0.011
C-reactive protein, mg/l, median (IR)	8 (4-23)	7 (3-14)	0.444
methylenetetrahydrofolate reductase-677T-allele, %	53	42	0.107
Clinical parameters			
Gender, male, %	30	29	0.991
Age, years, mean (SD)	54 (14)	52 (16)	0.299
VAS, mm, mean (SD)	53 (22)	54 (26)	0.704
28 tender joint count, median (IR)	6 (3-10)	4 (1-8)	<0.001
28 swollen joint count, median (IR)	6 (3-10)	3 (1-7)	<0.001
DAS28, mean (SD)	4.94 (1.15)	4.26 (1.43)	<0.001
Medication			
Methotrexate dosage, mean (SD)	25 (1)	15 (2)	<0.001
NSAIDs, %	14	36	<0.001
Other DMARDs, %	62	57	0.408
Oral glucocorticoids, %	62	11	<0.001
Parenteral glucocorticoids, %	32	3	<0.001
Subcutaneous methotrexate injections, %	0	6	<0.001

IR, interquartile range; SD, standard deviation; VAS, patient global assessment of general health on a visual analogue scale; DAS28, disease activity score in 28 joints; NSAID, non-steroidal anti-inflammatory drug; DMARD, disease-modifying antirheumatic drug.

Treatment response

In both cohorts, disease activity decreased over time. In the derivation cohort, the mean ± SD DAS28 was 4.94 ±1.15 at baseline and decreased to 3.12 ±1.19 after 3 months. In the validation cohort, the DAS28 decreased from 4.26 ±1.43 at baseline to 2.92 ±1.23 at 3 months. When treatment response was determined according to the EULAR response criteria, 46% of patients were good-responders, 38% were moderate-responders, and 16% were non-responders after 3 months of MTX treatment in the derivation cohort. In the validation cohort, 43% were good-responders, 39% were moderate-responders, and 18% were non-responders. In comparing the derivation cohort with the validation cohort, the DAS28 (mean 3.12 versus 2.92, p=0.174) and EULAR non-response rate (16% versus 18%, p=0.879) were comparable after 3 months.

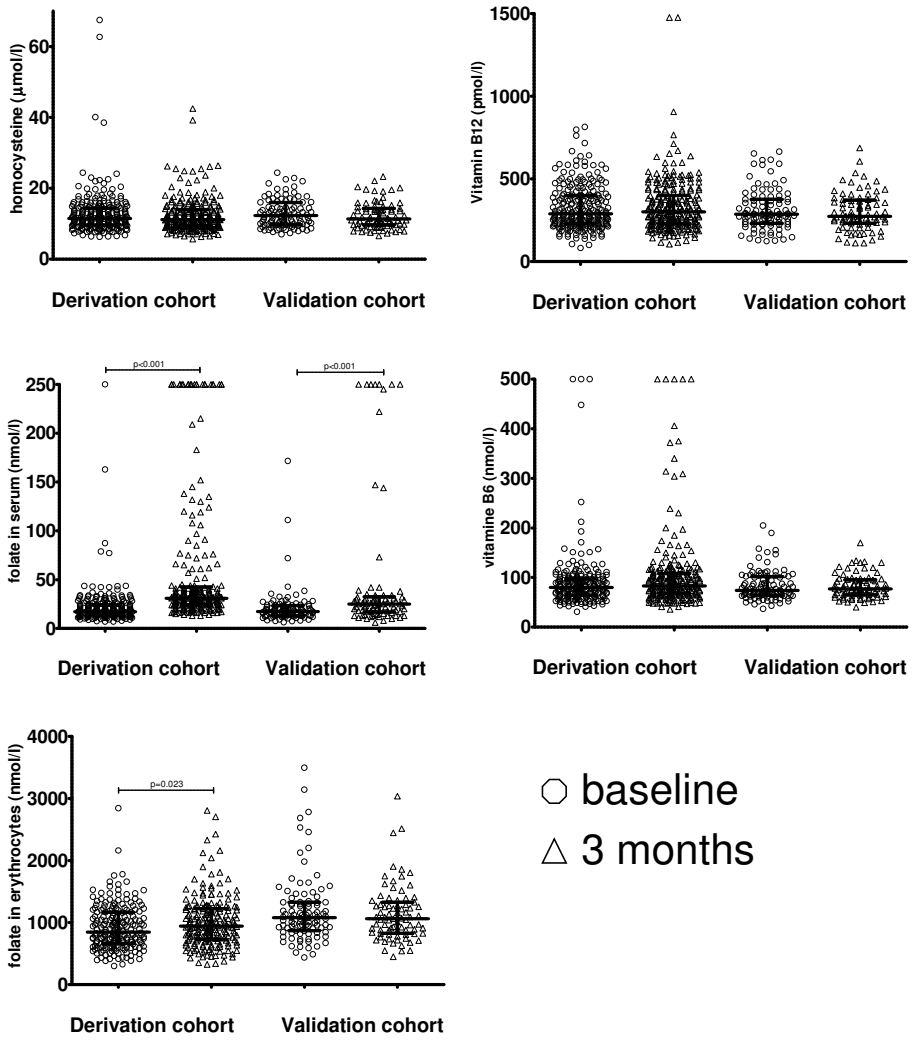

Figure 2 Concentrations of one-carbon metabolism biomarkers at baseline and after 3 months of methotrexate treatment in the 2 cohorts. Results are shown as the median with interquartile range.

A lower baseline level of erythrocyte-folate was associated with a higher DAS28 after 3 months (derivation cohort, ß=-0.15, p=0.037; validation cohort, ß=-0.20, p=0.048) (table 2). In line with these results, the results of logistic regression analyses, in which the erythrocyte-folate level (stratified into quintiles) was the independent variable and a DAS28 >3.2 was the dependent variable, showed a linear trend toward association in

both the derivation cohort (p=0.049) and the validation cohort (p=0.021) (figure 3). In summary, a low concentration of erythrocyte-folate at baseline was associated with MTX non-response (a higher DAS28) at 3 months of treatment. There were no significant interaction terms.

Table 2 Linear regression analysis of associations between one-carbon biomarker levels and the DAS28 after 3 months of MTX treatment in both cohorts.

Baseline	**Derivation cohort Standardized-ß (p-value)**	**Validation cohort Standardized-ß (p-value)**
Plasma-homocysteine	-0.07 (0.310)	0.16 (0.106)
Serum-vitamine B12	0.08 (0.243)	-0.04 (0.728)
Serum-folate	-0.06 (0.362)	-0.12 (0.223)
Erythrocyte-vitamine B6	0.01 (0.861)	-0.16 (0.083)
Erythrocyte-folate	-0.15 (0.037)	-0.20 (0.048)
Change from baseline to 3 months		
Plasma-homocysteine	-0.01 (0.842)	0.00 (0.973)
Serum-vitamine B12	-0.04 (0.541)	0.08 (0.430)
Serum-folate	-0.01 (0.924)	-0.06 (0.520)
Erythrocyte-Vitamine B6	-0.04 (0.583)	0.00 (0.998)
Erythrocyte-folate	0.02 (0.748)	0.09 (0.333)

Analyses were corrected for age, gender, baseline DAS28, MTX dose, use of other DMARDs, use of glucocorticoids, and presence of the *MTHFR* 677TT genotype. DAS28, disease activity score in 28 joints; MTX, methotrexate; DMARD, disease-modifying antirheumatic drug; *MTHFR*, methylenetetrahydrofolate reductase.

In the derivation cohort, there was a trend toward a linear association of the baseline erythrocyte-folate level with non-response to treatment according to the EULAR criteria at 3 months (p=0.066), and in the validation cohort, this association was significant (p=0.027). There were no associations between the baseline levels of homocysteine, vitamin B12, serum-folate or vitamin B6 and EULAR-defined non-response to MTX at 3 months.

Except for erythrocyte-folate, none of the other one-carbon metabolism biomarkers, measured at baseline, were associated with the DAS28 at 3 months (table 2). Moreover, changes in the levels of any of the one-carbon metabolism biomarkers over time (from baseline to 3 months) showed no association with either the DAS28 or the treatment non-response at 3 months. We also performed the analyses of the association between erythrocyte-folate levels and MTX response using 2 different versions of the DAS28, the DAS28-ESR and the DAS28-CRP, in the pooled cohort. Both results were comparable (DAS28-ESR, β=-0.15, p=0.009; DAS28-CRP, β=-0.13, p=0.038).

In the pooled cohort, the concentration of erythrocyte-folate was associated with the DAS28 (β=-0.15). When the pooled cohort was stratified according to genotype, the association of erythrocyte folate levels was similar between patients with the *MTHFR*

677CC genotype and those with the *MTHFR* CT genotype (β=-0.16 and β=-0.15, respectively). In the *MTHFR* TT stratum, the effect was smaller (β=-0.11). However, the interaction term (*MTHFR* 677CC versus *MTHFR* CT versus *MTHFR* TT x baseline erythrocyte-folate) was not significant (p=0.45). The interaction terms with the *MTHFR* 677 genotype, when divided into 2 categories (CC/CT versus TT and CC versus CT/TT x baseline erythrocyte-folate) were also not significant (p=0.31 and p=0.26, respectively). Thus, there was no evidence of an effect modification of the *MTHFR* 677 genotype on baseline erythrocyte-folate levels in this study.

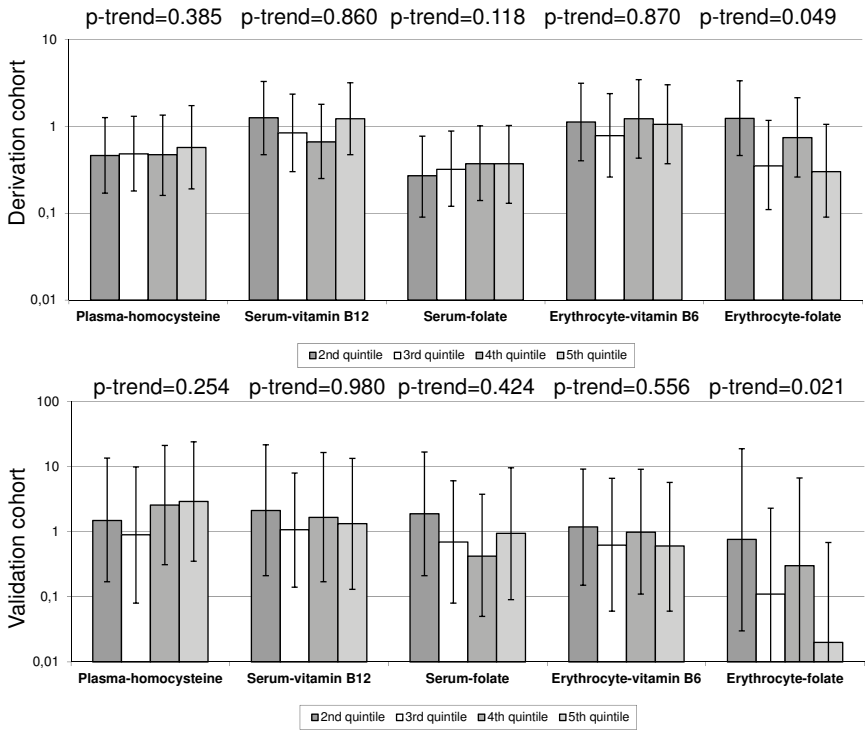

Figure 3 Logistic regression analyses of associations between biomarker concentrations in (stratified as second to fifth quintiles, from lowest to highest levels) and a DAS28 of >3.2 after 3 months of MTX treatment in the 2 cohorts. Results are shown as the OR with 95% CI, relative to the first quintile (set as an OR of 1), corrected for age, gender, baseline DAS28, MTX dose, use of other DMARDs, use of glucocorticoids and presence of the *MTHFR* 677TT-genotype. DAS28, disease activity score in 28 joints; MTX, methotrexate; DMARD, disease-modifying antirheumatic drug; *MTHFR*, methylenetetrahydrofolate reductase.

Adverse events

Figure 4 shows the percentage of patients with adverse events in both cohorts. The percentage of patients with any adverse event over 3 months was comparable in both cohorts (78% in the derivation cohort versus 80% in the validation cohort). Only the percentage of patients with malaise after 3 months of MTX treatment was significantly higher in the validation cohort compared to the derivation cohort (49% versus 32%, p=0.004). No significant associations between baseline levels of the one-carbon metabolism biomarkers and incidence of adverse events were found after 3 months. Furthermore, changes in the biomarkers between baseline and 3 months were not associated with the occurrence of any adverse events.

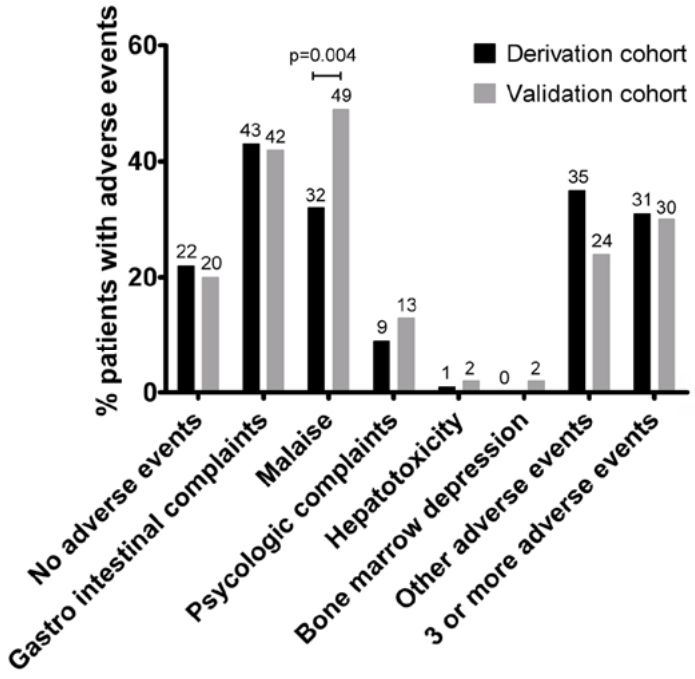

Figure 4 Percentage of patients who reported experiencing no adverse event, ≥3 adverse events, or specific categories of adverse events over 3 months of follow-up in the 2 cohorts. Percentage values are shown over the bars.

DISCUSSION

In RA patients, low baseline erythrocyte-folate levels of erythrocyte folate were linearly associated with non-response to short-term MTX treatment in 2 independent cohorts. None of the one-carbon metabolism biomarkers were associated with incidence of adverse events over 3 months.

Our study is the first to demonstrate that a low level of erythrocyte-folate at baseline is associated with non-response in 2 independent prospective cohorts of RA patients receiving treatment with MTX. We showed that the effect was similar between the derivation cohort and the validation cohort and was independent of the response criteria used. Erythrocyte-folate levels have been associated before with MTX outcome in 2 cross-sectional studies in RA patients.[8,9] In these studies, a higher erythrocyte-folate level was associated with higher disease activity. However, those patients were being treated with MTX and folic acid at the time of blood collection. Therefore, these associations could not be used for prediction of MTX outcome, since erythrocyte-folate concentrations may be influenced by MTX competition and folate supplementation. In our cohorts, patients were not receiving folic acid or MTX at baseline. Studies on the effects of folic acid supplementation on the MTX response have reported either no effects[19] or a negative association.[20] Taken together, these results suggest that lower concentrations of folate during MTX treatment facilitate higher effectiveness of MTX in the competition with folate for transporter proteins, polyglutamylation proteins, and target enzymes for MTX.

In contrast, we found that a lower baseline erythrocyte-folate concentration was associated with MTX non-response in the 2 independent cohorts. A possible explanation for this finding may be that in individuals with lower concentrations of folate, the absorption, transportation, cellular uptake, and retention of folates may be less effective. Since MTX is structurally similar to folate and uses the same means of transportation and metabolism, patients with low baseline levels of intracellular folate may less easily accumulate MTX intracellularly during therapy. In this sense, measurement of the baseline erythrocyte-folate level is a sort of functional assay for the body's capacity to accumulate and retain cellular folate, and thereby predicts how much MTX will be taken up and accumulated during therapy.

This hypothesis is supported in different ways. First, to test this hypothesis, we measured the total concentration of MTX in the erythrocytes of all patients in our 2 cohorts, using a recently described isotope-dilution LC-MS/MS assay.[21] The median total MTX concentration after 3 months of MTX treatment was 130 nmol/L packed erythrocytes (interquartile range [IR] 92-167) in the derivation cohort and 117 nmol/L packed erythrocytes (IR 78-157) in the validation cohort. Patients with lower baseline erythrocyte-folate concentrations achieved lower total erythrocyte-MTX concentrations after 3 months of MTX therapy, and vice-versa (ß=0.17, p=0.011), when analyses were corrected for gender, age, MTX-dose MTX administration route and cohort. In line with this result, others have also shown that erythrocyte-folate levels were positively associated with erythrocyte-MTX levels.[22]

Second, in a recent study of patients with juvenile arthritis, we observed that a genetic polymorphism in the influx transporter solute carrier 19A1 was associated with a diminished response to MTX treatment, and efflux transporter polymorphisms were associated with an improved response.[23] This finding underscores the need for effective uptake and cellular retention of MTX.

Levels of homocysteine and vitamin B12 were not associated with MTX-outcome in this study. An earlier study also reported no association of the baseline homocysteine concentration or the 3 months change in homocysteine concentration with MTX response or toxicity in RA.[24] An increased homocysteine concentration after MTX initiation was observed in earlier studies.[24-27] A decrease in homocysteine levels was observed in groups of patients who received supplementation with folic or folinic acid.[24,26] In the present study, we observed no significant change in the homocysteine concentration between baseline and 3 months of treatment. This may be explained by the fact that all patients in our study received folic acid and MTX. The baseline levels of folate in the serum were not associated with MTX non-response in our study. We did find a significant increase in serum-folate after 3 months. This can be explained by folic acid supplementation. The skewed distribution is probably a result of the combination of the short-elimination half-life of folic acid and the variation in time span between folic acid intake and sample collection. The increase in the erythrocyte-folate levels in the derivation cohort could not be replicated in the smaller validation cohort. Erythrocytes live approximately 3 months, and therefore the 3 month data could be diluted.

We observed no association between a lower erythrocyte-vitamin B6 concentration and MTX non-response. However, earlier research showed that vitamin B6 levels are inversely associated with systemic markers of inflammation.[28] In addition, mild vitamin B6 deficiency characterizes a subclinical at-risk condition in inflammation-related diseases.[29] Patients with lower vitamin B6 concentrations could thus have higher levels of inflammatory disease and, accordingly, a higher chance of being a non-responder. However, baseline vitamin B6 levels were not related to the baseline CRP level ($p=0.319$) or baseline DAS28 ($p=0.755$) in our study, and there was no significant association between the baseline vitamin B6 level and the DAS28 after 3 months.

The derivation cohort had lower baseline erythrocyte-folate concentrations compared to the validation cohort. Higher disease activity will cause higher activity of the immune system, and this could have caused higher usage of folate and may also have influenced the body's capacity to accumulate cellular folate. This might explain the lower baseline erythrocyte-folate level in the cohort with higher baseline disease activity. Erythrocyte-folate levels increased after 3 months of therapy only in the derivation cohort. Patients in both cohorts received 10 mg/week folic acid. The patients in the derivation cohort had lower erythrocyte-folate concentration compared to the patients in the validation cohort. It is plausible that the erythrocyte-folate levels in the patients in the derivation cohort increased to within the same range as those in the validation cohort after 3 months of treatment with 10 mg/week folic acid.

In a systematic review that included 3,463 RA patients who had received long-term treatment with MTX,[30] the authors reported that 72.9% of patients experienced any adverse event, 30.8% had gastrointestinal adverse events, 18.5% developed liver toxicity, 5.5% developed central nervous system toxicity and, 5.2% cytopenia. In our derivation and validation cohort after 3 months of MTX treatment, the percentages of patients with any adverse event (78% and 80%, respectively), gastro-intestinal

symptoms (43% and 42%, respectively), psychological disorders (9% and 13%, respectively), malaise (32% and 49%, respectively), and "other" adverse events (35% and 24%, respectively) were slightly higher than the values reported in the systematic review. In contrast, the percentage of patients who developed hepatotoxicity (1% and 2%, respectively) and bone marrow depression (0% and 1%, respectively) after MTX treatment appeared to be lower in the present study.

There are some differences between the systematic review and our study. First, the adverse events reported herein were measured after 3 months of treatment, whereas in the systematic review, the adverse events were measured after a mean of 36.5 months (range, 27-132 months). When we determined the incidence of these events after 9 months of treatment in our derivation and validation cohorts, we found that 14% and 31% of the patients, respectively, had experienced gastrointestinal symptoms and 4% in each cohort had experienced psychological disorders (results not shown), indicating that fewer patients had developed adverse events after long-term treatment.

Second, there were differences in dosages used. The mean MTX dosage in the systematic review was 8.8 mg/week, whereas in our cohorts, the mean dosages were 15 mg/week and 25 mg/week.

Third, the conditions reported as gastrointestinal symptoms in our cohorts (diarrhoea, vomiting, sickness, and abdominal pain) differed from those in the systematic review (stomatitis, ulcer, abdominal pain, gastrointestinal bleed, dyspepsia, nausea, vomiting, diarrhoea, weight loss, appetite loss).

Fourth, the low incidence of hepatotoxicity and bone marrow depression in our cohorts could have be attributed to our use of a strict definition of these toxicities. We used the definitions recommended by the Dutch Association of Rheumatologists, in which hepatotoxicity is defined as an ALAT level 3 times the upper limit of normal, and those bone marrow depression is defined as a leucocyte count of $<3.0 \times 10^9$/L or thrombocyte count of $<100 \times 10^9$/L. In the systematic review, liver toxicity was defined as an increase in the aspartate aminotransferase and/or ALAT level above the upper limit of normal, and presence of cytopenia (defined as a decrease of >2 g/dl in the haemoglobin level, or a platelet count of $<150 \times 10^9$/L, or white blood cell count of $<3.5 \times 10^9$/L.

Most of the studies in the systematic review did not report or insufficiently reported the use of folic acid. However, a total coverage of folic acid use would probably only have resulted in fewer adverse events being reported in the systematic review, because another systematic review reported a 79% reduction in mucosal and gastrointestinal side effects with the use of folic acid (OR 0.21, 95% CI 0.10-0.44).[19] In summary, we acknowledge that there are some differences in the incidence of adverse events between the literature and our cohorts, but these could be attributed to the above mentioned differences in definition and population characteristics.

None of the investigated baseline biomarkers levels were associated with occurrence of adverse events after 3 months of treatment. In contrast to this finding, 12 patients with juvenile arthritis who had a history of intolerance to MTX treatment were

shown to have significantly lower cellular folate concentrations when compared to 81 patients who had never been treated with MTX.[31] In addition, low-to-normal initial levels of plasma folate and red blood cell folate have been associated with the future toxicity of MTX in RA patients.[32] In our cohorts, all patients were treated with folic acid. This treatment has been proven to reduce MTX-related adverse events in RA patients.[19] This could have diluted the relationship between the investigated biomarker concentrations and adverse events in our study.

The percentages of patients with adverse events in the 2 cohorts were similar when the groups of patients were compared according to their different dosing schemes. A significantly higher percentage of patients experienced malaise in the validation cohort, although the MTX dosing scheme was lower (15 mg/week) than in the derivation cohort (25 mg/week). Malaise is a subjective parameter that is collected in patient self-report questionnaires. The questionnaires in the derivation cohort had closed questions, such as “Were you tired last week?” In contrast, the questionnaires in the validation cohort had a more open character, such as “Write down all adverse events from last week.” However, with closed questions one would suspect that the scores would be higher, because patients would be more aware of possible adverse events. However, this difference in questionnaire designs probably did not have an influence, since we observed a lower percentage of patients with malaise only in the group who responded to closed questions on the health status questionnaires. Taken together, these results indicate that a higher MTX dosing scheme did not lead to more adverse events in our study.

There are some limitations to the present study. First, the hypothesis was based on the MTX working mechanism, and therefore MTX monotherapy would be ideal. However, more than one-half of the patients in both cohorts received other DMARDs in addition to MTX. These drugs also cause a response in terms of modulating disease activity, and can produce similar side effects as those related to MTX. Therefore, we corrected all of the analyses for the use of other DMARDs. The corrected results were not significantly different from the uncorrected results.

Second, the difference in MTX dosage between the 2 cohorts was considerable (25 versus 15 mg/week, $p<0.001$), but all of our analyses were done in the 2 cohorts separately. Nevertheless, the difference in MTX dosage within each cohort was minimal, with standard deviations of 1 mg/week in the derivation cohort (median 25 mg/week, range 10-25) and 2 mg/week in the validation cohort (median 15 mg/week, range 5-25). We have corrected for the MTX dosage in all of our analyses.

Third, the levels of erythrocyte-folate at baseline were not linearly associated with treatment non-response according to the EULAR criteria at 3 months ($p=0.066$) in the derivation cohort, although there was a trend toward association. This might be explained by the smaller sample size of the derivation cohort, due to the restriction that only patients with a baseline DAS28 ≥3.3 could be assessed when applying the EULAR response criteria.

Fourth, unfortunately, we did not register information on the time relationship between folate supplementation and administration of the MTX dose and blood sample withdrawal. Patients in both cohorts were advised to take folate supplementation 2 days after receiving the MTX dosage. However, it would be extremely difficult to monitor in what way patients followed this instruction.

Moreover, the time between blood sample withdrawal and MTX dose or folate supplementation was not registered. The timing of folate supplementation was not registered. The timing of folate supplementation and blood sample withdrawal could have an impact on the 3 month serum-folate concentrations, but would have less impact on the erythrocyte-folate concentrations at 3 months. We are most interested in predicting the clinical response at the start of treatment (baseline), prior to administration of any medication. Our results showed that the baseline erythrocyte-folate level was predictive of the clinical response to MTX after 3 months of treatment. If the time between folate supplementation and blood sample withdrawal were to be standardized, it could be that there might be an effect of the 3 month serum-folate concentration on disease activity. In contrast, the 3 month erythrocyte-folate concentration did not show an effect on the DAS28 after 3 months (derivation cohort, β=-0.08, p=0.283; validation cohort, β=-0.12, p=0.260).

In conclusion, our study is the first prospective study to show that a lower baseline erythrocyte-folate level was associated with non-response to MTX after 3 months of treatment, as measured according to the DAS28, in 2 independent cohorts. Thus, the baseline erythrocyte-folate level may be a promising new biomarker for prediction models of MTX non-response. In contrast, baseline levels of plasma-homocysteine, serum-vitamin B12, serum-folate and erythrocyte-vitamin B6 were unrelated to MTX non-response in RA. None of the investigated folate biomarkers were associated with occurrence of adverse events after 3 months.

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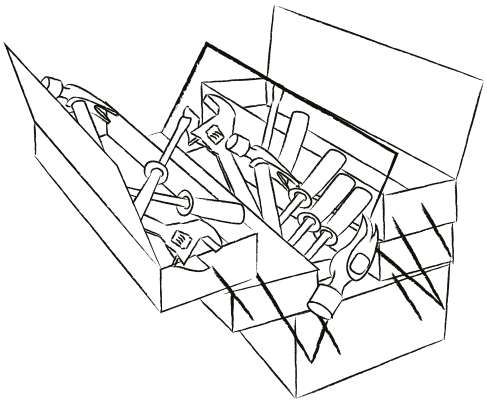

CHAPTER 5

Is baseline Erythrocyte-Folate-Polyglutamate Distribution Associated with 3 Months Erythrocyte-Methotrexate-Polyglutamate Distribution in Rheumatoid Arthritis Patients?

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ABSTRACT

Objective

We investigated whether baseline erythrocyte folate polyglutamates (folate-PG) are associated with erythrocyte-methotrexate polyglutamates (MTX-PG) in rheumatoid arthritis (RA) patients on MTX after 3 months of treatment.

Methods

Sixty-seven RA patients on MTX therapy were selected from 2 prospective cohort studies and analsed for baseline erythrocyte folate-PG and 3 months erythrocyte MTX-PG. Erythrocyte folate-PGs were determined on an affinity HPLC system with four-channel coulometric electrochemical detection. Erythrocyte MTX-PGs were analysed from cell-pellet aliquots with a liquid chromatography-electrospray ionization-tandem mass spectrometry-based assay using stable-isotope-labelled internal standards.

Results

Both baseline short chain folate-PG5-7 and medium/long-chain folate-PG6-9 were positively associated with 3 months short chain MTX-PG1 (β=0.25-0.36, $p<0.040$) and medium/long chain MTX-PG3-5 (β=0.26-0.48, $p<0.037$), respectively.

Conclusion

Baseline erythrocytes folate-PGs in RA patients on MTX are associated with 3 months erythrocyte-MTX-PG concentrations. This finding is consistent with our hypothesis that erythrocyte folate is a reflection of the body's capacity to accumulate and retain cellular MTX.

INTRODUCTION

Methotrexate (MTX) is the cornerstone disease-modifying anti-rheumatic drug (DMARD) in the treatment of rheumatoid arthritis (RA). In significant numbers of patients, MTX fails to achieve adequate suppression of disease activity and induces adverse events, which impacts the ability to increase or even continue the therapeutic dose.[1] This could cause irreversible joint damage and may be prevented by offering patients the right therapy at forehand. Therefore prediction of MTX outcome is needed. New predictors could be identified by thoroughly understanding of the mechanisms of MTX (non)response and adverse events.

Recently we showed that MTX polyglutamates (PG) in erythrocytes are associated with low disease activity over the first 9 months treatment.[2] MTX-PGs have a large inter-patient variability.[3] A part of this inter-patient variability is predicted by baseline erythrocyte folate.[4] We also showed that low baseline erythrocyte folate is a predictor of higher disease activity score (DAS) in 28 joints in RA patients who were treated with MTX for 3 months.[5] A possible explanation for this finding may be that individuals with lower concentrations of folate may be less efficient in absorbing, transporting and retention of folates or structural analogs. Since MTX is structurally similar to folate and uses the same means of transport and cellular retention mechanisms, patients with low baseline intracellular folate are less likely to accumulate MTX intracellularly during therapy. Consistent with this possibility are our recent findings that polymorphism in the influx transporter *SLC19A1* was associated with less response and efflux transporter polymorphisms were associated with improved response in juvenile arthritis[6] underlining the need for effective uptake and cellular retention of MTX.

We also showed recently that a folylpolyglutamate synthetase (*FPGS*) polymorphism is associated with erythrocyte MTX-PG concentrations.[4] *FPGS* is an intracellular enzyme responsible for folate and MTX polyglutamation. Polyglutamation is responsible of preventing cellular escape of folate and MTX. In the present study we investigated the relationship between baseline erythrocyte folate polyglutamates (folate-PG) and MTX-PGs in RA patients after 3 months of MTX treatment.

METHODS

Study design and patients

Data from two prospective cohorts with all Caucasian patients was used for this study. The treatment in Rotterdam Early Arthritis Cohort (tREACH) was a clinical multicentre, stratified single-blinded trial (ISRCTN26791028) described elsewhere.[7] The methotrexate in Rotterdam, Netherlands cohort (MTX-R) consisted of patients who started MTX between January 2006 and March 2011 in the department of Rheumatology, Erasmus University Medical Center, Rotterdam (Erasmus MC), Netherlands.[5] The medical ethics committee from the Erasmus MC approved both studies and patients gave written informed consent before inclusion.

The tREACH included patients on MTX who fulfilled the 2010 ACR/EULAR criteria for RA.[8] Patients in the MTX-R were included when diagnosed with RA by the physician.

Patients from the tREACH started with 25 mg/week MTX and were randomized to treatment with or without sulfasalazine, hydroxychloroquine and glucocorticoids[7] whereas in the MTX-R dosage and co-medication was chosen by the physician. In both cohorts, patients received folic acid (10 mg/week) during MTX treatment. In both cohorts, patients were assessed at baseline and after three months of treatment.

Biochemical analyses

Two additional EDTA blood sample-tubes were obtained from patients during every study visit besides routine EDTA and serum samples. One EDTA tube was immediately put on ice after collection and centrifuged for 10 min at 1700 g at 4 ^{o}C. Plasma and erythrocyte pellet aliquots were stored at -80 ^{o}C. One EDTA tube was kept at room temperature and whole-blood was divided into aliquots and stored at -80 ^{o}C.

Total Erythrocyte-folate was measured in whole blood from the room temp EDTA tube within 24 hours after sample collection as described earlier.[5] Erythrocyte-folate stability at room temperature has been proven op to 24 hours.[9] For the erythrocyte-folate assay, 100 µl whole blood was diluted with 1600 µl of a 10 g/l, pH 4, ascorbic acid solution and incubated 3 hours at room temperature. Tubes were centrifuged at 2000 g and analysed with an electrochemiluminescence immunoassay for folate (Modular E170, Roche).

Folate-PGs were determined according to our updated method for determination of folate polyglutamation.[10,11] Folates were heat-extracted from cell-pellet aliquots from EDTA samples. An affinity HPLC system with four-channel coulometric electrochemical detection was used for analysis. An affinity column was used first to purify folates from the extract. Purified folates were eluted from the affinity column using an acetonitrile gradient. The folate forms separated on a phenyl analytical column and quantified using an electrochemical detector. Folate-PGs were reported in pmol/g hemoglobin (Hb).

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 earlier by us.[12] Concentrations of MTX-PGs were reported in nmol/l packed erythrocytes.

Statistical analysis

Statistical comparisons were made by Student's t-test, X^2-test or Mann-Witney U-test when appropriate. Multivariate regression analysis was used to examine the associations between folate-PGs at baseline and MTX-PGs at three months. Results are expressed as standardised betas.

Statistics were performed with SPSS Statistics Version 21.0.0.1 (SPSS Inc. Chicago, USA).

RESULTS

Patient characteristics

Baseline folate-PGs and 3 months MTX-PGs were determined for 67 patients. Twenty-eight patients were from the tREACH and 39 patients were from the MTX-R cohort. Table 1 shows the baseline characteristics including the folate-PG concentrations. Age, gender, erythrocyte-folate and serum-folate in these 67 patients were not significantly different from the whole tREACH and MTX-R cohorts.[5]

Table 1 Baseline characteristics.

Characteristic	Baseline value
Age, years, mean (SE)	53 (2)
Female, n (%)	44 (66)
Total erythrocyte-folate, nmol/L, mean (SE)	1210 (87)
Serum-folate, nmol/L, mean (SE)	24 (3)
Folate-PG4, pmol/g Hb, mean (SE)	1170 (145)
Folate-PG5, pmol/g Hb, mean (SE)	9019 (1474)
Folate-PG6, pmol/g Hb, mean (SE)	6461 (835)
Folate-PG7, pmol/g Hb, mean (SE)	1851 (213)
Folate-PG8, pmol/g Hb, mean (SE)	408 (73)
Folate-PG9, pmol/g Hb, mean (SE)	142 (28)
Total Folate-PG, pmol/g Hb, mean (SE)	19109 (2632)
MTX dose, mg/week, mean (SE)	19 (1)
DAS28, mean (SE)	4.49 (0.18)

SE, standard error; PG, polyglutamates; Hb, hemoglobin; MTX, methotrexate; DAS28, disease activity score in 28 joints.

Folate polyglutamates

Folate-PG5 was the predominant folate-PG and was on average 41% (SE=1) of the total methyl folate-PG (table 1). Folate-PG4 accounted on average for 7% (SE=1), folate-PG6 for 35% (SE=1), folate-PG7 for 12% (SE=1), folate-PG8 for 3% (SE=0.3) and folate-PG9 for 1% (SE=0.2) of the total methyl folate-PG. Total folate-PG was significantly associated with total erythrocyte-folate (β=0.47, p<0.001). After 3 months MTX therapy mean (SE) concentrations for MTX-PG1, 2, 3, 4, 5 and total MTX-PG were respectively: 44 (6), 25 (2), 46 (2), 16 (1), 4 (1), 134 (8) nmol/L packed erythrocytes.

Table 2 shows that higher baseline short-chain folate-PG5 (β=0.36, p=0.003), folate-PG6 (β=0.36, p=0.003) and folate-PG7 (β=0.25, p=0.040), as well as total folate-PG (β=0.35, p=0.003) were associated with higher 3 months short-chain MTX-PG1. None of the folate-PGs was associated with MTX-PG2. Medium-chain folate-PG6 (β=0.26, p=0.031) and folate-PG7 (β=0.41, p=0.001) were associated with higher concentrations of medium-chain MTX-PG3. Long-chain folate-PG8 (β=0.30, p=0.013) and folate-PG9 (β=0.26, p=0.037) as wel as total folate-PG (β=0.25, p=0.043) were also associated with higher MTX-PG3 concentrations. Medium-chain folate-PG7 (β=0.41, p=0.001) and long-chain folate-PG8 (β=0.44, p<0.001) and folate-PG9 (β=0.38,

p=0.002) were associated with higher long-chain MTX-PG4. Also for the other long-chain MTX-PG, MTX-PG5, medium chain folate-PG7 (β=0.41, p=0.001) and long-chain folate-PG8 (β=0.48, p<0.001) and folate-PG9 (β=0.38, p=0.001) were significantly associated with higher MTX-PG concentrations. Folate-PG5 (β=0.34, p=0.005), folate-PG6 (β=0.37, p=0.002), folate-PG7 (β=0.35, p=0.003), folate-PG8 (β=0.30, p=0.015), folate-PG9 (β=0.27, p=0.028) and total folate-PG (β=0.36, p=0.003) were associated with higher total MTX-PG.

Table 2 Linear regression of baseline folate-PGs with 3 months MTX-PGs

	MTX-PG1 β (p-value)	**MTX-PG2 β (p-value)**	**MTX-PG3 β (p-value)**	**MTX-PG4 β (p-value)**	**MTX-PG5 β (p-value)**	**Total MTX-PG β (p-value)**
Folate-PG4	0.17 (0.163)	-0.04 (0.748)	0.17 (0.165)	0.17 (0.172)	0.09 (0.468)	0.19 (0.126)
Folate-PG5	0.36 (0.003)*	0.01 (0.922)	0.20 (0.102)	0.18 (0.143)	0.11 (0.399)	0.34 (0.005)*
Folate-PG6	0.36 (0.003)*	0.03 (0.786)	0.26 (0.031)*	0.24 (0.053)	0.16 (0.184)	0.37 (0.002)*
Folate-PG7	0.25 (0.040)*	-0.02 (0.882)	0.34 (0.005)*	0.41 (0.001)*	0.41 (0.001)*	0.35 (0.003)*
Folate-PG8	0.20 (0.106)	-0.12 (0.326)	0.30 (0.013)*	0.44 (<0.001)*	0.48 (<0.001)*	0.30 (0.015)*
Folate-PG9	0.20 (0.101)	-0.12 (0.344)	0.26 (0.037)*	0.38 (0.002)*	0.38 (0.001)*	0.27 (0.028)*
Total Folate-PG	0.35 (0.003)*	0.01 (0.941)	0.25 (0.043)*	0.24 (0.050)	0.17 (0.164)	0.36 (0.003)*

*p<0.05. MTX, methotrexate; PG, polyglutamate.

DISCUSSION

We showed in this first prospective study in RA patients, that baseline folate-PG distribution status is associated with 3 months MTX polyglutamation.

Cellular folate metabolism consists of several reactions that involve the transfer of one-carbon groups, leading to the interconversion of folate forms that differ by oxidation state and one-carbon substitutions. Intracellular folates also vary in the number of glutamate residues attached. Folates are transported across cell membranes in their mono- or diglutamyl forms. After entry into the cell, glutamate residues are sequentially added to the folate molecule by the enzyme *FPGS*. The rate of elongation of the glutamate chain length of folates is a function of both the pteridine ring structure of the folate and the number of glutamate residues. Unsubstituted, reduced folates are the preferred substrates for folylpolyglutamate synthetase.[10] With the preferred substrate tetrahydrofolate (THF),1 glutamate chain-length elongation is rapid up to a glutamate chain length of five glutamate residues. The addition of glutamate residues to THF with more than five glutamate residues occurs very slowly. Thus, folate glutamate chain lengths, particularly those greater than five residues, can indicate intracellular residence time of the folate molecule. Other factors that have been reported to affect the glutamate chain length of tissue and cell folates are folate, methionine, and vitamin B12 status.[10]

We showed that baseline short-chain folate-PG concentration is associated with higher 3 months short-chain MTX-PG concentrations, medium-chain folate-PG with

medium-chain MTX-PG and long-chain folate-PG is associated with long-chain MTX-PG. This finding further supports our hypothesis that the capacity of an individual to polyglutamate and retain cellular folate predicts the extents of intake, polyglutamation and accumulation of MTX during therapy. This capacity is likely to reflect both enzyme genetics like *FPGS* polymorphisms[4] and folate/MTX transporter mechanisms,[6] as well as dietary folate intake.

Erythrocyte-folate has been associated before with MTX outcome in two cross-sectional studies in RA patients.[13,14] In these studies, higher erythrocyte-folate was associated with higher disease activity. However, these patients were treated with MTX and folic acid at the time of blood collection. Hence these associations cannot be used for prediction of MTX outcome, and the erythrocyte-folate concentrations are influenced by MTX competition and folate supplementation. Studies on the effects of folic acid supplementation on MTX response report no effect[15] or a negative association.[16] Taken together, these results from literature may suggest that lower concentrations of folate during MTX treatment facilitate higher effectiveness of MTX in the competition with folate for transporter proteins, polyglutamylation proteins and target enzymes for MTX. The results from the present study, suggest that the body's capacity to accumulate and retain cellular MTX is of more importance for non-response than the competition between MTX and folate for transporter proteins, polyglutamylation proteins and target enzymes for MTX.

Like earlier described in JIA patients we also found that folate-PG5 was the predominant folate-PG followed by folate-PG6.[17]

An increase in erythrocyte-MTX-PG concentrations was associated with a decreased DAS28 over 9 months in two longitudinal RA cohorts.[2] Subsequently in JIA, long chain MTX-PGs were associated with lower disease activity during 1 year of MTX treatment.[18] Differences in MTX-PG concentrations between patients may explain MTX non-response in some patients. We showed that differences in MTX-PG concentrations between patients could partially be explained with differences in baseline folate-PG concentrations.

We acknowledge that our study has limitations. The sample size with 67 patients is very small. It is a sample from 2 larger studies, but we showed that baseline characteristics were not significantly different from the 2 original cohorts. We choose to pool the small amount of patients from 2 original studies in order to have greater sample size despite losing the ability of checking our findings in a validation cohort.

In conclusion, baseline erythrocytes folate-PGs in RA patients on MTX are related to 3 months MTX-PG concentrations. This finding further supports our hypothesis that erythrocyte folate is a reflection of the body's capacity to accumulate and retain cellular MTX. Further studies are needed to determine if erythrocytes folate polyglutamates distribution could help predict non-responders to MTX therapy.

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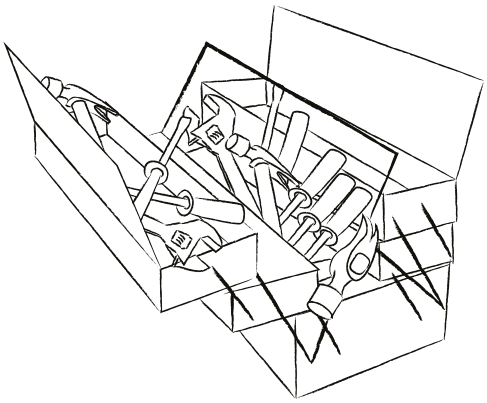

CHAPTER 6

Clinical, Metabolic and Genetic Determinants of Erythrocyte-Methotrexate-Polyglutamate Concentrations at 3 months of Treatment in Rheumatoid Arthritis

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ABSTRACT

Objective

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).

Conclusion

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 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 ^{o}C. Plasma and erythrocyte cell-pellet aliquots were stored at -80 ^{o}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 and genotyping

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. 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, X^2 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

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.

Table 1 Baseline characteristics of MTX-R and tREACH cohorts

Patient demographics	**Derivation cohort** (n=93)	**Validation cohort** (n=247)
Age, years, mean (SD)	51.2 (16.1)	52.8 (14.2)
Female (%)	72.0	69.2
BSA, m3, mean (SD)		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*		
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	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/1.73m3, 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	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	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.*

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).

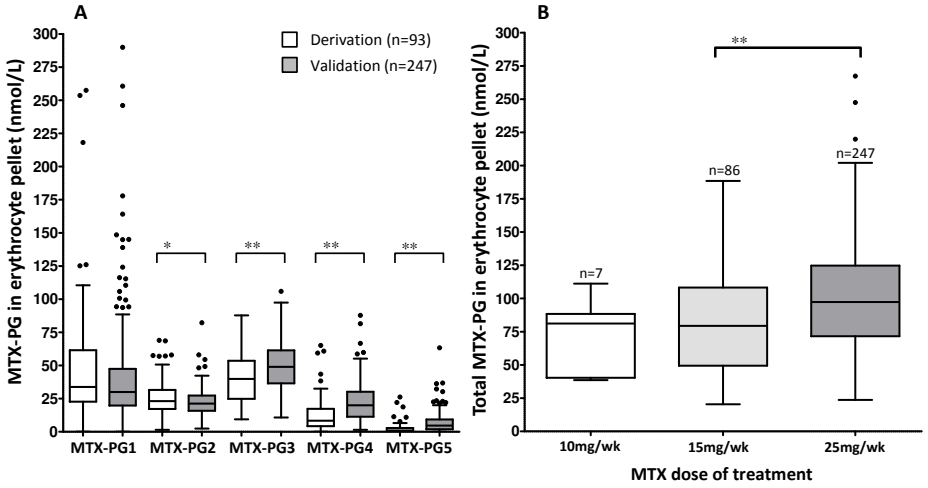

Figure 1 A) Concentrations of the separate MTX-PGs in the derivation (white bars, n=93) and validation (grey bars, n=247) cohorts. Brackets denote significant differences between cohorts, p-values are noted above the brackets. MTX-PG2 is significantly lower in the validation cohort, while MTX-PG3, MTX-PG4, 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.

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).

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).

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) (Figure2b). 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).

Table 2 Clinical, socio-demographic and biochemical determinants of erythrocyte MTX-PG levels at 3 months of treatment (multivariate analysis).

		Derivation cohort						Validation cohort				
		St.β					St.β					
Variable	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 (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 MTX	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	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/BSA	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, >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, >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, >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, >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, 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, >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 DAS28-ESR 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 glucocorticoid route. MTX-PG, methotrexate polyglutamate; ln: natural logarithm; DAS28-ESR, ESR based disease activity score in 28 joints; Anti-CCP, anti-cyclic citrullinated peptide antibody; DMARD, Disease-modifying antirheumatic agent; ESR, erythrocyte sedimentation rate; GFR, Glomerular Filtration Rate.

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

			Derivation Estimated marginal means (SE)						Validation Estimated marginal means (SE)					
			N	MTX-PG1	MTX-PG2	MTX-PG3	MTX-PG4+5	Total MTX-PG	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)

Table 3 Continued.

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)
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)
rs3785911 *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. Only SNPs with a significant correlation with any MTX-PG in the derivation or validation cohort are shown, full table in supplementary table 2. WT, wildtype; Het, heterozygous; Var, homozygous variant; Rs, reference SNP number; *ABCB1*, adenosine triphosphate-binding cassette transporter B1; *FPGS,* folylpolyglutamate synthetase; *FOLR1*, folate receptor 1/2; *GGH*, gamma glutamyl hydrolase; *SLC19A*, solute carrier 19A1.

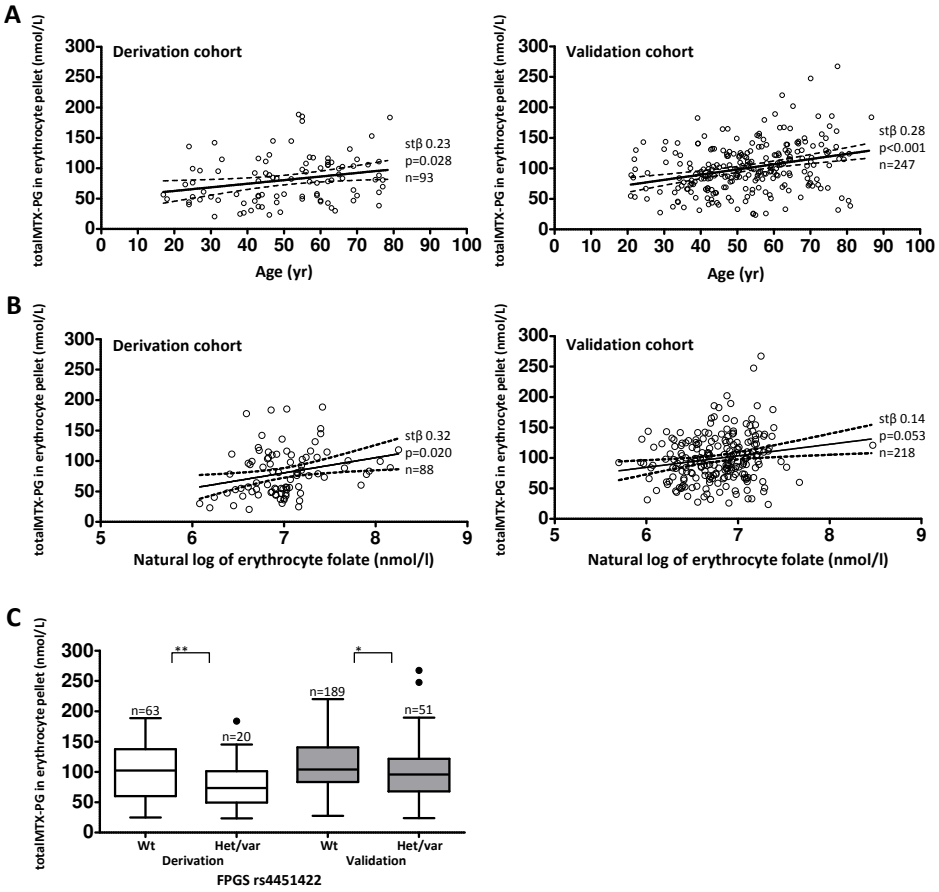

Figure 2 A) Linear regression of age and total MTX-PG. Solid line represents a trendline with its 95% confidence interval (dotted line). In both cohorts age is positively associated with total MTX-PG. Regression analysis is plotted from the unadjusted data, Stβ and p-values are from the confounder adjusted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

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 (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.

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.

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*	-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*	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*	-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	

**FPGS* rs4451422 was dichotomized into wild-type versus heterozygous/variant genotype. Variables which, after correction for confounders, had a significant correlation in both cohorts were included in 1 multivariate regression model. Correlations with p<0.05 were considered significant. Confounders were not shown and were serum folate (for erythrocyte folate) and gender (for FPGS rs4451422). MTX-PG, methotrexate polyglutamate; *FPGS*, folylpolyglutamate synthetase.

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.

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

	Derivation cohort						Validation cohort					
		St.β						St.β				
Variable	**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*	-0,1**8
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/BSA	93	0,02	-0,02	-0,02	-0,15	-0,09	98	-0,11	-0,03	-0,12	-0,22*	-0,17#
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*

Supplemental table 1 Continued.

Alcohol consumption, >4 vs<4 glasses/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/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/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/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. 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.

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.

			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)8
		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)

Supplemental table 2 Continued.

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)
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)

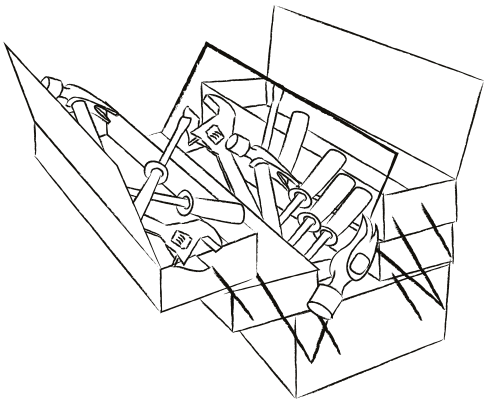
Supplemental table 2 Continued.

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	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)
rs4148396 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)

Supplemental table 2 Continued.

rs2239907 SLC46A1	a>	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 7

Methotrexate-Polyglutamates in Erythrocytes are Associated with Lower Disease Activity in Patients with Rheumatoid Arthritis

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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 erythrocyte-MTX 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 erythrocyte-MTX-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 erythrocyte-MTX-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.

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 ^{o}C. Plasma and cell-pellet aliquots were stored at -80 ^{o}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<$3.0x10^9$/l or thrombocytes<$100x10^9$/l.

Statistical analyses

Comparisons of patient characteristics between derivation and validation cohorts were made by Student's t-test, X^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.

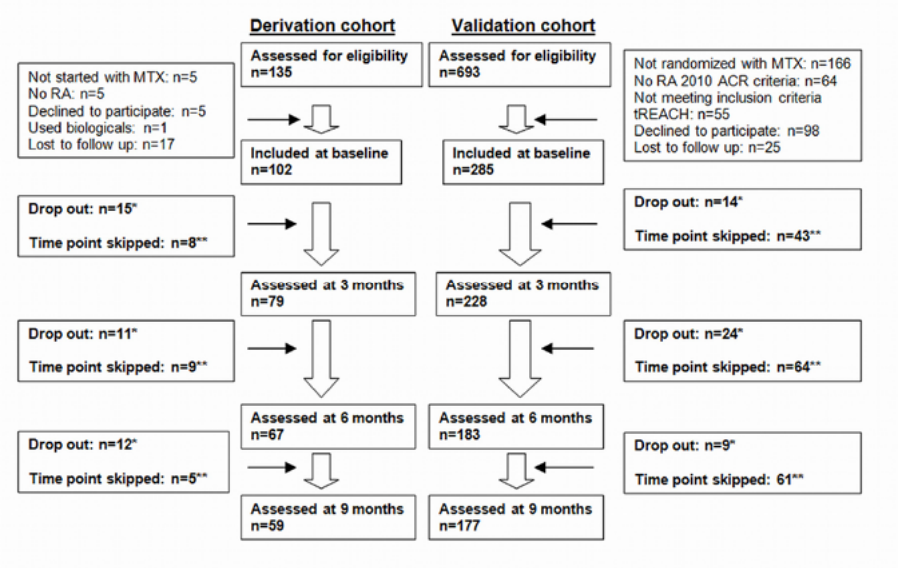

Figure 1 Flow chart of patient follow up for both cohorts. *Reasons for drop out were: patient refusal, adverse events, communication problems, no compliance, lost to follow up, and MTX stopped; **Reasons for time point skipped were: there was insufficient material available for determinations of MTX-PG 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.

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. Table 2 shows medians and ranges of erythrocyte MTX-PG concentrations at 3, 6 and 9 months in both cohorts. Supplemental 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.

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.

Supplemental table 2a/b shows the correlations between all MTX-PG's 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.

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. Supplemental 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 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 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			
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.

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.

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)
Derivation cohort	**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				
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 DAS28, 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; DAS28, disease activity score in 28 joints; DMARD, disease modifying anti-rheumatic drug; NSAID, non-steroid anti-inflammatory drug.

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).

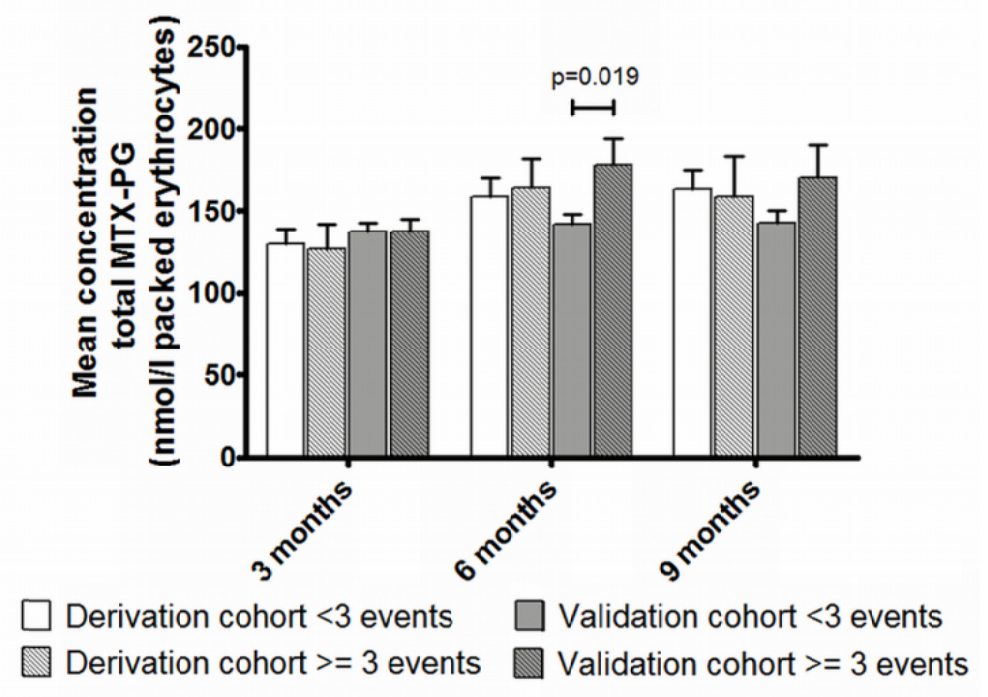

Figure 2 Mean concentration total erythrocyte-MTX-PG in both cohorts for patients with < 3 adverse events and patients with ≥ 3 adverse events after 3, 6 and 9 months. MTX-PG, methotrexate-polyglutamate.

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 (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-PG's 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 erythrocyte-MTX-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 ($p \leq 0.001$). 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 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-PG's 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-PG_{1-5}. 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 erythrocyte-MTX-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.

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Supplemental 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.

Supplemental 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).

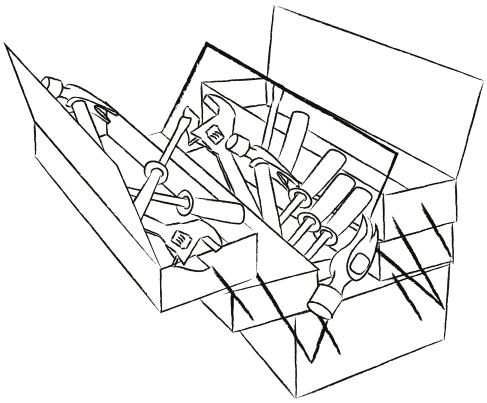
Supplemental table 2b Spearman's correlations in the validation cohort at 3 months

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

Supplemental 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

Effect of Methotrexate Use and Erythrocyte-Methotrexate-Polyglutamate on Glycosylated Hemoglobin in Rheumatoid Arthritis

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ABSTRACT

Objective

We investigated whether methotrexate (MTX) use and erythrocyte-methotrexate-polyglutamate (MTX-PG) concentrations are associated with changes in glycosylated hemoglobin (HbA_{1c}) in rheumatoid arthritis (RA) patients, compared with other therapies.

Methods

For the derivation cohort, RA patients according to the 2010 classification criteria were selected from the treatment in the Rotterdam Early Arthritis Cohort. Data were used from patients randomized into 6 treatment arms: Triple (MTX, sulphasalazine, hydroxychloroquine (HCQ)) disease-modifying antirheumatic drug therapy with intramuscular-glucocorticoids (TDT+IM-GC), TDT+oral-GC, MTX+oral-GC, MTX, oral-GC, and HCQ. HbA_{1c} was determined at baseline and after 3 months. Erythrocyte-MTX-PG1-5 concentrations were measured after 3 months. Within treatment-arms, paired t-test was used to compare HbA_{1c}-change. Associations of MTX-PG concentrations with HbA_{1c}-change were tested with multiple linear regression analysis, adjusted for age, gender, body mass index, and co-medication. Significant associations were validated in RA patients on MTX from the Methotrexate-Rotterdam cohort.

Results

In the derivation cohort, mean HbA_{1c}-change was -1.9 mmol/mol [-0.18%] (p=0.001). This decrease in HbA_{1c} after 3 months of treatment was observed in treatment-arms TDT+IM-GC: -5.5 mmol/mol [-0.50%] (p<0.001), TDT+oral-GC: -3.7 mmol/mol [-0.34%] (p<0.001), MTX: -0.8 mmol/mol [-0.08%] (p=0.018), and HCQ: -2.0 mmol/mol [-0.19%] (p=0.175). In the derivation cohort, MTX-PG2 (β=-0.20; p=0.005), MTX-PG3 (β=-0.31; p<0.001), MTX-PG4 (β=-0.33; p<0.001), MTX-PG5 (β=-0.39; p<0.001) and total MTX-PG (β=-0.29; p<0.001) were associated with decreased HBA_{1c}. In the validation cohort, HbA_{1c} decreased -2.6 mmol/mol [-0.23%] (p<0.001) and MTX-PG3 was associated with decreased HbA_{1c} (β=-0.26; p=0.018).

Conclusion

MTX use and higher erythrocyte-MTX-PG concentration are associated with decreased HbA_{1c} in RA patients. TDT and HCQ reduced and GC increased HbA_{1c}.

INTRODUCTION

Patients with rheumatoid arthritis (RA) experience higher rates of cardiovascular disease.[1,2] This could be explained by a direct effect of inflammation on atherosclerosis[3,4] and/or an increase in cardiovascular risk factors, like diabetes mellitus (DM).[5,6] Increased inflammation as with high disease activity in RA accelerates development of several cardiovascular risk factors such as DM.[7] RA predisposes patients to insulin resistance and places patients at risk for DM.[8-10] This raises the question if RA treatment is also effective in lowering DM incidence and lowering glycosylated hemoglobin (HbA_{1c}). Knowing which RA treatment could be effective in lowering HbA_{1c} may help in preventing diabetes in RA.

Immunosuppression in RA therapies has been related to DM incidence and HbA_{1c} concentrations. Hydroxychloroquine (HCQ) reduces the risk of incident DM[11,12] and reduces HbA_{1c} in RA.[13] In type 2 DM, HCQ has been associated with reduced HbA_{1c}.[13-15] Also, in new onset type 1 DM, which is considered to be an auto-immune disease,[16] immunosuppression with cyclosporin and MTX induced remission and decreased the amount of required insulin.[17] However, glucocorticoids (GC) in RA therapy may increase plasma glucose, as they induce hepatic and peripheral insulin resistance and reduce insulin secretion.[18,19]

MTX is the anchor drug in the treatment of RA. Besides reducing the risk for DM through the effects of systemic immunosuppression, direct effects of MTX on glucose metabolism have recently been found.[20] Chronic treatment with low doses of MTX increases skeletal muscle GLUT4 glucose transporter expression in experimentally-induced diabetic mice and was also associated with significant reduction of glucose and insulin serum concentrations in control and diabetic mice.[20] Therefore, MTX treatment in RA may decrease HbA_{1c} compared to other disease-modifying antirheumatic drugs (DMARD) and in addition this decrease may be controlled by MTX dose. Higher MTX dose leads to higher intracellular erythrocyte-MTX polyglutamate (MTX-PG) concentrations.[21] Circulating MTX contains 1 glutamate moiety (MTX-PG1). Once inside cells, up to 4 additional glutamates (PG2-PG5) are added.

We investigated if treatment with MTX among RA patients changed HbA_{1c} compared to treatment with different DMARDs such as TDT, GC and HCQ. The association between erythrocyte-MTX-PG concentrations and change of HbA_{1c} was also investigated. We hypothesized that the use of MTX and the associated increased erythrocyte-MTX-PG concentrations were associated with reduced HbA_{1c}.

PATIENTS AND METHODS

Patients

Patients from the treatment in Rotterdam Early Arthritis Cohort (tREACH) were used for the derivation cohort.[22,23] This multicentre, stratified single-blinded clinical trial (ISRCTN26791028) was performed in 8 rheumatology centers. Medical ethics committees at each participating centre approved study protocol, and all patients gave written informed consent before inclusion. The validation cohort consisted of patients

from the methotrexate-Rotterdam, Netherlands cohort (MTX-R)[24] who started MTX between January 2006 and March 2011 in the department of Rheumatology from the Erasmus MC, Rotterdam, Netherlands. The medical ethics committee from the Erasmus MC approved the MTX-R study and patients gave written informed consent before inclusion. In the derivation cohort, patients were stratified into 3 groups according to their likelihood of progressing to persistent arthritis based on the Visser prediction model.[25] For this study, we selected those patients who were newly diagnosed with RA, according to 2010 classification criteria.[26] Only patients who were stratified to the high and intermediate risk group were selected. At the time we selected the patients, inclusion for the intermediate risk group was still on going. In the derivation cohort, patients were randomized into one of following treatment strategies in the first 3 months for high risk: 1) triple DMARD therapy (TDT) (MTX, sulphasalazine and HCQ) with intramuscular-GC (TDT+IM-GC), 2) TDT+oral-GC, 3) MTX+oral-GC, and for intermediate risk: 4) MTX, 5) oral-GC and 6) HCQ. For low risk the treatment strategies were 1) non-steroidal anti-inflammatory drugs (NSAID), 2) IM-GC and 3) HCQ. In the validation cohort, patients with RA were included on the moment they started with MTX. Baseline blood sample and question forms were taken just before the first dose of MTX. The second study visit was 3 months after the first dose.

In the derivation cohort DMARD dosages were: MTX: 25 mg/week orally (dosage reached after 3 weeks), sulfasalazine 2 g/day and HCQ 400 mg/day. GC were either given IM (methylprednisolone 120 mg or triamcinolone 80 mg) or in oral tapering scheme (week 1-4: 15 mg/day, week 5-6: 10 mg/day, week 7-8: 5 mg/day, and week 9-10: 2.5 mg/day). In the validation cohort, the physician was free to choose dosing and co-medication. In both cohorts, all patients received folic acid (10 mg/week) during MTX prescription. In the derivation cohort body mass index (BMI) was calculated at baseline. BMI was not measured in the validation cohort. Data were used of the baseline and 3 months assessments.

Patients with diabetes were not registered in this study. However, in both cohorts patients with a random glucose concentration ≥11.1 mmol/L according to the diagnostic criteria for DM from the practice guideline 'DM type 2' from the Dutch College of General Practitioners[27] and the American Diabetes Association were identified as diabetics. In addition, in the validation cohort, patients with diabetes medication were registered. We repeated all analyses in the cohorts without these patients.

Biochemical parameters

Two blood EDTA-tubes were obtained during every study visit besides the routine blood samples for erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Alanine-aminotransferase, haemoglobin, leukocytes and thrombocytes. One EDTA tube was immediately put on ice after collection, centrifuged for 10 min at 1700 g, 4 ^{o}C, and plasma and cell-pellet aliquots were stored at -80 ^{o}C. The other EDTA tube was kept at room temperature and whole-blood was divided into aliquots and stored at -80 ^{o}C.

HBA_{1c} was determined in EDTA-whole blood using high-pressure liquid chromatography (Menarini 8160, Valkenswaard, Netherlands). HbA_{1c} measurements were done in batches of 30 samples. Intraday precision was <0.1% for an average of 42.1 mmol/mol [6.0%] and <0.1% for 96.3 mmol/mol [11.0%]. MTX-PG1-5 in nmol/l packed erythrocytes were analysed from the cell-pellet aliquots with liquid chromatography-electrospray ionization-tandem mass spectrometry-based assay using stable-isotope-labelled internal standards, as described earlier.[28] MTX-PG measurements were done in batches of 50-100 samples. Median intraday precision was 2.1% for all MTX-PGs at all concentrations.[28] In order to compare with HbA_{1c} from literature we converted mmol/mol to percentages.[29]

Statistical analyses

Statistical comparisons for baseline characteristics were made by ANOVA, Kruskall-Wallis, t-test, chi-square-test and Mann-Witney U-test. HbA_{1c}-change was defined as 3 months minus baseline HbA_{1c}. Paired t-test was used to test if HbA_{1c}-change was significant. To examine associations between MTX-PGs at 3 months and HbA_{1c}-change multiple linear regression analysis was used. In these linear regression models for MTX-PG concentrations and HbA_{1c}-change, age, gender and BMI, if available, were added as covariates. Co-medication covariates were added only in the analyses for the total cohorts and were added in two variables: 'HCQ yes/no' and 'GC yes/no'. DAS28-change, ESR-change and CRP-change were not added as covariates in the final model, because these covariates for inflammation were a mediator and not a confounder. Results were expressed as standardised betas. In order to compare the stratified results from the validation cohort with the derivation cohort, the validation cohort was stratified into patients on MTX mono-therapy, TDT, and other combinations of therapy with MTX, HCQ, sulfasalazine and GC.

In order to test if a possible effect of MTX-PG concentrations on HbA_{1c}-change was mediated by inflammation, the strength of the association between MTX-PGs and HbA_{1c}-change must be >10% reduced after controlling for inflammation.[30] This condition was tested for MTX-PG3 because it has the highest concentrations and was associated with response in both cohorts.[31] These analyses were performed with 3 months change of disease activity score 28 (DAS28)[32], ESR-change and CRP-change as inflammation covariates.

HbA_{1c}-change was also analysed in BMI-strata of normal weighted (BMI≤25 Kg/m^2), over weighted (BMI>25≤30 Kg/m^2) and obese (BMI>30 Kg/m^2) patients.

A post-hoc power calculation[33] was done to assess statistical power for a clinical relevant difference of 5.5 mmol/mol [0.50%] HbA_{1c}.[34] Statistics were performed with SPSS Statistics Version 21.0.0.1 (SPSS Inc. Chicago, USA). A $p<0.05$ was considered statistically significant.

RESULTS

Patients

In the derivation cohort, 299 patients were randomly assigned to all treatment arms in the high and intermediate probability groups (figure 1). Only 12 RA patients with low probability were included at baseline. HbA_{1c} was not measured in the low probability group because power would be too small. After 3 months, HbA_{1c} was measured in 277 patients, and MTX-PGs were determined in 196 patients. MTX-PGs were not determined for patients not on MTX in treatment-arms oral-GC and HCQ. There were no baseline differences between treatment-arms (table 1). In the derivation cohort, 69% of patients was female, mean age was 53 years, mean BMI was 26 kg/m^2 and baseline DAS28 was 4.86. At baseline, 99 patients were included in the validation cohort and after 3 months HbA_{1c} was assessed in 79 patients and MTX-PG in 77 patients. In the validation cohort, there were no baseline differences between the treatment groups. In the derivation cohort 22 (7%) patients who were included at baseline dropped out before inclusion at 3 months, and in the validation cohort 20 (20%) patients dropped out (figure 1). There were no significant changes in baseline characteristics between patients included at 3 months and patients who dropped out after being included at baseline.

In the validation cohort, 69% were female, mean age was 52 years and mean baseline DAS28 was 4.24. Of the 99 patients at baseline, 36% used NSAIDs, 48% HCQ, 42% sulfasalazine, 100% MTX, 3% IM-GC, 10% oral-GC, 36% was on MTX monotherapy and 32% on TDT. In the validation cohort, mean MTX dose was 15 mg/week (SE=0.2), mean HCQ dose was 306 mg/day (SE=47), mean sulfasalazine dose was 883 mg/day (SE=63) and mean dose of GC was 7 mg/day (SE=1). In the validation cohort, GC were used by none of the patients on MTX mono-therapy, 19% of the patients on TDT and 23% of the patients on other combinations of therapy. Between derivation and validation cohort, baseline age ($p=0.407$), gender ($p=0.965$) and HbA_{1c} ($p=0.761$) did not differ, whereas baseline DAS28 ($p<0.001$) and MTX dose ($p<0.001$) were significantly lower in the validation cohort.

In the derivation cohort there were 2 (15.3 mmol/L in the TDT+oral-GC arm and 24.3 mmol/L in the TDT+IM-GC arm) and in the validation cohort 1 patient (21.4 mmol/L in the arm with other medication) with a random glucose concentration >=11.1 mmol/L at baseline. In the validation cohort 5 extra patients used diabetes medication. 2 on MTX monotherapy (1 used pioglitazone and 1 used metformin), 2 on TDT (1 used metformin and glibenclamide and 1 used glimepiride) and 1 with other RA medication (patient used metformin and tolbutamide). Diabetes medication was not monitored in the derivation cohort and could therefore not be used to identify diabetic patients.

There were no changes between baseline and 3 months in observed diabetes medication and BMI. In both cohorts we did not give lifestyle advises to patients.

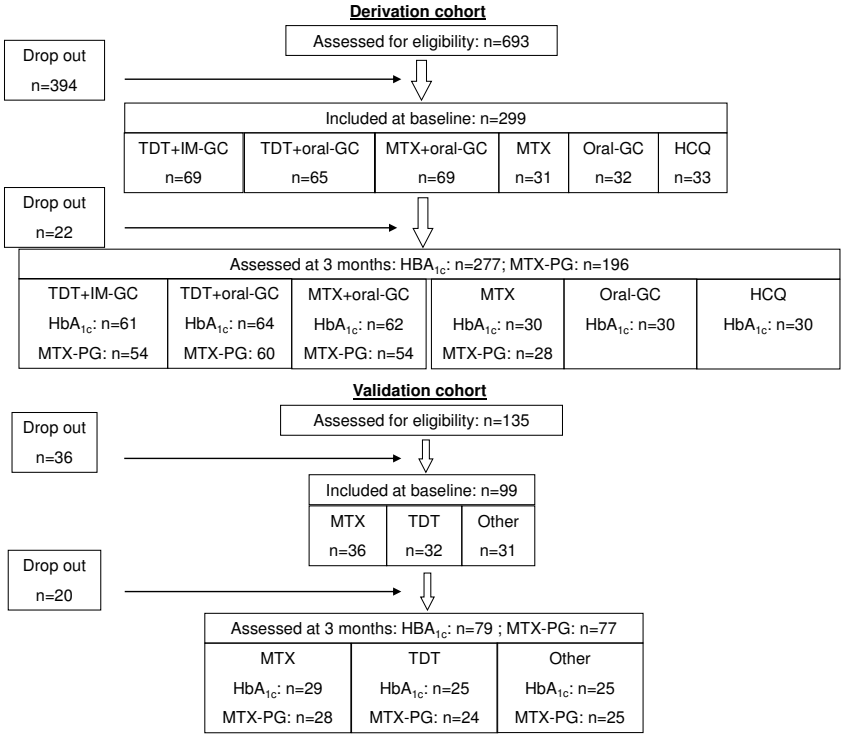

Figure 1 Flow chart of patient follow up in derivation and validation cohort. Drop outs before baseline included: not sufficient material, no RA 2010 ACR criteria, no inclusion criteria tREACH, declined to participate, lost to follow up and used biologicals. In the derivation cohort, from the 22 drop outs after baseline, 8 patients declined to participate, 3 patients stopped MTX because of adverse events, 1 patient was excluded because of communication problems, 2 patients were excluded because of noncompliance and for 8 patients there was insufficient material for HbA_{1c} determination. From the 20 drop outs in the validation cohort 11 patients declined to participate, 2 patients stopped MTX because of adverse events, 1 patient stopped MTX without reasons and for 6 patients there was insufficient material for HbA_{1c} determination. TDT, triple DMARD therapy with MTX, sulfasalazine and HCQ; IM, intramuscular; GC, glucocorticoids; MTX, methotrexate; HCQ, hydroxychloroquine; HbA_{1c}, glycosylated hemoglobin; MTX-PG, methotrexate polyglutamate; Other, different combinations of therapy with MTX, hydroxychloroquine, sulfasalazine and glucocorticoids; DMARD, disease-modifying antirheumatic drug.

HbA_{1c}

In the derivation cohort, mean HbA_{1c} was 36.5 mmol/mol [5.49%] at baseline and 34.6 mmol/mol [5.31%] after 3 months of treatment (table 2). Mean HbA_{1c}-change was -1.9 mmol/mol [-0.18%] ($p<0.001$). HbA_{1c} decreased significantly in TDT treatment-arms TDT+IM-GC and TDT+oral-GC with mean change -5.5 mmol/mol [-0.50%] ($p<0.001$) and -3.7 mmol/mol [-0.34%] ($p<0.001$) respectively.

Table 1 Baseline characteristics (mean (SE)) of the rheumatoid arthritis patients at the time of initiation with therapy.

Derivation cohort	**TDT+IM-GC**	**TDT+oral-GC**	**MTX+oral-GC**	**MTX**	**Oral-GC**	**HCQ**
Age, years	51 (2)	53 (2)	52 (2)	56 (3)	54 (3)	55 (3)
Women, %	58	74	74	81	66	67
BMI, Kg/m^2	26.0 (0.5)	26.2 (0.7)	26.7 (0.6)	26.8 (1.0)	25.8 (0.7)	27.7 (0.9)
DAS28	4.84 (0.13)	4.93 (0.14)	4.78 (0.14)	4.86 (0.23)	5.05 (0.21)	4.70 (0.17)
Validation cohort	**MTX**	**TDT**	**Other**			
Age, years	48 (2)	51 (3)	57 (3)			
Women, %	64	81	65			
DAS28	4.10 (0.23)	4.51 (0.25)	4.16 (0.28)			

TDT, triple DMARD therapy with MTX, sulfasalazine and HCQ; IM, intramuscular; GC, glucocorticoids; MTX, methotrexate; HCQ, hydroxychloroquine; DAS, disease activity score; HbA_{1c}, glycosylated hemoglobin; DMARD, disease-modifying antirheumatic drug; SE, standard error, Other, different combinations of therapy with MTX, sulfasalazine, HCQ and GC.

Table 2 Paired t-test of HbA_{1c} (mean (SE)) at baseline and after 3 months.

	Baseline HbA_{1c}		**3 months**		**Change from baseline to 3 months**		
Derivation cohort	**mmol/mol**	**%**	**mmol/mol**	**%**	**mmol/mol**	**%**	**p-value**
TDT+IM-GC	37.0 (1.1)	5.54 (0.10)	31.4 (0.7)	5.03 (0.06)	-5.5 (1.1)	-0.50 (0.10)	<0.001
TDT+oral-GC	36.5 (0.9)	5.49 (0.08)	33.0 (0.9)	5.17 (0.08)	-3.7 (0.7)	-0.34 (0.06)	<0.001
MTX+oral-GC	35.5 (0.7)	5.40 (0.07)	36.6 (1.0)	5.50 (0.09)	1.3 (0.3)	0.12 (0.03)	0.001
MTX	36.8 (0.7)	5.51 (0.07)	35.9 (0.7)	5.43 (0.06)	-0.8 (0.3)	-0.08 (0.03)	0.018
Oral-GC	34.7 (0.9)	5.32 (0.08)	36.0 (1.1)	5.45 (0.10)	1.3 (0.6)	0.12 (0.05)	0.022
HCQ	39.2 (2.2)	5.74 (0.21)	37.3 (1.3)	5.56 (0.12)	-2.0 (1.5)	-0.19 (0.13)	0.175
Total cohort	36.5 (0.5)	5.49 (0.04)	34.6 (0.4)	5.31 (0.04)	-1.9 (0.4)	-0.18 (0.03)	<0.001
Validation cohort							
MTX	36.6 (0.8)	5.50 (0.07)	35.9 (0.8)	5.43 (0.08)	-1.3 (0.6)	-0.12 (0.05)	0.028
TDT	36.4 (1.6)	5.48 (0.15)	31.4 (0.8)	5.02 (0.08)	-4.8 (1.8)	-0.44 (0.16)	0.012
Other	37.7 (1.8)	5.59 (0.17)	36.5 (1.8)	5.49 (0.16)	-1.8 (0.7)	-0.17 (0.07)	0.017
Total cohort	36.8 (0.8)	5.52 (0.07)	34.7 (0.7)	5.32 (0.07)	-2.6 (0.6)	-0.23 (0.06)	<0.001

TDT, triple DMARD therapy with MTX, sulfasalazine and HCQ IM, intramuscular; GC, glucocorticoids; MTX, methotrexate; HCQ, hydroxychloroquine; HbA_{1c}, glycosylated hemoglobin; DMARD, disease-modifying antirheumatic drug; SE, standard error; Other, different combinations of therapy with MTX, sulfasalazine, HCQ and glucocorticoids.

HbA_{1c} decreased less strong, but significant, in patients treated with MTX mono-therapy with mean -0.8 mmol/mol [-0.08%] (p=0.018). HbA_{1c} increased significantly in GC treatment-arms: MTX+oral-GC and oral-GC with mean 1.3 mmol/mol [0.12%] (p=0.001) and 1.3 mmol/mol [0.12%] (p=0.022). In patients in the HCQ treatment arm, HbA_{1c} decreased after 3 months treatment with mean -2.0 mmol/mol [-0.19%] (p=0.175). In the validation cohort, mean HbA_{1c} decreased from 36.8 mmol/mol [5.52%] at baseline to 34.7 mmol/mol [5.32%] after 3 months and mean HbA_{1c}-change was -2.6 mmol/mol [-

0.23%] (p<0.001). In patients on MTX-mono-therapy, mean HbA_{1c}-change was -1.3 mmol/mol [-0.12%] (p=0.028) and in the patients on TDT mean HbA_{1c}-change was -4.8 mmol/mol [-0.44%] (p=0.012).

BMI, adjusted for gender and age, was not associated with HbA_{1c}-change in the derivation cohort. Also, age and gender were not associated with HbA_{1c}-change. In the derivation cohort at baseline, there were 118 patients with BMI≤25 Kg/m^2, 97 with BMI>25≤30 Kg/m^2, 60 with BMI>30 Kg/m^2 and for 2 patients BMI was not registered. Table 3 shows the mean baseline, 3 months HbA_{1c} and HbA_{1c}-change of all BMI strata. HbA_{1c}-change did not differ between BMI strata (p=0.503).

Table 3 Paired t-test of HbA_{1c} (mean (SE)) at baseline and after 3 months stratified in BMI strata.

	Baseline		3 months		Change from baseline to 3 months		
	mmol/mol	%	mmol/mol	%	mmol/mol	%	p
BMI≤25 Kg/m^2	34.5 (0.5)	5.3 (0.04)	32.8 (0.5)	5.2 (0.1)	1.7 (0.4)	0.2 (0.03)	<0.001
BMI>25≤30 Kg/m^2	37.2 (0.8)	5.6 (0.1)	34.7 (0.5)	5.3 (0.04)	2.6 (0.8)	0.2 (0.1)	0.002
BMI>30 Kg/m^2	39.2 (1.4)	5.7 (0.1)	37.7 (1.4)	5.6 (0.1)	1.5 (0.9)	0.1 (0.1)	0.100

BMI, body mass index; HbA_{1c}, glycosylated hemoglobin; SE, standard error.

After multivariate analyses in the derivation cohort and adjusting for age, gender and BMI, MTX-use (β=-0.12, p=0.046) and HCQ-use (β=-0.38, p<0.001) were associated with decreased HbA_{1c}.

Supplemental table 1 shows the power to detect a clinical relevant difference of 5.5 mmol/mol [0.50%] between baseline and 3 months HbA_{1c} in each treatment-arm. TDT+IM-GC (0.71), HCQ (0.48) in the derivation cohort and the TDT group (0.35) in the validation cohort had insufficient (<0.80) power.

There were no significant changes between all analyses done in both our cohorts with identified diabetics and without.

Erythrocyte-MTX-PG concentration

In the derivation cohort, MTX-PG concentrations did not differ between treatment-arms (table 4). Also in the validation cohort, MTX-PG concentrations did not differ between the treatment groups. In the validation cohort, MTX-PG2 (p=0.007) was significantly higher; and MTX-PG3-5 (p≤0.003) were significantly lower compared to the derivation cohort. In the (total) derivation cohort, MTX-PG2 (β=-0.20, p=0.005), MTX-PG3 (β=-0.31, p<0.001), MTX-PG4 (β=-0.33, p<0.001), MTX-PG5 (β=-0.39, p<0.001) and total MTX-PG (β=-0.29, p<0.001) were associated with decreased HbA_{1c} (table 5).

Table 4 Median (minimum-maximum), erythrocyte-MTX-PG concentrations per treatment arm.

Derivation cohort	**TDT+IM-GC**	**TDT+oral-GC**	**MTX+oral-GC**	**MTX**	**Total cohort**
MTX-PG1	28 (0-164)	27 (0-110)	30 (0-290)	32 (0-337)	28 (0-337)
MTX-PG2	19 (2-82)	21 (0-49)	23 (3-42)	23 (0-39)	21 (0-82)
MTX-PG3	40 (0-97)	48 (0-89)	50 (14-90)	49 (0-76)	48 (0-97)
MTX-PG4	15 (0-88)	18 (0-88)	20 (2-67)	18 (0-50)	18 (0-88)
MTX-PG5	3 (0-63)	5 (0-64)	5 (0-30)	4 (0-23)	4 (0-64)
Total MTX-PG	111 (9-303)	125 (0-338)	142 (45-405)	133 (0-476)	128 (0-476)
Validation cohort	**MTX**	**TDT**	**Other**		**Total cohort**
MTX-PG1	33 (0-111)	33 (0-254)	47 (0-258)		35 (0-258)
MTX-PG2	24 (0-58)	22 (2-69)	27 (0-57)		23 (0-69)
MTX-PG3	31 (0-88)	44 (3-70)	38 (14-78)		39 (0-88)
MTX-PG4	7 (0-27)	12 (0-44)	9 (0-61)		8 (0-61)
MTX-PG5	0 (0-6)	3 (0-11)	1 (0-26)		1 (0-26)
Total MTX-PG	102 (0-254)	123 (28-396)	139 (27-358)		116 (0-396)

TDT, triple DMARD therapy with MTX, hydroxychloroquine and sulfasalazine; IM, intramuscular; GC, glucocorticoids; MTX, methotrexate; MTX-PG, methotrexate polyglutamate; DMARD, disease-modifying antirheumatic drug; Other, different combinations of therapy with MTX, sulfasalazine, HCQ and glucocorticoids.

In the (total) validation cohort, only MTX-PG3 (β=-0.26, p=0.018) was associated with decreased HbA_{1c}. In the derivation cohort, in TDT treatment-arm TDT+IM-GC, MTX-PG3 (β=-0.41, p=0.004), MTX-PG4 (β=-0.68, p<0.001), MTX-PG5 (β=-0.84, p<0.001) and total MTX-PG (β=-0.47, p=0.001) were associated with decreased HbA_{1c}.

In TDT treatment-arm TDT+oral-GC, MTX-PG2 (β=-0.39, p=0.005), MTX-PG3 (β=-0.47, p<0.001), MTX-PG4 (β=-0.27, p=0.039) and total MTX-PG (β=-0.40, p=0.004) were associated with decreased HbA_{1c}. There was no association between MTX-PGs and HbA_{1c}-change in treatment-arm MTX+oral-GC and in treatment-arm MTX. Treatment arms oral-GC and HCQ did not contain MTX and hence, could not be tested. In the validation cohort containing patients on MTX monotherapy, MTX-PG2 (β=-0.42, p=0.035), MTX-PG3 (β=-0.49, p=0.015) and total MTX-PG (β=-0.43, p=0.031) were associated with HbA_{1c}-change; in patients on TDT, there was a trend towards association of MTX-PG3 (β=-0.30, p=0.107) with decreased HbA_{1c}.

In the derivation cohort, the association between MTX-PG3 and HbA_{1c}-change reduced 19% to β=-0.25 (p=0.001) when DAS28-change was added as covariate. When ESR-change was added, the β reduced 19% to β=-0.25 (p=0.001) and when CRP-change was added the β changed 23% to β=-0.24 (p=0.001). In the validation cohort, β increased 12% to β=-0.29 (p=0.019) when DAS28-change was added, β reduced 4% to β=-0.25 (p=0.031) when ESR-change was added and when CRP-change was added β reduced 19% to β=-0.24 (p=0.031).

Table 5 Linear regression for associations (β (p-value)) of MTX-PG concentrations with change in HbA_{1c} between baseline and 3 months in different treatment arms.

Derivation cohort	TDT+IM-GC	TDT+oral-GC	MTX+oral-GC	MTX	Total cohort
MTX-PG1	-0.07 (0.616)	-0.25 (0.100)	-0.12 (0.418)	0.33 (0.105)	-0.04 (0.592)
MTX-PG2	-0.23 (0.091)	-0.39 (0.005)	0.004 (0.974)	-0.06 (0.805)	-0.20 (0.005)
MTX-PG3	-0.41 (0.004)	-0.47 (<0.001)	-0.02 (0.907)	0.12 (0.599)	-0.31 (<0.001)
MTX-PG4	-0.68 (<0.001)	-0.27 (0.039)	-0.05 (0.711)	0.30 (0.194)	-0.33 (<0.001)
MTX-PG5	-0.84 (<0.001)	-0.11 (0.410)	-0.03 (0.828)	0.28 (0.199)	-0.39 (<0.001)
Total MTX-PG	-0.47 (0.001)	-0.40 (0.004)	-0.13 (0.412)	0.37 (0.088)	-0.29 (<0.001)
Validation cohort	**MTX**	**TDT**	**Other**		**Total cohort**
MTX-PG1	-0.21 (0.298)	-0.01 (0.974)	0.06 (0.779)		0.03 (0.819)
MTX-PG2	-0.42 (0.035)	0.00 (0.996)	-0.12 (0.587)		-0.08 (0.490)
MTX-PG3	-0.49 (0.015)	-0.30 (0.107)	-0.20 (0.371)		-0.26 (0.018)
MTX-PG4	-0.37 (0.075)	-0.25 (0.180)	0.00 (0.998)		-0.14 (0.205)
MTX-PG5	-0.32 (0.109)	-0.19 (0.313)	0.15 (0.486)		-0.02 (0.865)
Total MTX-PG	-0.43 (0.031)	-0.11 (0.568)	-0.02 (0.921)		-0.10 (0.406)

There was corrected for age, gender and BMI in the individual treatment-arms and in the total cohort also for HCQ-yes/no and GC-yes/no. In the validation cohort BMI was not available. TDT, triple DMARD therapy with MTX, sulfasalazine and HCQ; IM, intramuscular; GC, glucocorticoids; MTX, methotrexate; HbA_{1c}, glycosylated hemoglobin; MTX-PG, methotrexate polyglutamate; DMARD, disease-modifying antirheumatic drug; Other, different combinations of therapy with MTX, sulfasalazine, HCQ and glucocorticoids.

DISCUSSION

To the best of our knowledge, we show for the first time in a prospective study that TDT or MTX monotherapy initiation and subsequent increased erythrocyte-MTX-PG concentrations are associated with reduced HbA_{1c} over the first 3 months treatment in RA patients. Furthermore, we confirmed earlier studies that HCQ therapy reduced and GC increased HbA_{1c}.

Change in HbA_{1c} among diabetes patients with rheumatic disease was investigated previously. A retrospective study compared pretreatment levels with those within 1 year following drug initiation.[13] HCQ produced a reduction in HbA_{1c} (60.8 to 53.6 = -7.2 mmol/mol [7.71% to 7.05%]) that was significantly larger than the change associated with MTX (57.2 to 56.0 = -1.2 mmol/mol [7.38% to 7.27%]).[13] In 11 type 2 DM patients, 6 months HCQ treatment decreased HbA_{1c} by -36.1 mmol/mol [-3.3%].[14] Similarly, in a placebo-controlled study in 135 type 2 DM patients, HCQ decreased HbA_{1c} by an absolute amount of -11.1 mmol/mol [-1.02%].[15] Treatment with HCQ in RA was also associated with a reduced risk of incident DM.[11,12] In our study, however, mono-therapy with HCQ did not produce a significant reduction in HbA_{1c}, which is probably due to the reduced power (0.48) in this treatment arm. A previous study also showed that MTX reduced HbA_{1c} by -1.2 mmol/mol [-0.66%] in DM patients with RA patients, although this decrease was not significant (p=0.45).[13] We confirm this observation in RA patients and also add evidence of this relationship by demonstrating

an association between higher erythrocyte-MTX-PG levels and reduced HbA_{1c}. A HbA_{1c} reduction of 4.8 to 5.5 mmol/mol [0.44 to 0.50% point] HbA_{1c} is considered clinically relevant.[34,35] In addition, data from the Atherosclerosis Risk in Communities (ARIC) study showed that the relative risk of a cardiovascular event was 1.38 (95% CI 1.22-1.56) for every one % point increase in HbA_{1C} for subjects without diabetes.[36]

The association between MTX-use and decreased HbA_{1c} could be mediated via decreased inflammation and/or a direct effect on glucose control. Increased inflammation as with high disease activity in RA accelerates development of several cardiovascular risk factors such as DM.[7,8] In our study, the decrease in HbA_{1c} was largest in treatment-arms with patients on TDT. Earlier, we showed that disease activity after 3 months was lower in patients receiving TDT than in those receiving MTX monotherapy.[23] This might suggest that the association between MTX-use and decreased HbA_{1c} is mediated via decreased disease activity. Also, associations between MTX-PG concentrations and decreased HbA_{1c} found in our study could be mediated via decrease in disease activity since MTX-PGs are associated with decreased disease activity in RA in both our cohorts.[31] The β of the association of MTX-PG3 with HbA_{1c}-change reduced 4-23% (>10%) when an inflammation covariate was added. Thus, the effect of MTX on HbA_{1c}-change was partly mediated through a decrease in inflammation explaining why other DMARDS also reduced HbA_{1c}. Additionally, there might be an additional direct effect of MTX on decrease in HbA_{1c}.

A direct effect of MTX on glucose metabolism has recently been described.[20] Chronic treatment of experimental type 2 DM in mice with low doses of MTX increased skeletal muscle GLUT4 glucose transporter expression and improved metabolic control.[20] MTX treatment was also associated with significant reduction of glucose and insulin serum concentrations in diabetic mice, and glucose levels in controls.[20] MTX inhibits 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) transformylase.[37] The inhibition of this enzyme may lead to an upstream accumulation of AICAR,[38] a well-known activator of 5'-AMP-activated kinase (AMPK) and of its downstream pathways, which regulates insulin-independent GLUT4 expression and glucose metabolism.[39,40] Skeletal muscle glucose uptake is the rate-limiting step of glucose utilization, and it is physiologically regulated by an insulin-dependent and an insulin-independent signaling pathways, both leading to translocation of GLUT4 glucose transporter to the plasma membrane.[41] MTX mediated increase of GLUT4 expression via increase in AICAR may explain our partially direct effect of MTX-PG concentrations on HbA_{1c}-change.

The largest decrease in HbA_{1c} was observed in treatment-arms with TDT. This may be explained by an additive effect above MTX of HCQ and/or sulfasalazine. HCQ reduced risk of incident DM[11,12] and decreased HbA_{1c}[13-15] as we mentioned earlier. Moreover, insulin sensitivity in obese non-diabetic individuals was improved by HCQ therapy[42] and a glucose lowering side effect of HCQ in a non-diabetic patient, who presented with severe hypoglycemia, was reported.[43] Glucose lowering effects of sulfasalazine in type 2 DM have also been reported.[44] However, we have to be careful with the interpretations from treatment-arms TDT+IM-GC and TDT+oral-GC because

the fall in HbA_{1c} may be a result of increased erythropoiesis as a product of drug-induced hemolysis by sulfasalazine.[45] Our results confirm the glucose lowering effects of HCQ (-2.0 mmol/l) and suggest that combination of HCQ, sulfasalazine and MTX therapy results in even more HbA_{1c} decrease (-5.5 and -3.7 mmol/mol [-0.50 and -0.34%]). DM diagnose was not registered in our cohorts. There are studies reporting a higher incidence rate of diabetes in RA compared to healthy persons and studies reporting a similar incidence rate of diabetes in RA.[46,47] Diabetes medication was not monitored in the derivation cohort and could therefore not be used to identify diabetic patients. Therefore, 2 patients (0.7%) with baseline random-glucose ≥11.1 is probably an underestimation. On the contrary, in the validation cohort diabetes medication was registered. There was 1 patient with baseline random-glucose ≥11.1 and 5 other patients used diabetes medication. The 6 diabetic patients (7.6%) in the validation cohort is probably a better estimation of the true number of diabetics in our study in RA patients.

HbA_{1c} increased after 3 months in patients who used oral-GC combined with MTX and in patients with oral-GC-mono-therapy, but not in patients on TDT and GC. In addition, HbA_{1c} decreased more in patients who received one injection of intramuscular-GC than in patients who received 3 months oral-GC in combination with TDT. This may be explained by a higher overall dose in the patients on oral-GC compared to patients who received IM-GC because GC in RA therapy may increase plasma glucose[18,19] and effects of GC on insulin sensitivity are dose dependent.[48] However, studies assessing the risk of diabetes associated with low-dose GC treatment of RA have provided conflicting results.[49,50] To support our results, the same pattern was present in associations between MTX-PGs and HbA_{1c}-change: the effect of total MTX-PG on HbA_{1c}-change was largest in treatment-arm with IM-GC and TDT, smaller in treatment-arm with oral-GC and TDT, and not significant in the treatment-arm without HCQ and sulfasalazine in addition to MTX and oral-GC. In summary, we show that multiplicative effects might exist between HbA_{1c} lowering effects of MTX, HCQ and/or sulfasalazine and that GC may have opposite effects.

We validated the decrease in HbA_{1c} after initiation of MTX therapy in a validation cohort. In this cohort, HbA_{1c} also significantly decreased after 3 months treatment. The HbA_{1c} lowering effect was also strongest in patients on TDT compared to patients on MTX mono-therapy, which could be due to additional HCQ and/or sulfasalazine. The association between MTX-PGs and decreased HbA_{1c} was weaker and only present for MTX-PG3 in the validation cohort compared to the derivation cohort. This may be explained by the lower MTX dose and the resulting lower medium- and long-chain erythrocyte-MTX-PG concentrations in the validation cohort compared to the derivation cohort (Table 2) as higher MTX-PG concentrations are function of MTX dose.[21] In the validation cohort, MTX-PGs seem to be stronger associated with decreased HbA_{1c} in patients on MTX mono-therapy compared to patients on TDT. This can be explained because none of the patients on MTX mono-therapy used GC opposed to patients from the other treatment-groups of the validation cohort. Furthermore, differences in power

between the derivation (n=196) and validation (n=77) cohorts may also have played a role.

Our strengths are the use of a derivation and validation cohort and its design namely a prospective rather than a cross-sectional study. We also used different treatments, with commonly used DMARD (combinations) and GC regimens. One of the weaknesses of this study was that we did not identify diabetics at forehand. HbA_{1c} is higher in patients with DM.[13] This could have influenced our results and to adjust for DM would have been better. We have repeated our analyses in the cohorts without patients who were retrospectively identified as diabetics, and these results did not differ from those including these patients. Second, there was no power calculation at forehand. However, we performed a post-hoc power calculation. Supplemental table 1 reveals that treatment-arms TDT+IM-GC and HCQ in the derivation cohort and TDT in the validation cohort had power calculations <0.80. Non-significant results from these treatment-arms, as in the HCQ treatment-arm (HbA_{1c}-change, p=0.175), might be significant when tested in a larger sample. Third, BMI, a possible confounder, was not registered in the validation cohort. It has been shown that insulin sensitivity in RA patients receiving anti-tumor necrosis factor therapy improved in those of normal weight but not in the obese.[51] However, BMI has no effect on our results since mean change in HbA_{1c}-change was not significantly different between obese, over weighted and normally weighted RA patients in the derivation cohort (p=0.503). The low sample size in the obese group (n=60) compared to the none obese groups (n=118, n=97) probably caused the none significant HbA_{1c}-change in the obese group (table 3). Finally, there were no significant changes between baseline characteristics of the drop out patients and patients who were included at 3 months. Therefore we have no reasons to believe that drop outs could have led to an important bias.

In conclusion, our study is the first prospective study showing that TDT or MTX monotherapy and increased erythrocyte-MTX-PG concentrations are associated with reduced HbA_{1c} after 3 months treatment in RA patients. HCQ also reduced HbA_{1c} while the opposite effect was seen with oral-GC. Since MTX use and erythrocyte-MTX-PG concentration via MTX dose are modifiable, our findings may have implications for the prevention and treatment of cardiovascular disease and diabetes in RA.

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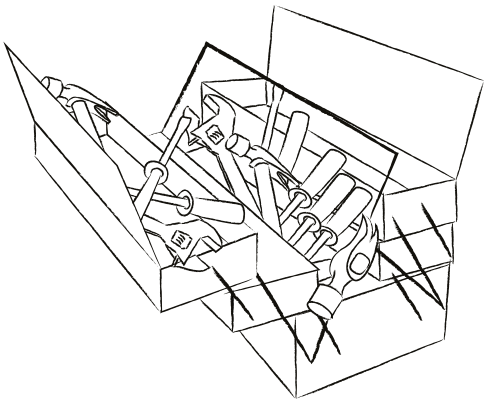
Supplemental table 1 Power calculation for a clinical relevant difference of 5.5 mmol/mol (0.5%).

Derivation cohort	SD HbA1C difference	Standardized difference	n	Power
TDT+IM-GC	8.8	0.63	61	0.71
TDT+oral-GC	5.2	1.06	64	0.99
MTX+oral-GC	2.7	2.04	62	1.00
MTX	1.8	3.06	30	1.00
Oral-GC	3.0	1.83	30	1.00
HCQ	8.0	0.69	30	0.48
Total cohort	6.3	0.87	277	1.00
Validation cohort				
MTX	3.0	1.83	29	1.00
TDT	8.8	0.63	25	0.35
Other	3.6	1.53	25	0.96
Total cohort	5.8	0.95	79	0.99

TDT, triple DMARD therapy with MTX, sulfasalazine and HCQ; IM, intramuscular; GC, glucocorticoids; MTX, methotrexate; HCQ, hydroxychloroquine; HbA_{1c}, glycosylated hemoglobin; DMARD, disease-modifying antirheumatic drug; SE, standard error; Other, different combinations of therapy with MTX, sulfasalazine, HCQ and glucocorticoids.



CHAPTER 9

Prediction of Clinical Non-Response to Methotrexate Treatment in Rheumatoid Arthritis

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ABSTRACT

Objective

To identify rheumatoid arthritis (RA) patients, before disease modifying anti-rheumatic drug (DMARD) initiation, with a high chance of non-response or adverse events after 3 months of methotrexate (MTX) treatment.

Methods

In a prospective derivation (n=285) and validation cohort (n=102) with RA patients on MTX clinical characteristics, lifestyle variables, genetic and metabolic biomarkers involved in the mechanism of action of MTX were determined at baseline. These variables were used to construct two prediction models with disease activity score (DAS)28 >3.2 and three or more adverse events as outcome measures.

Results

The final prediction model for non-response, after multivariable logistic regression with backward selection in the derivation cohort, included: DAS28>5.1, HAQ>0.6, current smoking, BMI>25 kg/m^2, adenosine triphosphate binding cassette transporter (*ABC*) *B1* rs1045642 genotype, *ABCC3* rs4793665 genotype, and erythrocyte-folate <750 nmol/L. Area under receiver operating characteristics curve was 0.80 (95%CI: 0.73-0.86) in the derivation cohort and 0.80 (95%CI: 0.69-0.91) in the validation cohort. The prediction model was transformed into a total risk score (range 0-8). At a cutoff of ≥4, probability for non-response was 0.44, sensitivity was 71%, specificity 72%, with a positive and a negative predictive value of respectively 72% and 71%. None of the investigated variables was significantly associated with adverse events at 3 months and therefore a prediction model for adverse events could not be developed.

Conclusion

A prediction model for non-response to MTX in 2 prospective RA cohorts by combining genetic, metabolic, clinical and lifestyle variables was developed and validated. This model satisfactorily identified RA patients with a high risk of non-response to MTX and may be a tool for personalized RA-treatment.

INTRODUCTION

Methotrexate (MTX) is anchor-drug for the treatment of rheumatoid arthritis (RA). In significant numbers of patients, however, MTX fails to achieve adequate suppression of disease activity and induces adverse events, which prevents the ability to increase or even continue therapeutic dose.[1] Patients who do not respond to MTX or develop severe adverse events within 3 months, after MTX initiation are frequently given biologicals, alone or in combination with MTX.[2] Prediction of MTX non-response and MTX-induced adverse events before MTX start is paramount since the first months upon diagnosis represent a window of opportunity during which outcomes can be more effectively modulated by therapy.[3] It is necessary to identify at baseline non-responders and patients prone to experience adverse events in order to ensure that only patients unresponsive to MTX receive early additional treatment with biologicals and those responsive to MTX are spared costly biologicals.[2]

Earlier, prediction models for MTX non-response have been developed for juvenile idiopathic arthritis (JIA)[4] and for RA.[5-8] However, these models did not use metabolic predictors,[5-7] were not validated[6] or the model was developed in patients on MTX monotherapy[5-7] rather than in therapy with a combination of disease-modifying anti-rheumatic drugs (DMARD). Therefore, the aim of this study was to develop and validate a prediction model for non-response and a prediction model for adverse events. These prediction models, may discriminate at baseline between RA patients who do not respond or have adverse events after 3 months of MTX treatment and those who do respond or have no adverse events.

Genetic, metabolic, clinical and lifestyle parameters were combined to build a prediction model with highest sensitivity and specificity in two independent prospective cohorts of RA patients.

METHODS

Study design and patients

Data from two prospective cohorts with only Caucasian patients were used. The derivation cohort consisted of patients who were enrolled in the treatment in Rotterdam Early Arthritis Cohort (tREACH). This is a clinical multicentre, stratified single-blinded trial (ISRCTN26791028) described elsewhere.[9] The validation cohort consisted of patients from the Methotrexate in Rotterdam (Netherlands) cohort (MTX-R) and were patients who started MTX between January 2006 and March 2011 in the department of Rheumatology, Erasmus University Medical Center, Rotterdam (Erasmus MC), Netherlands.[10] The medical ethics committee from the Erasmus MC approved both studies and patients gave written informed consent before inclusion.

The derivation cohort included patients on MTX who fulfilled the 2010 ACR/EULAR criteria for RA.[11] Patients in the validation cohort were included when diagnosed with RA by the physician. Inclusion and exclusion criteria for both cohorts were shown in supplemental table 1. Patients from the derivation cohort started with 25 mg/week MTX and were randomized to treatment with or without sulfasalazine,

hydroxychloroquine and glucocorticoids,[9] whereas in the validation cohort, dosage and co-medication was chosen by the physician. In both cohorts, patients received folic acid (10 mg/week) during MTX treatment. In both cohorts, patients were assessed at baseline and after 3 months.

Assessment of non-response and adverse events

Primary outcome was the disease activity score with 28-joint count (DAS28) and ESR[12] which was assessed at 3 months follow-up. In rheumatology practices of the present studies, physicians used the cut-off values of DAS28>3.2 to step-up therapy after 3 months. Therefore, non-response was defined as DAS28>3.2 after three months of treatment with MTX.

Adverse events were assessed with biochemical and self-reported measures. Gastrointestinal complaints, malaise, psychological complaints, hepatotoxicity, bone marrow depression and other complaints were counted as an adverse event. Three or more (versus two or less) adverse events was used as the outcome variable for adverse events because these patients are more prone to stop MTX therapy then patients with less adverse events. Adverse events in both cohorts were described more thoroughly earlier.[10]

Data collection for potential predictors

All potential predictors that we examined, have been shown to be associated with non-response or adverse events in previous association studies or were prone to be associated by physiology. All predictors were dichotomized according to commonly used cutoff values or by dividing them into quartiles. The quartile-divider-value were the percentage non-responders differed the most from the percentage non-responders in the adjacent quartile was chosen as dichotomizing value.

Clinical and lifestyle variables

Before treatment was started, we collected blood at the hospital from each RA patient and determined erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), tender joint count (TJC), swollen joint count (SJC), visual analog scale (VAS) for general health and DAS28. DAS28>5.1 was used as dichotomous variable for high disease activity at baseline according to the European League Against Rheumatism (EULAR) criteria for response.[13] The Health assessment questionnaire (HAQ) was added as variable as it could possibly predict MTX response since mild functional impairment was associated with RA remission.[14]

Lifestyle variables included smoking, alcohol consumption and body mass index (weight (kg) / (height (m))^2). Cola, coffee and tea are caffeine extracts. Caffeine is an adenosine receptor antagonist which can decrease the effect of increased adenosine caused by MTX and therefore possibly decreases MTX response.[15]

Metabolic variables were erythrocyte-folate, serum-folate, plasma homocysteine, serum vitamin B_6, serum vitamin B_{12}, and estimated glomerular filtration rate (eGFR)

calculated with the modification of diet in renal disease (MDRD) formula.[16] The selection, material collection and determination of metabolic variables were described earlier.[10] EGFR was added as possible predictor of MTX outcome because it influences intracellular MTX polyglutamate concentrations.[17]

The genetic variables consisted of single nucleotide polymorphisms (SNP) and were selected based on their involvement in the MTX metabolic pathways, their high polymorphic allele frequency and documented functional effects. DNA was obtained from whole blood. SNP selection, DNA isolation and genotyping were performed as we described earlier.[4,18] The following SNPs were determined using real-time PCR with Taqman technique: Adenosine triphosphate binding cassette transporter (*ABC*) family B member 1 (*ABCB1* rs1045642, rs1128503 and rs2032582), *(ABCC1* rs35592 and rs3784862), (*ABCC2* rs717620 and rs4148396), (*ABCC3* rs3785911 and rs4793665), (*ABCC4* rs868853 and rs2139560), (*ABCC5* rs2139560), (*ABCG2* rs2231142 and rs13120400), adenosine-deaminase (*ADA* rs73598374), adenosine A2A receptor (*ADORA2A* rs5751876), adenosine monophosphate deaminase 1 (*AMPD1* rs17602729*)*, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (*ATIC* rs2372536), Folate receptor 2 (*FOLR2* rs514933), folylpolyglutamate synthetase (*FPGS* rs4451422), Gamma glutamyl hydrolase (*GGH* rs3758149 and rs10106587), inosine triphosphatase (*ITPA* rs27354), methylenetetrahydrofolate reductase (*MTHFR* rs1801131 and rs1801133), methionine synthase reductase (*MTRR* rs1801394), solute carrier (*SLC*) 19A1 (SLC19A1 rs1051266) and (*SLC46A1* rs2239907).

Statistical analysis

To construct a model to predict 3 months non-response and adverse events, backward logistic regression analysis was performed in several stages. First, all continuous variables were dichotomized to facilitate the use of the models in daily clinical practice. Second, univariable odds ratios (ORs) with 95% confidence intervals (CI) were calculated. Third, potential predictors (p<0.200) were combined into a multivariable logistic regression model. The full model was simplified according to statistical strength (exclusion if p≥0.200, in each step deleting the variable with the highest p-value), correlations between predictors and practical considerations. If two potential predictors correlated strongly (Spearman's r≥0.40), the variable that was clinically more relevant or stronger associated with the outcome measure in univariate analysis was given preference. Fourth, to obtain the final prediction model, clinical and genetic variables with a p value <0.200 on the log-likelihood test were combined in the multivariable logistic regression analysis.

To calculate predicted probabilities of 3 months DAS28>3.2 or ≥3 adverse events, we used the following formula: $P_{MTXoutcome} = \frac{e^{(\beta_0+\beta_1 x_1+\beta_2 x_2+\ldots+\beta_p x_p)}}{1+e^{(\beta_0+\beta_1 x_1+\beta_2 x_2+\ldots+\beta_p x_p)}}$ were P is the predicted probability of achieving 3 months DAS28>3.2 or ≥3 adverse events, β_0 is the constant and β_1, β_2 and β_p represent the regression coefficients for each of the predictors x_1, x_2 and x_p.

To evaluate the predictive power of the model, we used the predicted probabilities of non-response or adverse events to construct a receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) measures the concordance of predicted values with actual outcomes, with an AUC of 0.5 reflecting no predictive power and an AUC of 1.0 reflecting perfect prediction. To assess whether the models fit the data well, we used the Hosmer-Lemeshow test.

To compute the risk score of being an MTX non-responder for individual patients, the regression coefficients (β) of the predictors in the final model were transformed into simple scores that sum up to a total risk score. The total risk scores and probabilities of MTX non-response for each patient from the derivation cohort was computed. Mean probabilities for each risk score were calculated. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for each risk score cut-off by using the ROC curve of the derivation cohort.

The prediction model was externally validated in the validation cohort. The regression coefficients of the predictors obtained from the derivation cohort were entered in the above-mentioned formula. This was used to construct a ROC curve for the validation cohort. All statistical analyses were carried out with SPSS V.21.0.0.1 (SPSS, Chicago, Illinois, USA).

RESULTS

Patient characteristics

Three months data of the derivation cohort were published earlier[19] and both cohorts were used earlier as derivation and validation cohort.[10] The baseline characteristics from that study are shown in supplemental table 2.[10] For the present study, 285 patients from the derivation cohort were included at baseline and 270 participated after 3 months. From the validation cohort 102 patients were included at baseline and 84 participated after 3 months. MTX dose was higher in the derivation cohort (25 versus 15 mg/week) as compared to the validation cohort (supplemental table 2). Patients in the validation cohort had lower DAS28, used more non-steroidal anti-inflammatory drugs (NSAID), less glucocorticoids and received more often MTX as subcutaneous injections than patients in the derivation cohort.[10]

In both cohorts, disease activity decreased over time.[10] In the derivation cohort, mean DAS28 was 4.94 (SD=1.15) at baseline and decreased to 3.12 (SD=1.19) after three months. In the validation cohort, DAS28 decreased from 4.26 (SD=1.43) to 2.92 (SD=1.23). In the derivation cohort and validation cohort, DAS28 (3.12 versus 2.92; p=0.174) was comparable after three months. In the derivation cohort 116 patients (43%) had a DAS28>3.2 after 3 months and in the validation cohort 32 patients (38%).

The percentage patients with ≥ 3 adverse events was comparable in both cohorts (31% versus 30%).[10]

Prediction model for MTX non-responders

The following baseline variables were, univariately associated (p≤0.200) (table 1) with non-response, after 3 months of therapy in the derivation cohort: gender, age, triple DMARD therapy, *ABCB1* rs1045642, *ABCB1* rs2032582, *ABCC3* rs4793665, *ABCG2* rs2231142, *ADA* rs73598374, *ATIC* rs2372536, *GGH* rs3758149, *MTHFR* rs1801133, *MTRR* rs1801394, *SLC46A1* rs2239907, folate in erythrocytes, baseline DAS28, baseline HAQ, smoking, alcohol use, tea consumption and BMI. These variables were included in the multivariate logistic regression model with backward selection. The multivariate logistic regression variables that stayed in the final prediction model after backward selection were *ABCB1* rs1045642 genotype, *ABCC3* rs4793665 genotype, erythrocyte-folate<750 nmol/L, baseline DAS28>5.1, baseline HAQ>0.6, current smoking and BMI>25 kg/m^2. The AUC of the prediction model was 0.80 (95% CI: 0.73-0.86), indicating that it classified 80% of patients correctly (table 2) (figure 1). The Hosmer-Lemeshow goodness-of-fit test was not statistically significant (p=0.816), indicating that the model fit the data well.

These predictors were used to test the model in the validation cohort. Smoking and BMI were not determined in the validation cohort and therefore could not be tested. The AUC in the validation cohort was 0.80 (95% CI: 0.69-0.91) (table 2), indicating that 80% of patients were classified correctly.

To make our prediction model suitable for daily practice, we transformed the regression coefficients (β) of the model's predictors, into simple scores. Thereafter, individual risk scores for having a DAS28>3.2 after 3 months of therapy were computed (table 2). The constant (OR=0.01) of the multivariate model of -5 was suppressed in order to simplify the model and was therefore not used. The score ranged from 0 to 8 whereby a higher score reflects a higher chance at treatment failure (non-response) after 3 months. The risk score of a patient, who has all predictors of the final model, is calculated by adding up the simple scores, assigned to individual predictors: 1+1+1+1+1+2+1, which results in a risk score of 8. If all predictors are present the probability of 3 months DAS28>3.2 is 0.80. The risk score of a patient having no predictors would be equal to 0. If no predictors are present, the probability of non-response is 0.01. Within the 0-8 range, the diagnostic accuracy of different cut-offs for the prediction model was evaluated by calculating the risk scores, and probability of having non-response, for each individual patient in the derivation cohort.

Mean probabilities of having non-response for each risk score cut-off were shown in table 3. Table 3 also shows, sensitivity, specificity, PPV and NPV for each risk score cut-off. With DAS28>5.1 as only predictor the prediction model achieved a ROC curve AUC of 0.66 (95% CI: 0.59-0.72) in the derivation cohort and 0.66 (95% CI: 0.54-0.79) in the validation cohort. When HAQ>0.6, erythrocyte folate<750 nmol/L, *ABCB1 rs1045642* and *ABCC3* rs4793665 were added the AUC raised to 0.73 (95% CI: 0.66-0.80) in the derivation and to 0.80 (95% CI: 0.69-0.91) in the validation cohort.

Table 1 Prevalence, univariate OR (95% CI) for potential predictors of 3 months DAS28>3.2 non-response for derivation and validation cohorts.

Predictors		Derivation cohort		Validation cohort	
		n (%)	OR (95% CI)	n (%)	OR (95% CI)
Female		201 (71)	1.70 (0.99-2.91)*	72 (71)	2.29 (0.80-6.59)*
Age > 40 year		239 (84)	1.76 (0.89-3.50)*	78 (77)	1.44 (0.49-4.28)
Medication					
No HCQ		98 (38)	1.47 (0.87-2.48)*	53 (53)	1.05 (0.43-2.54)
No Sulfasalazine		99 (38)	1.42 (0.84-2.40)*	59 (58)	1.51 (0.61-3.77)
No TDT		99 (38)	1.42 (0.84-2.40)*	69 (68)	1.24 (0.47-3.26)
GC		242 (93)	1.68 (0.56-4.98)	14 (14)	2.22 (0.55-8.98)
No IM-GC		160 (62)	1.42 (0.82-2.46)	11 (11)	0.61 (0.04-10.07)
NSAID		37 (14)	1.53 (0.69-3.41)	36 (36)	1.69 (0.67-4.26)
SNPs					
ABCB1 rs1045642 G>A	AA vs GG/GA	190 (73)	1.77 (0.99-3.18)*	64 (67)	2.08 (0.71-6.06)*
ABCB1 rs1128503 G>A	AA vs GG/GA	217 (83)	1.49 (0.76-2.95)	73 (77)	1.21 (0.37-3.93)
ABCB1 rs2032582 C>A/T	AA/AT/TT vs CC/CA/CT	208 (79)	1.70 (0.90-3.22)*	72 (76)	0.96 (0.31-2.97)
ABCC1 rs35592 T>C	TC/CC vs TT	163 (62)	1.05 (0.63-1.75)	55 (58)	0.95 (0.37-2.43)
ABCC1 rs3784862 A>G	AG/GG vs AA	153 (58)	1.05 (0.63-1.75)	43 (45)	0.52 (0.20-1.36)*
ABCC2 rs717620 C>T	CC vs CT/TT	74 (28)	1.30 (0.75-2.25)	30 (32)	0.77 (0.28-2.11)
ABCC2 rs4148396 C>T	CC/CT vs TT	35 (13)	1.46 (0.71-3.01)	10 (11)	1.47 (0.30-7.10)
ABCC3 rs3785911 A>C	AC/CC vs AA	134 (51)	1.28 (0.78-2.11)	45 (47)	1.41 (0.55-3.59)
ABCC3 rs4793665 T>C	TT vs TC/CC	173 (66)	2.02 (1.17-3.49)*	68 (72)	0.36 (0.13-1.00)*
ABCC4 rs868853 T>C	TT vs TC/CC	38 (14)	1.21 (0.60-2.45)	20 (21)	1.53 (0.53-4.43)
ABCC4 rs2274407 C>A	CA/AA vs CC	232 (88)	1.09 (0.50-2.38)	81 (85)	0.59 (0.16-2.14)
ABCC5 rs2139560 G>A	AA vs GG/GA	212 (81)	1.52 (0.79-2.90)	78 (82)	1.23 (0.34-4.44)
ABCG2 rs2231142 G>T	GT/TT vs GG	204 (78)	1.54 (0.83-2.87)*	78 (82)	7.15 (0.87-58.73)*
ABCG2 rs13120400 T>C	TT vs TC/CC	110 (42)	1.08 (0.65-1.79)	37 (39)	1.56 (0.61-4.02)
ADA rs73598374 C>T	CC vs CT/TT	33 (13)	1.68 (0.80-3.54)*	13 (14)	1.22 (0.36-4.18)
ADORA2A rs5751876 C>T	TT vs CC/CT	213 (81)	1.17 (0.62-2.22)	81 (85)	0.18 (0.04-0.76)*
AMPD1 rs17602729 G>A	GG vs GA/AA	45 (17)	1.49 (0.78-2.86)	17 (18)	0.46 (0.12-1.80)
ATIC rs2372536 C>G	GG vs CC/CG	226 (86)	1.69 (0.79-3.61)*	86 (91)	4.14 (0.48-35.53)*
FOLR2 rs514933 T>C	TT/TC vs CC	42 (16)	1.24 (0.63-2.43)	16 (17)	1.06 (0.32-3.55)
FPGS rs4451422 A>C	AA/AC vs CC	61 (23)	1.46 (0.80-2.66)	19 (20)	1.73 (0.59-5.07)
GGH rs3758149 G>A	GG vs GA/AA	137 (52)	1.58 (0.95-2.61)*	41 (43)	0.90 (0.35-2.30)
GGH rs10106587 A>C	AC/CC vs AA	134 (51)	1.02 (0.62-1.69)	43 (45)	0.77 (0.30-1.99)
ITPA rs1127354 G>T	GT/TT vs GG	229 (87)	1.04 (0.50-2.16)	79 (83)	1.07 (0.29-3.94)
MTHFR rs1801131 A>C	AA vs AC/CC	139 (53)	1.37 (0.83-2.27)	42 (44)	0.97 (0.38-2.49)
MTHFR rs1801133 C>T	CT/TT vs CC	125 (48)	1.61 (0.97-2.66)*	55 (58)	0.68 (0.26-1.77)
MTRR rs1801394 A>G	GG vs AA/AG	174 (66)	1.45 (0.85-2.47)*	67 (71)	0.52 (0.19-1.44)
SLC19A1 rs1051266 C>T	TT vs CC/CT	220 (84)	1.48 (0.73-2.98)	77 (81)	2.20 (0.56-8.69)
SLC46A1 rs2239907 C>T	CC/CT vs TT	52 (20)	1.94 (1.04-3.60)*	13 (14)	1.30 (0.33-5.09)
Metabolic parameters					
Erythrocyte-folate<750 nmol/L		74 (37)	1.48 (0.82-2.69)*	14 (14)	2.80 (0.80-9.79)*
Serum-folate<13 nmol/L		57 (24)	1.43 (0.77-2.66)	25 (25)	0.95 (0.33-2.74)
Plasma homocysteine>14 µmol/L		64 (27)	1.16 (0.64-2.11)	34 (34)	1.10 (0.43-2.81)
Serum vitamin B_6<80 nmol/L		103 (50)	1.40 (0.80-2.47)	57 (58)	1.23 (0.50-3.03)
Serum vitamin B_{12}>400 pmol/L		61 (26)	1.11 (0.61-2.03)	20 (20)	2.30 (0.78-6.82)*
eGFR<80 ml/min/1.73m^2		63 (53)	1.43 (0.67-3.03)	39 (38)	1.35 (0.55-3.32)

Table 1 Continued.

Disease activity				
ESR>40 mm/hour	73 (26)	2.77 (1.58-4.85)*	18 (18)	1.83 (0.61-5.50)
CRP>10 mg/L	125 (44)	1.37 (0.74-2.54)	31 (30)	0.81 (0.31-2.13)
TJC>3 joints	221 (80)	3.81 (1.95-7.43)*	58 (57)	3.78 (1.44-9.97)
SJC>3 joints	229 (80)	2.05 (1.08-3.89)*	55 (54)	1.50 (0.62-3.64)
VAS>34 mm	215 (75)	3.82 (2.00-7.32)*	77 (76)	1.44 (0.49-4.58)
DAS28>5.1	125 (44)	3.70 (2.23-6.16)*	25 (25)	5.96 (1.98-17.93)*
HAQ>0.6	217 (76)	2.80 (1.51-5.18)*	88 (86)	1.27 (0.35-4.63)
Rheumatoid factor negative	85 (34)	1.58 (0.92-2.72)*	55 (59)	0.96 (0.38-2.47)
Anti-CCP negative	75 (30)	1.41 (0.81-2.46)	55 (59)	1.68 (0.65-4.37)
Disease duration>145 days	143 (51)	1.10 (0.68-1.78)	**	**
Life style				
Smoking	87 (33)	2.01 (1.19-3.41)*	**	**
Alcohol consumption<30 glasses/month	191 (73)	1.47 (0.84-2.58)*	34 (76)	2.25 (0.39-13.17)
Cola consumption>30 glasses/month	33 (13)	1.28 (0.62-1.67)	16 (16)	1.64 (0.53-5.08)
Coffee consumption<90 glasses/month	117 (45)	1.11 (0.68-1.83)	50 (51)	1.19 (0.48-2.93)
Tea consumption>90 glasses/month	57 (22)	1.58 (0.87-2.88)*	20 (20)	0.19 (0.04-0.90)*
BMI>25 kg/m^2	157 (56)	1.70 (1.04-2.79)*	**	**

*Variables significantly associated with MTX non-response (p<0.200) in the derivation cohort were included in the multivariate backward logistic regression analysis; **not determined; HCQ, hydroxychloroquine; TDT, triple disease-modifying antirheumatic drug therapy; GC, glucocorticoids; IM, intramuscular; NSAID, non-steroidal anti-inflammatory drugs; *ABCB1,* Adenosine triphosphate-binding cassette transporter B1; *ADA*, adenosine-deaminase; *ADORA2A*, adenosine A2A receptor; *AMPD1*, adenosine monophosphate deaminase 1; *ATIC*, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; *FOLR2*, Folate receptor 2; *FPGS*, folylpolyglutamate synthetase; *GGH*, γ-glutamyl hydrolase; *ITPA*, inosine triphosphatase; *MTHFR*, methylenetetrahydrofolate reductase; *MTRR*, methionine synthase reductase; *SLC19A1*, solute carrier 19A1; eGFR, estimated glomerular filtration rate; TJC, tender joint score in 28 joints; SJC, swollen joint score in 28 joints; HAQ, health assement questionnaire; CCP, cyclic citrulinated peptide; BMI, body mass index.

Table 2 Prediction model and scores for 3 months MTX non-response (DAS28>3.2).

Predictors		β	Score	OR (95% CI)	p
Baseline Das28	>5.1	1.13	1	3.08 (1.26-7.52)	0.014
HAQ	>0.6	1.26	1	3.53 (1.30-9.56)	0.013
ABCB1 rs1045642 G>A	AA vs GG/GA	1.34	1	3.82 (1.54-9.47)	0.004
ABCC3 rs4793665 T>C	TT vs TC/CC	1.24	1	3.45 (1.44-8.31)	0.006
Folate in erythrocytes	<750 nmol/L	0.82	1	2.28 (1.02-5.12)	0.046
Current Smoking		1.75	2	5.77 (2.34-14.24)	<0.001
BMI	>25 kg/m^2	1.11	1	3.02 (1.31-6.97)	0.009
Constant		-5.07	*	0.01	
AUC derivation cohort				0.80 (0.73-0.86)	<0.001
AUC validation cohort				0.80 (0.69-0.91)	<0.001
Hosmer-Lemeshow test				0.816	

Risk score of an RA patient having all predictors is calculated as follows: Add up scores of individual predictors, namely 1+1+1+1+1+2+1, which equals 8 points. *The constant was suppressed. MTX, methotrexate; DAS28, disease activity score in 28 joints; OR, odds ratio; CI, confidence interval; HAQ, health assessment questionnaire; *ABCB1*, adenosine triphosphate-binding cassette transporter B1; BMI, body mass index; AUC, area under the receiver operating characteristics curve.

When finally current smoking and BMI>25 were added to the prediction model in the derivation cohort the AUC raised to 0.80 (95% CI: 0.73-0.86). When the laboratory predictors were left out and only the easy to collect clinical predictors, DAS28>5.1, HAQ>0.6, current smoking and BMI>25 were added to the derivation cohort, the AUC was 0.76 (95% CI: 0.70-0.82).

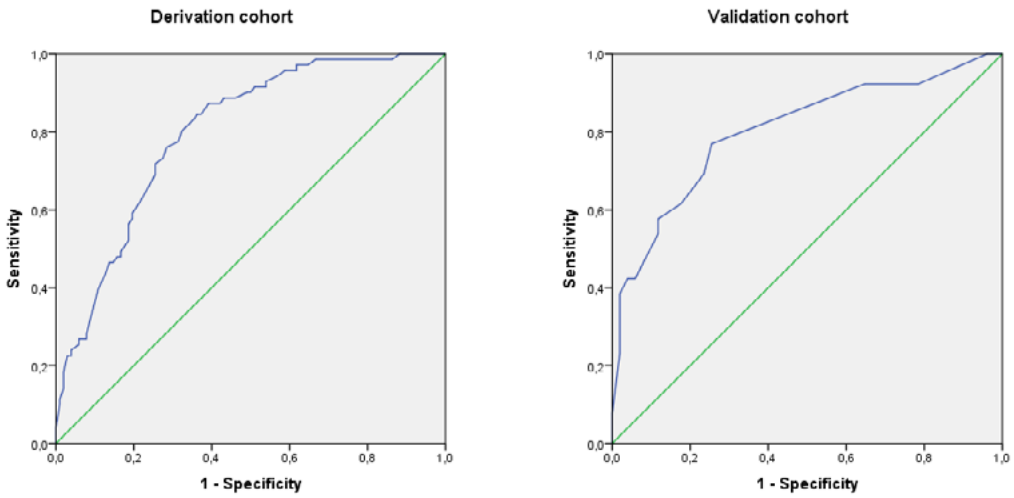

Figure 1 ROC curves for the prediction models of MTX non-response (DAS28>3.2) in the derivation (left panel) and validation (right panel) cohorts. Area under receiver operating characteristics curve was 0.80 (95%CI: 0.73-0.86) in the derivation cohort and 0.80 (95%CI: 0.69-0.91) in the validation cohort.

Prediction model for MTX adverse events

The following variables that were univariately associated with adverse events were included in the multivariable logistic regression: triple DMARD therapy, *ABCB1* rs2032582, *ABCC5* rs2139560, *ABCG2* rs2231142, swollen joint count, HAQ and alcohol use (table 4, $p<0.2$). All variables in the final prediction model had p-values≥0.050. Therefore, we did not further develop a prediction model for adverse events after 3 months of MTX therapy.

Table 3 Diagnostic parameters for various risk score cut-offs predicting MTX non-response (3 months DAS28>3.2) in the derivation cohort.

Cut-off	Probability*	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
≥1	0.10	99	27	58	96
≥2	0.20	97	38	61	93
≥3	0.33	86	63	70	82
≥4	0.44	71	72	72	71
≥5	0.58	47	85	76	62
≥6	0.56	47	85	76	62
≥7	0.85	11	99	92	53
≥8	0.80	14	98	88	53

Risk scores were calculated in each patient in the derivation cohort. *mean probability of all patients with the specific risk score for non-response (3 months DAS28>3.2). MTX, methotrexate; DAS, disease activity score; PPV, positive predictive value; NPV, negative predictive value.

Table 4 Prevalence, univariate OR (95% CI) for potential predictors of ≥3 adverse events at 3 months for derivation and validation cohorts.

		Derivation cohort		Validation cohort	
Variables		**n (%)**	**OR (95% CI)**	**n (%)**	**OR (95% CI)**
Female		201 (71)	1.24 (0.70-2.18)	66 (72)	1.67 (0.59-4.74)
Age < 40 year		239 (84)	1.39 (0.72-2.69)	71 (77)	1.19 (0.42-3.37)
Medication					
HCQ		98 (38)	1.64 (0.94-2.87)*	44 (48)	0.90 (0.37-2.18)
Sulfasalazine		99 (38)	1.68 (0.97-2.94)*	39 (42)	1.23 (0.50-3.02)
TDT		99 (38)	1.68 (0.97-2.94)*	30 (33)	0.58 (0.21-1.57)
GC		242 (93)	1.58 (0.50-4.99)	11 (12)	0.83 (0.20-3.37)
IM-GC		160 (62)	1.12 (0.64-1.95)	11 (11)	2.30 (0.14-38.08)
No NSAID		37 (14)	1.34 (0.57-3.15)	60 (66)	1.44 (0.55-3.78)
SNPs					
ABCB1 rs1045642 G>A	AA/GA vs GG	190 (73)	1.27 (0.69-2.32)	18 (21)	0.92 (0.29-2.93)
ABCB1 rs1128503 G>A	GG/GA vs AA	217 (83)	1.32 (0.68-2.56)	18 (21)	0.25 (0.05-1.16)*
ABCB1 rs2032582 C>A/T	CC/CA/CT vs AA/AT/TT	208 (79)	1.92 (1.04-3.54)*	19 (22)	0.23 (0.05-1.06)*
ABCC1 rs35592 T>C	TC/CC vs TT	163 (62)	1.05 (0.61-1.79)	51 (59)	1.32 (0.51-3.45)
ABCC1 rs3784862 A>G	AA vs AG/GG	153 (58)	1.11 (0.65-1.88)	45 (52)	1.55 (0.60-3.99)
ABCC2 rs717620 C>T	CT/TT vs CC	74 (28)	1.06 (0.59-1.89)	58 (67)	1.35 (0.49-3.75)
ABCC2 rs4148396 C>T	TT vs CC/CT	35 (13)	1.20 (0.55-2.63)	78 (91)	0.37 (0.08-1.61)*
ABCC3 rs3785911 A>C	AC/CC vs AA	134 (51)	1.35 (0.80-2.28)	43 (50)	1.77 (0.69-4.55)
ABCC3 rs4793665 T>C	TT/TC vs CC	173 (66)	1.21 (0.63-2.35)	20 (23)	0.54 (0.16-1.80)
ABCC4 rs868853 T>C	TC/CC vs TT	38 (14)	1.61 (0.73-3.58)	67 (78)	0.86 (0.28-2.59)
ABCC4 rs2274407 C>A	CA/AA vs CC	232 (88)	1.17 (0.51-2.66)	73 (85)	0.41 (0.12-1.38)*
ABCC5 rs2139560 G>A	GG/GA vs AA	212 (81)	1.84 (0.98-3.45)*	15 (17)	0.87 (0.25-3.03)
ABCG2 rs2231142 G>T	GG vs GT/TT	204 (78)	1.71 (0.94-3.13)*	16 (19)	1.14 (0.35-3.69)
ABCG2 rs13120400 T>C	TC/CC vs TT	110 (42)	1.11 (0.66-1.90)	51 (59)	1.32 (0.51-3.45)
ADA rs73598374 C>T	CT/TT vs CC	33 (13)	1.17 (0.51-2.66)	73 (85)	0.41 (0.12-1.38)*
ADORA2A rs5751876 C>T	CC/CT vs TT	213 (81)	1.00 (0.52-1.95)	13 (15)	1.66 (0.48-5.67)
AMPD1 rs17602729 G>A	GA/AA vs GG	45 (17)	1.56 (0.75-3.25)	70 (81)	1.99 (0.51-7.68)
ATIC rs2372536 C>G	CC vs CG/GG	226 (86)	1.11 (0.65-1.87)	9 (11)	0.75 (0.30-1.92)
FOLR2 rs514933 T>C	TT/TC vs CC	42 (16)	1.08 (0.54-2.18)	14 (16)	0.62 (0.16-2.44)
FPGS rs4451422 A>C	CC vs AA/AC	61 (23)	1.01 (0.54-1.87)	19 (20)	0.23 (0.08-0.68)*
GGH rs3758149 G>A	GG vs GA/AA	137 (52)	1.26 (0.74-2.13)	41 (43)	1.16 (0.46-2.96)
GGH rs10106587 A>C	AC/CC vs AA	134 (51)	1.09 (0.65-1.84)	43 (45)	0.46 (0.17-1.21)*
ITPA rs1127354 G>T	GT/TT vs GG	229 (87)	1.15 (0.52-2.52)	79 (83)	0.88 (0.27-2.86)
MTHFR rs1801131 A>C	AC/CC vs AA	139 (53)	1.16 (0.69-1.95)	42 (44)	1.08 (0.42-2.76)
MTHFR rs1801133 C>T	CC/C/T vs TT	125 (48)	1.51 (0.72-3.14)	55 (58)	1.53 (0.34-6.94)
MTRR rs1801394 A>G	AG/GG vs AA	174 (66)	1.08 (0.56-2.11)	67 (71)	0.77 (0.25-2.40)
SLC19A1 rs1051266 C>T	TT vs CC/CT	220 (84)	1.10 (0.54-2.24)	77 (81)	3.43 (0.72-16.36)*
SLC46A1 rs2239907 C>T	TT vs CC/CT	52 (20)	1.52 (0.76-3.04)	13 (14)	0.35 (0.10-1.20)*
Metabolic parameters					
Erythrocyte-folate>750 nmol/L		74 (37)	1.42 (0.77-2.64)	14 (14)	1.36 (0.34-5.47)
Serum-folate<13 nmol/L		57 (24)	1.16 (0.62-2.15)	25 (25)	0.94 (0.32-2.76)
Plasma homocysteine>14 µmol/L		64 (27)	1.22 (0.67-2.22)	34 (34)	0.79 (0.30-2.08)
Serum vitamin B_6< 80 nmol/L		103 (50)	1.31 (0.74-2.31)	57 (58)	0.81 (0.33-2.00)
Serum vitamin B_{12}< 400 pmol/L		61 (26)	1.03 (0.55-1.91)	20 (20)	1.28 (0.41-4.01)
eGFR<80 ml/min/1.73m^2		63 (53)	1.36 (0.58-3.16)	39 (38)	0.40 (0.15-1.08)*

Table 4 Continued

Disease activity				
ESR>40 mm/hour	73 (26)	1.24 (0.70-2.18)	18 (18)	0.47 (0.12-1.80)
CRP<10 mg/L	125 (44)	1.34 (0.73-2.45)	31 (30)	1.42 (0.55-3.66)
TJC>3 joints	221 (80)	1.31 (0.70-2.44)	58 (57)	0.97 (0.40-2.39)
SJC<3 joints	229 (80)	1.51 (0.82-2.79)*	55 (54)	1.71 (0.70-4.20)
VAS>34 mm	215 (75)	1.12 (0.62-2.04)	77 (76)	1.29 (0.41-4.00)
DAS28>5.1	125 (44)	1.00 (0.60-1.67)	25 (25)	0.34 (0.11-1.11)*
HAQ>0.6	217 (76)	1.61 (0.86-3.02)*	88 (86)	0.57 (0.16-1.96)
Rheumatoid factor positive	85 (34)	1.10 (0.63-1.94)	55 (59)	0.40 (0.15-1.11)*
Anti-CCP positive	75 (30)	1.19 (0.66-2.15)	55 (59)	0.40 (0.15-1.11)*
Disease duration>145 days	143 (51)	1.26 (0.76-2.09)	**	**
Life stile				
No smoking	87 (33)	1.37 (0.78-2.41)	**	**
Alcohol consumption<30 units/month	191 (73)	3.08 (1.55-6.11)*	34 (76)	1.33 (0.30-5.93)
Cola consumption<30 units/month	33 (13)	1.28 (0.57-2.89)	16 (16)	1.03 (0.32-3.32)
Coffee consumption<90 units/month	117 (45)	1.08 (0.64-1.83)	50 (51)	0.50 (0.20-1.24)*
Tea consumption>90 units/month	57 (22)	1.13 (0.60-2.11)	20 (20)	1.22 (0.40-3.70)
BMI>25 kg/m	157 (56)	1.25 (0.75-2.09)	**	**

*Variables significantly associated with adverse events (p<0.200) in the derivation cohort were included in the multivariate backward logistic regression analysis; **not determined; OR, odds ratio; CI, confidence interval; DAS, disease activity score; HCQ, hydroxychloroquine; TDT, triple disease-modifying antirheumatic drug therapy; GC, glucocorticoids; IM, intramuscular; NSAID, non-steroidal anti-inflammatory drugs; *ABCB1*, Adenosine triphosphate-binding cassette transporter B1; *ADA*, adenosine-deaminase; *ADORA2A*, adenosine A2A receptor; *AMPD1*, adenosine monophosphate deaminase 1; *ATIC*, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; *FOLR2*, Folate receptor 2; *FPGS*, folylpolyglutamate synthetase; *GGH*, gamma glutamyl hydrolase; *ITPA*, inosine triphosphatase; *MTHFR*, methylenetetrahydrofolate reductase; *MTRR*, methionine synthase reductase; *SLC19A1*, solute carrier 19A1; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; CRP, C-reactive proteïn; TJC, tender joint score in 28 joints; SJC, swollen joint score in 28 joints; HAQ, health assement questionnaire; CCP, cyclic citrulinated peptide; BMI, body mass index.

DISCUSSION

We developed and validated a model, which could predict treatment failure after 3 months of therapy at baseline in 2 prospective RA cohorts. The model consisted of the following variables: baseline DAS28>5.1, HAQ>0.6, *ABCB1* rs1045642 genotype, *ABCC3* rs4793665 genotype, erythrocyte-folate<750 nmol/L, current smoking and BMI>25 kg/m^2. The model classified 80% of patients correctly in the derivation and without current smoking and BMI data, 80% in the validation cohort. Unfortunately we could not build a prediction model for adverse events because none of the baseline variables were significantly associated with adverse events.

Prediction models for MTX nonresponse in RA have been reported before.[5-7] These prediction models confirm high baseline DAS28, mild functional impairment as in the HAQ>0.6 and current smoking as variables predicting MTX non-response. However, most studies into prediction of MTX non-response looked at clinical predictors[5-7] and some also at genetic predictors[5,7] but they did not include metabolic predictors[5-7] and one lacked a validation cohort.[6] Our study is the first validated and prospective study on

prediction of MTX non-response that also incorporated metabolic predictors next to clinical and genetic predictors.

One of the earlier reported models to predict MTX efficacy in MTX monotherapy classified 85% of patients correctly.[5] This model contained 4 clinical variables: gender, rheumatoid factor, smoking status, DAS and 4 genetic variables encoding *AMPD1, ATIC*, *ITPA* and *MTHFD1* genotypes. We did found a possible association of *ATIC* in our univariate analyses in both cohorts, but this association was not strong enough to survive the multivariate logistic regression model. The outcome variable in this prediction model was a 6 months DAS≤2.4. The DAS is more a research parameter and consists of 44 joints while the DAS28 is more routinely used. In addition, an early response is associated with a better clinical outcome, therefore predicting treatment response as early as possible with as few variables would be preferable. The same group showed later that their model performed better in DMARD naïve recent-onset RA patients than in patients with a prior history of DMARD failure.[7] Others[6] developed a prediction model using the EULAR response criteria[13] as dependent variable and current smoking, female gender, longer symptom duration and younger age as independent variables. EULAR response criteria can only be used in patients with baseline DAS28≥3.3 and therefore, the model cannot be used in a considerable number of patients.[13] In addition, unlike our model, this model did not use genetic or metabolic predictors. In a systematic review,[14] the following variables were found to be independent predictors of RA remission: male gender, young age, late-onset RA, short disease duration, non-smoker, low baseline disease activity, mild functional impairment, low baseline radiographic damage, absence of rheumatoid factor and anti-citrullinated peptide, low serum level of acute-phase reactant, interleukin-2, and RANKL at baseline, *MTHFR* 677T alleles and *MTHFR* 1298C alleles in the methotrexate treated patients.

Recently, the following clinical predictors of response to MTX and other DMARDs were summarized in an editorial review: male gender, non-smoking, early RA, DMARD naïve and disease activity.[8] Also, glucocorticoid response at 2 weeks is a useful tool for recognizing those patients who will probably have active disease after 3 months of DMARD treatment.[20] We reported earlier an association of *ABCB1* rs1045642 and *ABCC3* rs4793665 in JIA patients with MTX response.[18] These SNPS were also included in our final prediction model. Recently we reported an association of low baseline erythrocyte folate with non-response.[10] Conflicting results on the association of BMI and disease activity in RA has been reported, underweight and obesity both have been associated with worse disease activity in RA.[21,22]

Readers can decide for themselves which cut-off values to choose with the supplied ROC curves, PPVs and NPVs from figure 1 and table 3. For instance, if the goal is to correctly identify as many MTX non-responders at 3 months as possible (high sensitivity) and to avoid misidentification of responders as much as possible (reasonable specificity) the cut-off could be ≥4 with a probability for having 3 months DAS28>3.2 of 0.44 (table 3). At this cut-off 71% of non-responders and 72% of responders are identified correctly in the derivation cohort. Therefore, 71% non-

responders could have received other therapy (i.e. biologicals) at forehand, which may prevent irreversible joint destruction. However, 28% of responders will receive other medication while MTX would have worked. Whether the cost reduction of less irreversible joint destruction exceed the extra costs that will be made for biologicals should be further investigated.

The AUC of the ROC curve of the final model without current smoking and BMI>25 was lower in the derivation cohort 0.73 (95% CI: 0.66-0.80) compared to the validation cohort 0.80 (95% CI: 0.69-0.91). This was unexpected, because the power of the derivation cohort should be higher with the larger sample size compared to the validation cohort. However, because of the smaller sample in the validation cohort, the confidence interval is larger and therefore the differences in AUC between the cohorts should be interpreted with caution.

A pitfall of this study was that current smoking and BMI>25 could not be replicated in the validation cohort. These variables have to be replicated in other cohorts. However, we decided to keep these predictors in the prediction model, because they were of major importance for the predictive value of the model (AUC from 0.73 to 0.80). In addition, current smoking and BMI>25 are easy to collect with a fast result compared to laboratory predictors. With current smoking and BMI>25 in addition to DAS28>5.1 and HAQ>0.6 without laboratory predictors the AUC was still 0.76 in the derivation cohorts. This is still a good predictive value, but the addition of the laboratory predictors, erythrocyte-folate<750 nmol/L, *ABCB1 rs1045642* and *ABCC3* rs4793665 added a valuable 0.04 to the AUC of the derivation cohort. In addition, erythrocyte-folate is a routine laboratory test and genotyping might be transformed to fast-test if useful.

Another pitfall of this study was that MTX-dose could not be added as predictor because all patients in the derivation were started on 25 mg/week and all patients in the validation cohorts were started on 15 mg/week. There was no variation in MTX-dose within the cohorts. This predictor should be added in new studies. In addition, triple DMARD therapy was added to the backwards logistic regression, but did not survive this method unless triple DMARD therapy is better than MTX monotherapy after 3 months in the derivation cohort.[19] This may also be a result of the low frequency (38%) of no triple DMARD therapy in the derivation cohort. Furthermore the low frequency of glucocorticoids use in the derivation cohort (7%), also caused low power for this possible predictor.

A strong point for this study was that this model was externally validated and that the predictive value remained equal. However, to further confirm this model, validation should be performed in large international cohorts prior to its implementation in daily clinical practice.

In this study we could not build a prediction model for MTX adverse events because none of the variables was significantly associated with ≥3 adverse events. There is no standard in reporting adverse events[23] like there is for disease activity as the DAS28.[12] Therefore it is hard to compare association and prediction studies for MTX adverse events in RA. *MTHFR* genotypes have been associated to elevation of transaminases

of RA patients on MTX. Also *GGH* genotypes have been associated to bone marrow toxicity. No prediction models for adverse events in RA have been developed.

In conclusion we developed and validated a prediction model for non-response to MTX in 2 prospective RA cohorts by combining genetic, metabolic, clinical and lifestyle variables. This model can satisfactorily identify RA patients with a high risk of non-response to MTX, and can therefore be used by clinicians as a tool for personalized treatment. RA patients who are likely to be unresponsive to MTX therapy, may be (additionally) treated with biologicals.

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Supplemental table 1 Exclusion criteria for the derivation and validation cohort.

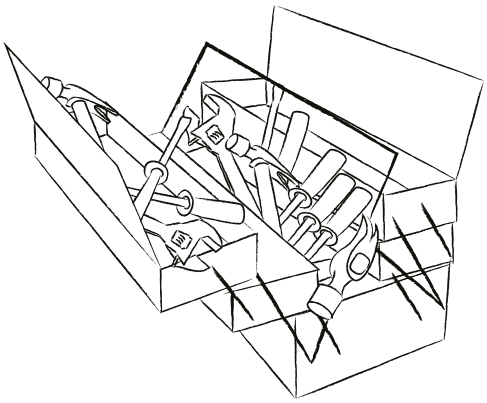
Cohort	Inclusion criteria	Exclusion criteria
Derivation	Arthritis in ≥ 1 joint	No possibility to communicate
	Duration of complaints < 12 months	Trauma
	Age ≥ 18 years	Gout, infectious arthritis or a systematic disease
	Informed consent	Former anti-rheumatic therapy
	2010 ACR/EULAR criteria for RA	Contra indication for study medication
Validation	Age ≥ 18 years	No possibility to communicate
	Informed consent	Trauma
	Diagnosed with RA by physician	Gout, infectious arthritis or a systematic disease
	MTX prescribed by physician	Former anti-rheumatic therapy

ACR, American College for Rheumatology; EULAR, European League Against Rheumatism; RA, rheumatoid arthritis; MTX, methotrexate.

Supplemental table 2 Baseline characteristics per cohort.[10]

Laboratory parameters	Derivation cohort (n=285)	Validation cohort (n=102)	p
Plasma-homocysteine (µmol/l), median (IR)	11 (10-14)	12 (10-16)	0.264
Serum-vitamin B12 (pmol/l), median (IR)	290 (231-404)	286 (230-376)	0.588
Serum-folate (nmol/l), median (IR)	17 (13-24)	17 (13-23)	0.742
Erythrocyte-vitamin B6 (nmol/l), median (IR)	80 (64-97)	74 (64-102)	0.485
Erythrocyte-folate (nmol/l), median (IR)	844 (662-1165)	1079 (868-1326)	<0.001
Rheumatoid factor positive	66%	41%	<0.001
Anti-cyclic citrullinated peptide antibody positive	70%	41%	<0.001
Erythrocyte sedimentation rate, mm/h, median (IR)	23 (13-40)	19 (9-33)	0.011
C-reactive protein, mg/l, median (IR)	8 (4-23)	7 (3-14)	0.444
Clinical parameters			
Gender, male	30%	29%	0.991
Age, mean (SD)	54 (14)	52 (16)	0.299
VAS mm, mean (SD)	53 (22)	54 (26)	0.704
28 tender joint count, median (IR)	6 (3-10)	4 (1-8)	<0.001
28 swollen joint count, median (IR)	6 (3-10)	3 (1-7)	<0.001
DAS28, mean (SD)	4.94 (1.15)	4.26 (1.43)	<0.001
Medication			
Methotrexate dose, mean (SD)	25 (1)	15 (2)	<0.001
NSAIDs	14%	36%	<0.001
Other DMARDs	62%	57%	0.408
Oral corticosteroids	62%	11%	<0.001
Parenteral corticosteroids	32%	3%	<0.001
Subcutaneous methotrexate injections	0%	6%	<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-steroidal anti-inflammatory drug; DMARD, disease modifying anti-rheumatic drug.



CHAPTER 10

General Discussion

Personalized medicine of methotrexate therapy in arthritis, a toolbox.
The aims of this thesis were: 1) To identify clinical, genetic and metabolic determinants of (non)response and adverse events of methotrexate (MTX) therapy in arthritis; 2) To better understand the mechanisms of (non)response and co-effects of MTX therapy in arthritis; 3) To investigate if erythrocyte-MTX-polyglutamate (PG) concentrations are related to (non)response or adverse events; 4) To assess metabolic co-effects of MTX therapy in arthritis. 5) To develop a prediction model for non-response and adverse events. The main findings of this thesis are summarized in figure 1.

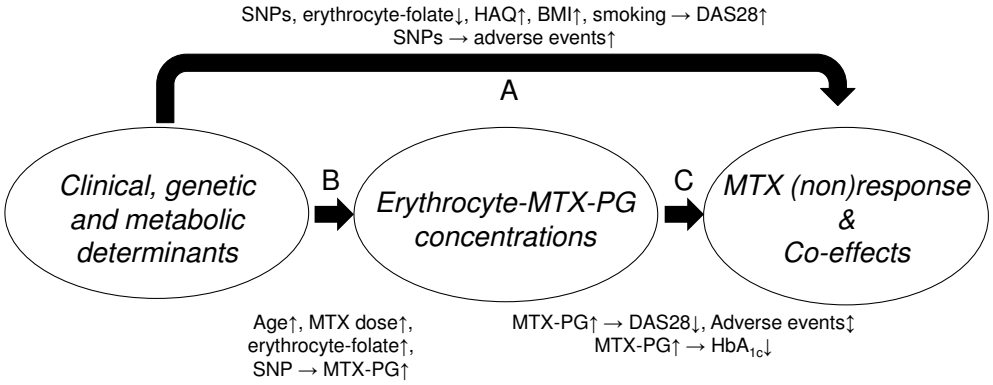


Figure 1 The relations A,B,C that were investigated in this thesis and their main findings. SNP, single nucleotide polymorphism; HAQ, health assessment questionnaire; BMI, body mass index; DAS28, disease activity score in 28 joints; MTX, methotrexate; PG, polyglutamate; HbA_{1c}, glycosylated hemoglobin.

Main findings

- Longitudinal designs are better suitable for MTX pharmacogenetic studies compared to cross-sectional designs (chapter 2).
- Adenosine triphosphate-binding cassette transporter B1 (*ABCB1)* rs1045642, *ABCC3* rs4793665 and solute carrier 19A1 (*SLC19A1*) rs1051266 single nucleotide polymorphisms (SNP) were associated with response to MTX in juvenile idiopathic arthritis (JIA) patients (chapter 3A, relation A).
- The *ABCC3* rs4793665 and *SLC19A1* rs1051266 SNPs were associated with fatigue in JIA patients on MTX and the *ABCB1* rs1045642 SNP was associated with malaise in rheumatoid arthritis (RA) patients on MTX (chapter 3B, relation A).
- Low baseline erythrocyte-folate was associated with non-response at 3 months after MTX start in RA patients (chapter 4, relation A).
- One-carbon metabolism biomarker concentrations were not associated with any adverse event (chapter 4, relation A).
- MTX in 25 mg/week dose does not cause more adverse events after the first 3 months than MTX in 15 mg/week dose in RA patients (chapter 4, relation A).

- Baseline erythrocytes-folate polyglutamates (folate-PG) in RA patients on MTX were associated with 3 months MTX-PG concentrations (chapter 5, relation B).
- Higher age, higher MTX-dose, higher erythrocyte folate status and the folylpolyglutamate synthetase (*FPGS*) rs4451422 wildtype genotype were associated with higher MTX-PG concentrations (chapter 6, relation B).
- Higher erythrocyte MTX-PG concentrations at three months of treatment were associated with a decreased disease activity score (DAS)28 over 9 months MTX therapy (chapter 7, relation C).
- Erythrocyte-MTX-PG concentrations were not associated with any adverse event (chapter 7, relation C).
- MTX use and higher erythrocyte-MTX-PG concentrations were associated with decreased glycosylated hemoglobin (HbA_{1c}) in RA patients (chapter 8, relation C).
- Baseline DAS28>5.1, health assessment questionnaire (HAQ) score>0.6, current smoking, body mass index (BMI)>25 kg/m^2, *ABCB1* rs1045642 wild type, *ABCC3* rs4793665 wild type and baseline erythrocyte-folate<750 nmol/L can be used in a prediction model for 3 months DAS28>3.2 (chapter 9, relation A).
- None of the investigated determinants could be used in a prediction model for adverse events (chapter 9, relation A).

Snapshot or longitudinal studies?

Are cross-sectional (snapshot) analyses sufficient for pharmacogenetic studies in RA? In order to explain the discrepant literature results concerning MTX pharmacogenetics in arthritis the typical treatment response patterns of patients with JIA were plotted in chapter 2. This chapter, showed, in JIA patients, that treatment response could be roughly divided into 3 profiles, depending on the outcome measure chosen: A) patients who will respond to treatment at any time point between start of treatment and 1-year follow-up and will stay in remission; B) patients who shift back and forth from responder to non-responder; and C), patients who did not show any response during first year of treatment. From a clinical point of view, prediction of treatment response at only one time point is less informative because, at the next hospital visit, a substantial number of responders may become non-responder and vice-versa.

This problem is solved in the longitudinal approach, because the results over time of each individual are used. In addition, the longitudinal approach has more power, because there are more data. Longitudinal data can be analyzed with a mixed model. Mixed models deal very elegantly with missing data, by using the results of an individual and the whole group over time. For instance, if for DAS28 visit 2 of an individual was absent and visit 1 and 3 were present, and the DAS28 was for the individual and the group decreasing, the mixed model still uses the missing time point. Mixed models look at the decrease/increase over time in an individual and in the whole group. This provides more power and can never be done in cross-sectional analysis. Future pharmacogenetic studies in arthritis research should preferably evaluate the treatment (non)response in a longitudinal way.

The longitudinal approach also suffers from a problem. In the treatment in Rotterdam Early Arthritis Cohort (tREACH) and in the methotrexate in Rotterdam (MTX-R) cohorts, study medication was switched every 3 months. Therefore it was hard to relate baseline determinants to longitudinal study outcomes such as disease activity and adverse events, because study medication will have a large influence. In addition, the first months upon diagnosis represent a window of opportunity during which outcomes can be more effectively modulated by therapy.[1] Therefore, prediction is mainly important for the first 3 months. A prospective approach was chosen with only baseline and 3 months as time points in chapters 4, 5, 6, 8 and 9.

In chapter 3A, study medication remained the same over whole study period. In chapter 7, both the independent (MTX-PGs) and the dependent (disease activity) covariate were measured longitudinally instead of only the dependent covariate in the prediction studies. Therefore, chapter 7 profits maximally from the longitudinal approach. This is clear in table 3 from this chapter, where the snapshot analyses in the tREACH (validation cohort) after 3 months therapy are not significant, but the longitudinal analyses over 9 months are significant.

Concluding, longitudinal designs are better suitable for MTX pharmacogenetic studies than cross-sectional designs.

Genetic determinants for (non)response and adverse events in arthritis

The presence of *ABCB1* rs1045642 or *ABCC3* rs4793665 variant genotypes increased the likelihood to become an MTX responder 2-3 fold or for *SLC19A1* rs1051266 decreased the likelihood 2-3 fold (chapter 3A).

ABCB1 belongs to the efflux transporters of the *ABC* super family, subfamily B, and was formerly referred to as multidrug resistance 1 (*MDR1)* gene. The product of the *ABCB1* gene is P-glycoprotein (P-gp).[2] Although the *ABCB1* rs1045642 polymorphism is synonymous (i.e.. not leading to amino acid exchange) it is associated with altered P-gp expression and reduced P-gp function.[3] Early in vitro experiments in cell lines with high levels of MTX resistance suggested that P-gp could transport MTX [4,5]. From this perspective, the *ABCB1* rs1045642 polymorphism may result in impaired cellular efflux of MTX in heterozygous and homozygous variants with concomitant increased intracellular MTX levels and increased MTX efficacy. However, recent research showed that MTX is unlikely to be a substrate of P-gp.[6,7] P-gp is expressed as a cell membrane-associated protein in natural killer cells, cluster of differentiation (CD)4-positive and CD8-positive lymphocytes and bone marrow progenitor cells[8] and plays a role in the transport of some inflammatory mediators, in particular bioactive lipids.[9] This could explain why *ABCB1* gene polymorphisms have been associated with increased response to MTX in adult RA[10,11] and in JIA in the present study; if the *ABCB1* rs1045642 polymorphism is associated with a diminished extrusion of inflammatory mediators, this could facilitate a better therapeutic effect of MTX. Collectively, changes in the physiological function of P-gp could provide an alternative explanation for the association between the *ABCB1* rs1045642 polymorphism and MTX response.

ABCC3 is involved in the efflux of MTX.[2,12] The rs4793665 SNP is located in the 5'-promoter region of the *ABCC3* gene and was associated with significantly lower *ABCC3* transcript levels, a trend towards lower protein expression in human liver, and it could affect the binding of nuclear proteins to the *ABCC3* promoter.[13] Less expression of *ABCC3* transporter could have a positive effect on the MTX cellular retention, leading to higher intracellular levels (figure 2, chapter 1). This could provide an explanation for our finding that the rs4793665 SNP was associated with response to MTX. However, others have shown that this polymorphism determined neither the expression of the *ABCC3* gene nor the response to MTX therapy in acute leukemia.[14] Nevertheless the treatment dosage is much lower in the JIA context, and thus these studies are not comparable. SNPs in efflux transporters may have a greater impact on low dose MTX therapy.
The membrane transporter *SLC19A1* is involved in the influx of MTX. Previously, we associated *SLC19A1* rs1051266 with an increased risk of pediatric acute lymphoblastic leukemia and elucidated the effects of this carrier on MTX metabolism.[15] SNPs in *SLC19A1* have been associated with response to MTX in RA[16] but not in JIA.[17]

GI adverse events shortly after starting MTX have the most influence on limiting dose increases or continuation. Therefore, GI adverse events 3 months after start of MTX were chosen as dependent variable in chapter 3B. The *SLC19A1* rs1051266 SNP showed a trend towards association with abdominal pain (OR 2.76, 95% CI 0.88–8.62, p = 0.081). The *ABCC3* rs4793665 (OR 0.33, 95% CI 0.12–0.91, p = 0.031) and *SLC19A1* rs1051266 (OR 2.94, 95% CI 1.37–6.31, p = 0.006) polymorphisms were associated with fatigue. The same SNPs were also investigated in relation to GI events and malaise in a cohort of 387 adult patients with RA (chapter 3B). The *ABCB1* rs1045642 SNP was associated with malaise (OR 2.57, 95% CI 1.59-4.15, p<0.001). Unfortunately, it is hard to compare these results with results from the literature, since adverse events are scored in many different ways. In order to correctly assess the associations of these genetic determinants and adverse events, large collaboration studies are needed with standardized measures for adverse events.

Adverse events, are important when they cause stopping MTX therapy. This loses precious time in the window of opportunity and raises the chance for irreversible joint damage. Therefore adverse events that cause stopping of MTX-therapy should be investigated first. Although effects of transporter gene polymorphisms on GI adverse events in JIA and RA were observed (chapter 3B), the findings of Ranganathan, et al.[18] could not be replicated. In addition, there were no validation cohorts available for the findings from chapter 3B. This underscores the need for meta analyses and collaborations between centers to build prediction models for outcomes of MTX therapy in pediatric and adult rheumatic diseases.

Concluding, *ABCB1* rs1045642, *ABCC3* rs4793665 and *SLC19A1* rs1051266 polymorphisms are associated with response to MTX in JIA patients. In addition, the *ABCC3* rs4793665 and *SLC19A1* rs1051266 SNPs were associated with fatigue in JIA patients on MTX and the *ABCB1* rs1045642 SNP was associated with malaise in RA patients on MTX.

Metabolic determinants for (non)response and adverse events in arthritis

Baseline low erythrocyte-folate was associated with 3 months non-response in two independent prospective cohorts of RA patients on MTX (chapter 4).

Erythrocyte-folate has been associated before with MTX outcome in two cross-sectional studies in RA patients.[19,20] In these studies, in contrast to chapter 4, higher erythrocyte-folate was associated with higher disease activity. Studies on the effects of folic acid supplementation on MTX response report no effect[21] or a negative association.[22] Taken together, these results from the literature suggest that lower concentrations of folate during MTX treatment facilitate higher effectiveness of MTX in the competition with folate for transporter proteins, polyglutamylation proteins and target enzymes for MTX. However, the cross-sectional design of these studies[19,20] cannot be compared with the prospective design from this thesis. In these cohorts[19,20] patients were on MTX and folic acid supplementation for varying time periods from 3 months to >10 years. Influences of MTX use and folic acid supplementation on erythrocyte-folate concentrations cannot be ignored. In chapter 4, only baseline erythrocyte-folate concentrations from MTX and folate supplement naïve patients were used.

None of the metabolic biomarkers from chapter 4 was associated with MTX adverse events. Contrary to these results, 12 juvenile arthritis patients with historical intolerance to MTX were shown to have significantly lower cellular folate concentrations than 81 patient's naive for MTX.[23] In addition, low-normal initial plasma and red blood cell folate levels have been associated with future toxicity of MTX in RA.[24] In the cohorts from chapter 4, all patients were treated with folic acid. This treatment has been proven to reduce MTX adverse events in RA patients.[21] This could have diluted the relationship between the investigated biomarker concentrations and adverse events in chapter 4.

Severe adverse events, bone marrow toxicity and hepatotoxicity which cause MTX treatment discontinuations were too low in frequency for proper statistical analysis. Studies with larger sample sizes are needed to find predictors for these adverse events.

Metabolic determinants were not associated with adverse events in chapter 4. However, another important conclusion could be drawn from the adverse events results from this chapter. The amount of reported adverse events in the tREACH where patients receive 25 mg/week MTX and in the MTX-R cohort where patients receive 15 mg/week MTX, were almost the same (figure 4, chapter 4). Only in the MTX-R more malaise was reported. Thus, starting with 25 mg/week MTX does not cause extra adverse events in the first 3 months compared to starting with 15 mg/week MTX. Together with the associations from chapter 6 between MTX-dose and MTX-PG concentrations and the associations from chapter 7 between MTX-PG concentrations and disease activity, this is an extra argument for starting with higher dose MTX in RA. Higher MTX-dose leads to higher MTX-PG concentrations (chapter 6). These higher MTX-PG concentrations lead to lower disease activity (chapter 7) without causing more adverse events (chapter 4).

In conclusion, low baseline erythrocyte-folate was associated with non-response at 3 months after MTX start in RA patients. Metabolic determinants were not associated with any adverse event. MTX in 25 mg/week dose does not cause more adverse events after the first 3 months than MTX in 15 mg/week dose in RA patients.

Mechanisms of MTX (non)response and adverse events in arthritis

Chapter 4 showed that lower baseline erythrocyte-folate concentration is associated with non-response in two independent cohorts. A possible explanation for this finding may be that individuals with lower concentrations of folate may be less effective in absorbing, transporting, cellular uptake and retention of folates. Since MTX is structurally similar to folate (figure 1, chapter 1) and uses the same means of transportation and metabolism, patients with low baseline intracellular folate may less easily accumulate MTX intracellularly during therapy. In this sense, baseline erythrocyte-folate is a sort of functional assay for the body's capacity to accumulate and retain cellular folate and thereby predicts how much MTX will be taken up and accumulated during therapy.

That uptake and accumulation of MTX is important for MTX non-response was shown in chapter 7. An increase in erythrocyte MTX-PG concentrations was associated with a decreased DAS28 over 9 months in the tREACH and MTX-R cohorts. This suggests that the mechanism for the relation between low baseline erythrocyte-folate and non-response may be revealed. Low baseline erythrocyte-folate reflects the poor capacity of an individual to retain and accumulate intracellular MTX which leads to low intracellular MTX. Low intracellular MTX leads to non-response. Thus, intracellular MTX and especially MTX-PG concentrations may be an intermediate for the relation between baseline erythrocyte-folate and non-response. Therefore, chapter 6 focused on the mechanisms behind the highly variable MTX-PG concentrations.

In chapter 6 clinical, genetic, socio-demographic, and biochemical determinants of erythrocyte MTX-PG concentrations in RA patients treated with MTX in the tREACH and MTX-R cohorts were investigated. Age, MTX dosage, erythrocyte folate content and the *FPGS* rs4451422 SNP were identified as the major determinants of MTX-PG concentrations in both cohorts. That erythrocyte-folate was associated with intracellular MTX-PG concentrations further supports the hypothesis that intracellular MTX is an intermediate in the relation between low erythrocyte-folate and non-response. Also others have shown that erythrocyte-folate levels were positively associated with erythrocyte-MTX levels.[25]

FPGS rs4451422 wild type was also associated with higher MTX-PG concentrations (chapter 6). *FPGS* is an intracellular enzyme responsible for folate and MTX polyglutamation. Any changes in function might therefore dramatically decrease the longer chain MTX-PGs, thereby leading to slower build-up of MTX-PGs and lower steady-state concentrations. If MTX polyglutamation is a feature of individuals partly controlled by *FPGS* polymorphisms, baseline folate polyglutamations should be related to 3 months MTX polyglutamylation. Therefore, in chapter 5 was investigated whether

baseline erythrocyte folate polyglutamates (folate-PG) were associated with 3 months MTX-PGs. We showed that baseline short-chain folate-PG concentrations were positively associated with 3 months short-chain MTX-PG concentrations, medium-chain folate-PGs with medium-chain MTX-PGs and long-chain folate-PGs were associated with long-chain MTX-PGs. These findings further supports the hypothesis that not only intracellular MTX but especially intracellular MTX-PGs are an intermediate in the relation between baseline erythrocyte-folate and non-response.

The observations from chapter 3A that a genetic polymorphism in the influx transporter SLC19A1 was associated with non-response and efflux transporter polymorphisms were associated with improved response in juvenile arthritis[26] further underscores the need for effective uptake and cellular retention of MTX. This suggests that intracellular MTX is also an intermediate for the relation between SNPs in genes for MTX transporters and non-response. However, chapter 6 did not reveal an association between *ABCB1* rs1045642, *ABCC3* rs4793665 or *SLC19A1* rs1051266 and erythrocyte-MTX-PG concentrations. Therefore it cannot be concluded that intracellular MTX is an intermediate in the relation between MTX transporter SNPs and non-response. A possible explanation could be that we determined the MTX-PG concentrations in erythrocytes and not in leukocytes. Leukocytes are possibly the effector cells for decreased inflammation and RA disease activity with MTX-use. We assumed a correlation between MTX-PG concentrations in erythrocytes and leukocytes.

Chapter 6 shows also that MTX-dose influences MTX-PG concentrations. Also others have shown this association before.[25,27] The higher MTX-dose (25 mg/week) in the tREACH caused higher MTX-PG3, MTX-PG4 and MTX-PG5 concentrations after 3 months ($p \leq 0.001$) compared to the lower MTX-dose (15 mg/week) in the MTX-R cohort (chapter 6). After 9 months, there were no differences in MTX-PG concentrations between cohorts (chapter 7). This may be explained because 28% of patients in the MTX-R cohort used 25 mg MTX/week after 9 months. Also, the longer MTX use could have caused higher MTX-PG concentrations.[28]

In chapter 6 was concluded that higher MTX-dose was associated with higher MTX-PG concentrations. However the tREACH and MTX-R cohorts had to be pooled in order to investigate the relation between MTX-dose and MTX-PG concentrations. MTX-dose did not differ within these 2 cohorts, but was different between the cohorts. Patients in the tREACH used 25 mg/week MTX and patients in the MTX-R used mainly 15 mg/week MTX. It is hard to tell whether the differences in MTX-PG concentrations between the cohorts are caused by MTX-dose or by other differences between these cohorts. For instance, both cohorts had large differences in co-medication like triple DMARD therapy and use of glucocorticoid bridging therapy. MTX-dose was associated with erythrocyte-MTX-PG concentrations in chapter 6. This conclusion has to be interpreted with caution. Further research is needed to confirm this relation.

In conclusion, baseline erythrocyte-folate may be considered a sort of functional assay for the body's capacity to accumulate and retain MTX and thereby predicts how much MTX will be taken up and accumulated during therapy. MTX-PG concentrations

are therefore an intermediate in the relation between erythrocyte folate and 3 months non-response that was found in chapter 4.

MTX-PGs as a tool for therapeutic drug monitoring in arthritis.

Plasma-MTX is eliminated from plasma within 24 hours[29] and is unrelated to non-response[28] and therefore, is not a reliable tool for therapeutic drug monitoring (TDM).[30] Higher MTX-dose leads to higher intracellular MTX-PG concentrations.[25,27] Erythrocyte-MTX-PGs could have a promising role as biomarkers of patients' non-response to MTX and in turn could be potentially used as TDM tool. In chapter 7, we showed that MTX-PG concentrations are related to disease activity over 9 months MTX therapy. Besides the results from the present study in adult RA, we also showed in a back-to-back publication that MTX-PG3, MTX-PG4, MTX-PG5 and total MTX-PG concentrations were related to lower disease activity in JIA.[31]

TDM of intracellular MTX concentrations in erythrocytes may help identifying refractoriness patients with non-response and high MTX-PG concentrations who need treatment with other DMARDs or biologicals. TDM of erythrocyte-MTX-PGs may also be beneficial for patients with a difficulty in accumulating MTX or non-compliance who may benefit from a dose increase or treatment of compliance issues.

Since MTX-PGs in erythrocytes is easy to measure, shows good correlation with MTX non-response and is well established in several laboratories, it may be a promising tool for TDM in low-dose MTX therapy. It is a more promising tool for TDM than measuring MTX-PGs in leukocytes. In leukocytes MTX-PGs were barely correlated with MTX non-response.[32] The pre-analytics for isolating leukocytes are difficult to control and hard to incorporate in routine laboratory procedures. In leukocytes, the concentrations are lower and the variations higher, causing higher confidence intervals. Erythrocytes are easy to isolate and MTX-PG concentrations are higher.[32]

In chapter 7, we showed cut off concentrations for MTX-PG2, MTX-PG3, MTX-PG4 and total MTX-PG. The AUC for the ROC curves were only significantly different from 50% for MTX-PG2 and total MTX-PG. However, there was a trend towards significance for all these MTX-PGs. Moreover, these cut-off concentrations were constructed in a small sample size (n-57) from the MTX-R. This small sample size resulted from the rule for EULAR response criteria that only patients with baseline DAS28≥3.3 can be included. Research in larger and other cohorts is needed, to confirm cut-off concentrations. Summarizing, chapter 7 showed that MTX-PG2-4 and total MTX-PG could be used to predict MTX non-response. Since, MTX-PG3 is the predominant MTX-PG with highest concentrations, this MTX-PG seems a good choice. However, the AUC is larger for total MTX-PG, therefore this choice has more predictive power.

Cut-off concentrations (total MTX-PG: ≥74 nmol/l) should be used in combination with DAS28 to identify those patients where MTX-PG and DAS28 are discrepant. If DAS28 is high and MTX-PG concentrations are low, MTX dose could be increased or compliance issues have to be solved. 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 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.

At 3 months, MTX-PG concentrations have almost reached steady state. Therefore, measuring at 4-8 weeks could provide more variation in MTX-PG concentrations.[28] In addition, it takes about 6 weeks before patients experience less disease activity by MTX. Exploring the capacities of earlier TDM is important since the first months upon diagnosis represent a window of opportunity during which outcomes can be more effectively modulated by therapy.[1]

MTX-PGs were not associated with adverse events in chapter 7. This was also found by others.[16,20,31,32] However, relationships between MTX-adverse events and higher concentrations of MTX-PG4 and MTX-PG5 have been reported.[33] Also, in JIA an association between elevated liver function tests and gastrointestinal adverse events and high MTX-PG3-5 concentrations has been found.[34] In chapter 7, all patients were treated with folic acid. This treatment has been proven to reduce MTX adverse events in RA patients.[21] This could have diluted the relationship between MTX-PG concentrations and adverse events.

In conclusion, higher erythrocyte MTX-PG concentrations were associated with lower DAS28 during 9 months MTX treatment in RA patients. 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.

Influence of MTX therapy in arthritis on decrease of HbA_{1c}, a co-effect of MTX ?

Patients with RA experience higher rates of cardiovascular disease.[35,36] This could be explained by a direct effect of inflammation on atherosclerosis[37,38] and/or an increase in cardiovascular risk factors, like diabetes mellitus (DM).[39,40] Knowing which RA treatment could be effective in lowering HbA_{1c} may help in preventing diabetes in RA. Whether lowering HbA_{1c} was a positive co-effect of MTX therapy was investigated in chapter 8. In chapter 8 it was concluded that triple DMARD therapy or MTX monotherapy initiation and subsequent increased erythrocyte-MTX-PG concentrations were associated with reduced HbA_{1c} over the first 3 months treatment in RA patients.

The association between MTX-use and decreased HbA_{1c} might be explained by decreased inflammation and/or a direct effect on glucose control. Increased inflammation as with high disease activity in RA accelerates development of several cardiovascular risk factors such as DM.[41,42] In chapter 8, the decrease in HbA_{1c} was largest in treatment-arms with patients on triple DMARD therapy. Earlier, we showed that disease activity after 3 months was lower in patients receiving triple DMARD therapy than in those receiving MTX monotherapy.[43] This might suggest that the association between MTX-use and decreased HbA_{1c} is mediated via decreased disease activity. Also, associations between MTX-PG concentrations and decreased HbA_{1c} found in our study could be mediated via decrease in disease activity since MTX-PGs

are associated with decreased disease activity in RA in both our cohorts.[44] The β of the association of MTX-PG3 with HbA_{1c}-change reduced 4-23% (>10%) when an inflammation covariate was added. Thus, the effect of MTX on HbA_{1c}-change was partly mediated through a decrease in inflammation explaining why other DMARDS also reduced HbA_{1c}. Additionally, there might be an additional direct effect of MTX on decrease in HbA_{1c}.

A direct effect of MTX on glucose metabolism has recently been described.[45] Chronic treatment of experimental type 2 DM in mice with low doses of MTX increased skeletal muscle GLUT4 glucose transporter expression and improved metabolic control.[45] MTX treatment was also associated with significant reduction of glucose and insulin serum concentrations in diabetic mice, and glucose levels in controls.[45] MTX inhibits 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) transformylase.[46] The inhibition of this enzyme may lead to an upstream accumulation of AICAR,[47] a well-known activator of 5'-AMP-activated kinase (AMPK) and of its downstream pathways, which regulate insulin-independent GLUT4 expression and glucose metabolism.[48,49] Skeletal muscle glucose uptake is the rate-limiting step of glucose utilization, and it is physiologically regulated by an insulin-dependent and an insulin-independent signaling pathways, both leading to translocation of GLUT4 glucose transporter to the plasma membrane.[50] MTX mediated increase of GLUT4 expression via increase in AICAR may explain our partially direct effect of MTX-PG concentrations on HbA_{1c}-change.
In conclusion, MTX use and MTX-PG concentrations are associated with decrease of HbA_{1c} in RA patients. This is a positive metabolic co-effect of MTX, partially facilitated via decreased disease activity and possibly via a direct effect.

A prediction model for MTX non-response and adverse events in arthritis.
To ensure that only patients unresponsive to MTX receive timely additional treatment with biologicals and those responsive to MTX are spared costly biologicals, it is necessary to identify non-responders and patients prone to experience adverse events at baseline. The aim of chapter 9 was to develop and validate prediction models for non-response and adverse events after 3 months of treatment with MTX. Clinical, genetic and metabolic determinants were combined in order to construct a prediction model with highest sensitivity and specificity.

In chapter 9, baseline DAS28>5.1, HAQ>0.6, *ABCB1* rs1045642 variant, *ABCC3* rs4793665 variant, baseline erythrocyte-folate<750 nmol/L, current smoking and BMI >25 kg/m^2 were incorporated in a prediction model for 3 months DAS28>3.2. The model classified 80% of patients correctly in the derivation and 80% in the validation cohort.

A strong point from this thesis, is that the associations from chapter 3A and chapter 4 were confirmed in our prediction model from chapter 9. The *ABCB1* rs1045642 and *ABCC3* rs4793665 SNPs from chapter 3A and the lower erythrocyte-folate from chapter 4 were included in the prediction model from chapter 9. The final variables in the prediction model were chosen by backwards logistic regression, with all possible clinical, genetic and metabolic determinants. In chapter 4 the same cohorts

were used as in the prediction model, but in the prediction model all variables were dichotomized. Dichotomizing, causes loss of power, but low erythrocyte-folate still predicted non-response in chapter 9. In chapter 3A a totally different population was used compared to the prediction model from chapter 9. The associations between the SNPs and response from chapter 3A were found in a JIA cohort and the prediction model from chapter 9 was built in 2 RA cohorts. This shows that defects in MTX transporter genes are associated with (non)response in pediatric and adult patients in 2 totally different diseases.

One of the weaknesses of chapter 9 was that the MTX-R cohort which was used as validation cohort did not contain BMI and smoking information of the RA patients. Therefore these important predictors which were responsible for 3 points of the prediction model could not be validated. Without these 3 predictors, the AUC of the ROC curve to predict 3 months DAS28>3.2 was 0.76 in the tREACH and 0.80 in the MTX-R. One would expect a lower AUC in the MTX-R with the smaller sample size (102 versus 285). However, the larger confidence intervals in the MTX-R cohort could explain the lower AUC. Although BMI and smoking were not validated they were kept in the prediction model, because they are easy to collect for a physician and are therefore important predictors. Whether physicians will incorporate the prediction model into their clinical practice depends on the benefits, but also on how easy it is to measure. Laboratory values like genetics and erythrocyte-folate take time for drawing blood and analysis. Our prediction model also proved to have a reasonable AUC without laboratory values. However, BMI and smoking have to be validated as predictors for non-response in another large cohort.

Unfortunately, a prediction model for adverse events could not be build because none of the possible determinants for a prediction model for adverse events in chapter 9 survived the longitudinal backwards regression. This is unfortunate since significant numbers of RA patients on MTX suffer from adverse events which prevent the ability to increase or even continue MTX treatment.[51] Predicting adverse events would therefore be beneficial. In conclusion, *ABCB1* rs1045642, *ABCC3* rs4793665 and *SLC19A1* rs1051266 polymorphisms are associated with response to MTX in JIA patients. However in chapter 3B, the *ABCC3* rs4793665 and *SLC19A1* rs1051266 SNPs were associated with fatigue in JIA patients on MTX and the *ABCB1* rs1045642 SNP was associated with malaise in RA patients on MTX. These associations were not strong enough to survive the longitudinal backwards regression. In addition, the findings from the literature[18] could not be replicated in chapter 3B. This underscores the need for meta analyses and collaborations between centers to build prediction models for adverse events of MTX therapy in arthritis.

In conclusion, baseline DAS28>5.1, HAQ>0.6, *ABCB1* rs1045642 variant, *ABCC3* rs4793665 variant, baseline erythrocyte-folate<750 nmol/L, current smoking and BMI >25 kg/m^2 were incorporated in a prediction model for 3 months DAS28>3.2. A prediction model for adverse events could not be realized.

Concluding remarks

In this thesis attempts were made to reach all 5 aims: 1) *ABCB1* rs1045642, *ABCC3* rs4793665 and *SLC19A1* rs1051266 SNPs were identified as possible genetic determinants of MTX non-response in arthritis. *ABCC3* rs4793665 and *SLC19A1* rs1051266 SNPs were identified as possible genetic determinants of fatigue in JIA patients on MTX and the *ABCB1* rs1045642 SNP was identified as possible genetic determinant of malaise in RA patients on MTX. Low baseline erythrocyte-folate was identified as metabolic determinant of non-response of MTX therapy in arthritis; 2) Intracellular MTX-PG concentrations might be an intermediate in the association between low baseline erythrocyte-folate and MTX non-response in arthritis. This could be the mechanism of action of low erythrocyte-folate as an metabolic determinant for non-response; 3) Higher erythrocyte-MTX-PG concentrations are associated with lower disease activity. Erythrocyte-MTX-PG concentrations are not associated with adverse events; 4) MTX-use and higher intracellular MTX-PG concentrations were associated with decrease of HbA_{1c}. This might be a positive metabolic co-effect of MTX therapy in arthritis; 5) Baseline DAS28>5.1, HAQ>0.6, *ABCB1* rs1045642 variant, *ABCC3* rs4793665 variant, baseline erythrocyte-folate<750 nmol/L, current smoking and BMI >25 kg/m^2 were incorporated in a prediction model for 3 months DAS28>3.2. A prediction model for adverse events could not be realized.

Future perspectives

Tools for personalized medicine in arthritis

- The prediction model for MTX non-response has to be validated in large multicenter studies were patients should be randomized into a group were the prediction model decides the therapy and a group were the physician chooses therapy.
- For validation studies of non-response prediction models, bone erosion scores from x-ray photos and disability scores should be used as end points for irreversible joint destruction.
- Clinical, genetic and metabolic results from hospital information systems should be used to provide the physician with an automated personalized medicine advice in that same hospital information system.

MTX dose and adverse events

- Measures for adverse events have to be standardized and large collaborations between centers realized to build prediction models for adverse events of MTX therapy in arthritis.
- Since 25 mg/week MTX does not cause more adverse events compared to 15 mg/week MTX (chapter 4) and MTX-dose is associated with MTX-PGs and MTX-PGs are associated with lower disease activity physicians should consider starting with higher MTX-dose for RA.

MTX use and HbA_{1c}

- Because MTX use, but especially triple DMARD therapy causes decrease in HbA_{1c} (chapter 8) physicians should choose these therapies for treating RA instead of other DMARDS in order to prevent more cardiovascular disease in RA.
- Starting with higher MTX dose should be considered since MTX-dose was associated with higher MTX-PG concentrations (chapter 6) and MTX-PG concentrations were associated with lower HbA_{1c}.

Co-medication, MTX-dose and MTX response

- A cohort with patients on different MTX dosing regimens with the same co-medication has to be designed to investigate the relations between MTX-dose and MTX-PG concentrations.
- A cohort with patients on different co-medication, with the same dosing regimen has to be designed to investigate the relations between co-medication and MTX-PG concentrations. For these new cohorts it is important that all variables have to be the same and only the variable for investigation must differ within the cohorts.

Tools for therapeutic drug monitoring of MTX therapy in arthritis

- Before erythrocyte MTX-PGs can be used for TDM the benefit has to be confirmed in large multicenter studies.
- Studies have to be performed in which erythrocyte-MTX-PG concentrations are measured at 4-8 weeks, because the first months upon diagnosis represent a window of opportunity for effective modulation of outcomes by changes in therapy.
- MTX-PG cut-off concentrations may be used in combination with DAS28 to assess ineffective MTX-dose and compliance issues.

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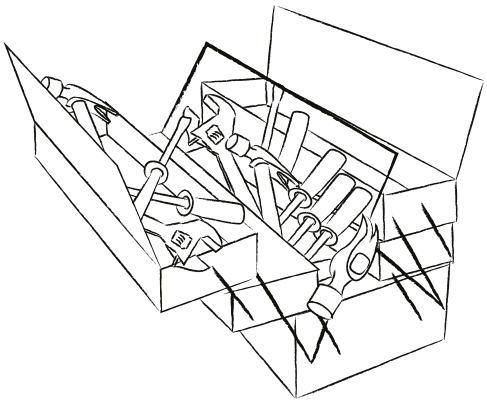


CHAPTER 11

Summary

SUMMARY

Methotrexate (MTX) is the cornerstone disease-modifying anti-rheumatic drug (DMARD) in the treatment of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA). In significant numbers of patients, MTX fails to achieve adequate suppression of disease activity and induces adverse events, which has its impact on the ability to increase or even continue the therapeutic dose. Patients who do not respond to MTX or who develop severe adverse events within 3 months after starting MTX, are frequently given expensive biologicals instead. The prediction of MTX non-response and MTX-induced adverse events before starting MTX, is paramount since the first months upon diagnosis represent a window of opportunity during which outcomes can be more effectively modulated by therapy. To ensure that only patients unresponsive to MTX receive early additional treatment with biologicals and those responsive to MTX are spared costly biologicals, it is necessary to identify non-responders and patients prone to experience adverse events at baseline. In order to predict MTX non-response and occurrence of adverse events, risk factors of these outcomes should be identified.

MTX is a folate antagonist that uses the same transport mechanisms as folate itself. Inside cells, MTX inhibits key-enzymes in one-carbon metabolism, which is responsible for its therapeutic effects as well as its adverse-event profile. The central hypothesis in this thesis states that derangements in the cellular MTX pathway and its metabolism influences MTX response as well as adverse events, through direct effects on the mechanism of MTX action or indirectly mediated via changes in intracellular methotrexate-polyglutamate (MTX-PG) accumulation.

The primary aim was to identify determinants for non-response and adverse events (co-effects in figure 1) of MTX therapy in arthritis, and subsequently develop a prediction model. Physicians could then use this model for more personalized medication for their patients. A simple blood test and filling out a questionnaire, before prescription, would provide the physician with the information necessary to determine which therapy to start with and in what dosage. A second aim of this thesis was to better understand the mechanism between determinants and MTX (non)response or co-effects. Co-effects can be adverse events, but can also be positive co-effects on comorbidities such as the prevention of cardiovascular diseases and diabetes with a decreased glucose homeostasis. We have investigated these mechanisms of (non)response or co-effects by looking at intracellular MTX-PG concentrations as an intermediate that caused the effects on non-response and adverse events (figure 1). A third aim of this thesis was to find out whether erythrocyte-MTX-PG concentrations are related to disease activity or adverse events in RA patients on MTX and thus whether MTX-PGs could be a tool for therapeutic drug monitoring. Besides adverse events, as stated above MTX may also have positive co-effects. The fourth aim was to assess metabolic co-effects of MTX therapy, especially decrease of glycosylated haemoglobin as marker for a diminished glucose homeostasis. Before all these aims were assessed, first the advantages and disadvantages of cross-sectional versus longitudinal study designs were investigated.

Figure 1 shows the aims and relations that were investigated in this thesis and the main outcomes. Relation A represents the associations between determinants and MTX (non)response / co-effects. Relation B represents the associations between determinants and intracellular MTX-PG concentrations. Relation C represents the associations between MTX-PGs and MTX (non)response and co-effects.

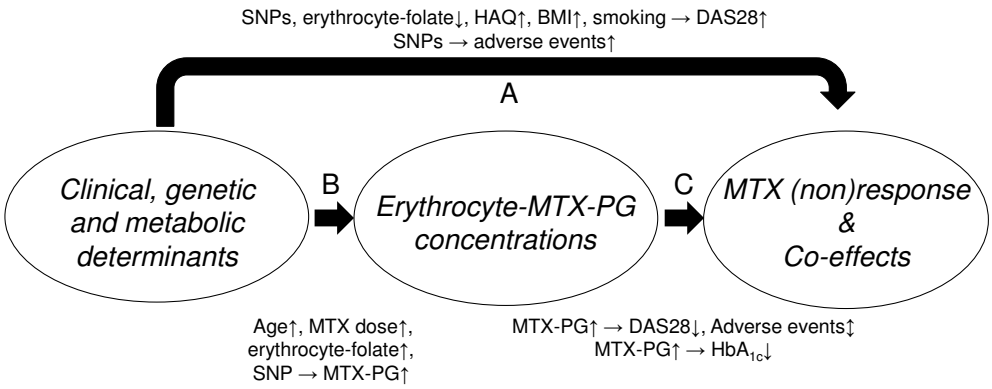


Figure 1 The relations A,B,C that were investigated in this thesis and their main findings. Co-effects include adverse events. A) Specific SNPs, a higher HAQ score, a higher BMI and current smoking were associated with a higher DAS28. Specific SNPs were associated with specific adverse events. B) Higher age, higher MTX-dose, higher erythrocyte-folate and a SNP were associated with higher MTX-PGs. C) Higher MTX-PGs were associated with lower DAS28, lower HbA_{1c} and not associated with adverse events. SNP, single nucleotide polymorphism; HAQ, health assessment questionnaire; BMI, body mass index; DAS28, disease activity score in 28 joints; MTX, methotrexate; PG, polyglutamate; HbA_{1c}, glycosylated hemoglobin.

Chapter 1 of this thesis summarizes the main aims and reviews the literature.

In **chapter 2**, the advantages of longitudinal analysis compared to cross-sectional (snapshot) analysis in pharmacogenetic studies in JIA patients were shown. In a prospective cohort of JIA patients, we show that examining treatment response at only 1 point in time is less informative because, at the next hospital visit, a substantial number of responders may have become non-responder and vice-versa. This problem is tackled in the longitudinal approach, since then the results over time of each individual are used.

Therefore, a longitudinal cohort of 287 JIA patients was used in **chapter 3A** to assess associations of single nucleotide polymorphisms (SNP) in genes involved in cellular MTX transport and polyglutamylation in relation to MTX response. The presence of adenosine triphosphate binding cassette transporter (*ABC*) *B1* rs1045642 or *ABCC3* rs4793665 variant genotypes increased the likelihood to become an MTX responder 2-3 fold while for solute carrier (*SLC*) *19A1* rs1051266 the likelihood was decreased 2-3 fold. Therefore, these SNPs may be used in prediction models for MTX non-response.
The relation between SNPs in MTX pathway genes and adverse events 3 months after start of MTX was investigated in **chapter 3B.** The *ABCC3* rs4793665 and *SLC19A1*

rs1051266 SNPs were associated with fatigue in the 287 JIA patients. The same SNPs were also investigated in relation to gastro intestinal events and malaise in a cohort of 387 adult patients with RA. The *ABCB1* rs1045642 SNP was associated with malaise. Unfortunately, it is difficult to compare these results with results from the literature, since adverse events are scored in many different ways. In order to correctly assess the associations of these genetic determinants and adverse events, large collaboration studies are needed with standardized measures for adverse events.

In **chapter 4** was investigated whether baseline one-carbon metabolism biomarkers were associated with non-response and adverse events in RA patients on MTX. Plasma-homocysteine, serum-vitamin B12, serum-folate, erythrocyte-vitamin B6 and erythrocyte-folate were determined at baseline and after three months in the treatment in Rotterdam Early Arthritis Cohort (tREACH, n=285) and the methotrexate in Rotterdam cohort (MTX-R, n=102). Low baseline erythrocyte-folate was associated with high disease activity 3 months after starting MTX in the tREACH and MTX-R cohorts. Therefore, baseline erythrocyte-folate could be used in prediction models for MTX outcome. None of the investigated one-carbon metabolism biomarkers was associated with adverse events.

The association between baseline erythrocyte-folate-polyglutamates (folate-PG) and 3 months erythrocyte-MTX-PGs in RA patients on MTX was investigated in **chapter 5**. Sixty-seven RA patients on MTX therapy were selected from the tREACH and MTX-R cohorts and analyzed for baseline erythrocyte folate-PG and 3 months erythrocyte MTX-PG. Both baseline short chain folate-PG5-7 and medium/long-chain folate-PG6-9 were positively associated with 3 months short chain MTX-PG1 and medium/long chain MTX-PG3-5, respectively. This finding is consistent with the hypothesis that erythrocyte folate is a reflection of the body's capacity to accumulate and retain cellular MTX, which was also investigated in chapter 6.

The aim of **chapter 6** was to define the determinants of 3 months erythrocyte MTX-PG concentrations in RA patients. Ninety-three RA patients from the MTX-R cohort, and 247 from the tREACH were used. This prospective study showed that higher age, higher MTX dose, higher erythrocyte folate status and the *FPGS* rs4451422 wildtype genotype were 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.

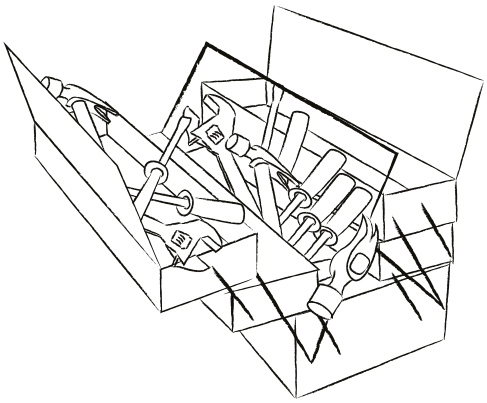
In **chapter 7** was investigated if erythrocyte-MTX-PG concentrations in RA patients are associated with disease activity or adverse events. Hundred and two RA patients from the MTX-R cohort and 285 from the tREACH were used. This first longitudinal study showed that an increase in erythrocyte MTX-PG concentrations was associated with a decreased DAS28 over 9 months in two cohorts, and is therefore a potential tool for therapeutic drug monitoring of MTX in RA. None of the MTX-PGs were associated with adverse events.

The aim of **chapter 8** was to investigate whether treatment with MTX and erythrocyte-MTX-PG concentrations were associated with changes in glycosylated hemoglobin

(HbA_{1c}) in RA patients, compared with other forms of therapy. In the tREACH, patients were randomized into 6 treatment arms. In the MTX-R, treatment was chosen by the physician. MTX treatment and higher erythrocyte-MTX-PG concentrations were associated with decreased HbA_{1c} in RA patients after 3 months therapy. In comparison, Triple DMARD therapy and hydroxychloroquine reduced and glucocorticoids increased HbA_{1c} concentrations.

In **chapter 9** all possible determinants for MTX non-response or adverse events were combined in order to build prediction models for 3 months MTX non-response and adverse events. Clinical characteristics, genetic and metabolic biomarkers involved in the mechanism of action of MTX were determined at baseline in the tREACH (n=285) and MTX-R cohorts (n=102). These variables were used to construct and validate 2 prediction models with disease activity score (DAS)28 >3.2 and 3 or more adverse events as outcome measures. The final prediction model for non-response in the tREACH, included: DAS28>5.1, HAQ>0.6, current smoking, BMI>25 kg/m^2, *ABCB1* rs1045642 genotype, *ABCC3* rs4793665 genotype, and erythrocyte-folate <750 nmol/L. This model satisfactorily identified RA patients with a high risk of non-response to MTX and may be a tool for personalized RA-treatment. None of the investigated variables was significantly associated with adverse events at 3 months and therefore a prediction model for adverse events could not be developed.

In this thesis attempts were made to reach all 5 aims: 1) *ABCB1* rs1045642, *ABCC3* rs4793665 and *SLC19A1* rs1051266 SNPs were identified as possible genetic determinants of MTX non-response in arthritis. *ABCC3* rs4793665 and *SLC19A1* rs1051266 SNPs were identified as possible genetic determinants of fatigue in JIA patients on MTX and the *ABCB1* rs1045642 SNP was identified as possible genetic determinant of malaise in RA patients on MTX. Low baseline erythrocyte-folate was identified as metabolic determinant of non-response of MTX therapy in arthritis; 2) Intracellular MTX-PG concentrations might be an intermediate in the association between low baseline erythrocyte-folate and MTX non-response in arthritis; 3) Higher erythrocyte-MTX-PG concentrations are associated with lower disease activity in RA; 4) MTX-use and higher intracellular MTX-PG concentrations were associated with decrease of HbA_{1c}; 5) Baseline DAS28>5.1, HAQ>0.6, *ABCB1* rs1045642 variant, *ABCC3* rs4793665 variant, baseline erythrocyte-folate<750 nmol/L, current smoking and BMI >25 kg/m^2 were incorporated in a prediction model for 3 months DAS28>3.2.



CHAPTER 12

Samenvatting

SAMENVATTING

Methotrexaat (MTX) is het belangrijkste geneesmiddel voor volwassen reumatoïde artritis (RA) en jeugdreuma dat het ziekteverloop beïnvloedt (disease-modifying antirheumatic drug, DMARD). Toch leidt behandeling met MTX niet altijd tot een goede reductie van de ziekteactiviteit (non-respons) of treden er ernstige bijwerkingen op, waardoor de behandeling gestaakt dient te worden. Patiënten die niet reageren op MTX of bijwerkingen ontwikkelen, krijgen meestal dure geneesmiddelen (biologicals) voorgeschreven. Het voorspellen van welke patiënten onvoldoende reageren op MTX of bijwerkingen ontwikkelen is belangrijk omdat zonder de juiste therapie de artritis in de eerste maanden onomkeerbare gewrichtsschade kan veroorzaken. Om ervoor te zorgen dat alleen patiënten met MTX non-respons dure biologicals voorgeschreven krijgen en patiënten waarbij MTX wel werkt, geen dure biologicals krijgen, moeten deze groepen patiënten voordat ze therapie krijgen geïdentificeerd worden. Om dit te kunnen doen moeten de risicofactoren voor non-respons van MTX en het ontwikkelen van bijwerkingen van MTX geïdentificeerd worden.

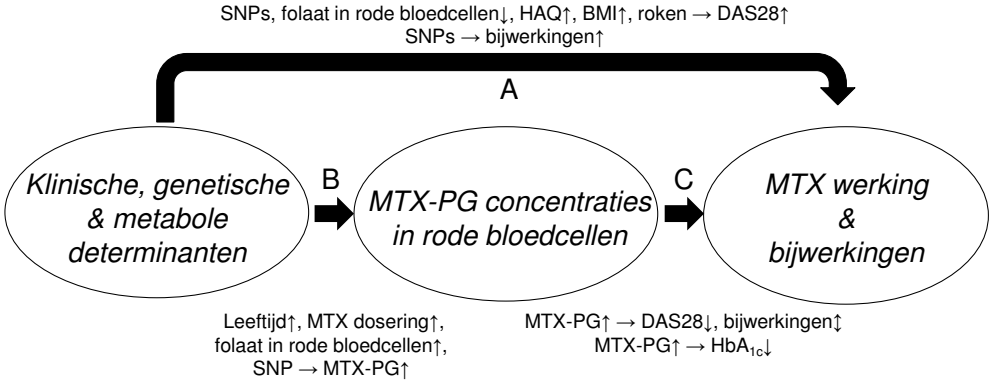
MTX is een folaatantagonist en wordt door dezelfde transporteiwitten de cel in getransporteerd als folaat. Folaat is de werkzame vorm van foliumzuur in het lichaam. In de cel remt MTX een aantal belangrijke enzymen in het folaatmetabolisme. Dit is waarschijnlijk de basis voor het werkingsmechanisme van MTX tegen artritis.

De centrale hypothese in dit proefschrift is dat individuele verschillen of verstoringen in de folaatstofwisseling bepalen of een patiënt niet reageert op MTX of bijwerkingen ontwikkelt door een direct effect op het werkingsmechanisme van MTX of door een indirect effect via de opbouw van MTX-polyglutamaat (MTX-PG) concentraties in de cel.

Het eerste doel was het identificeren van risicofactoren van MTX non-response en bijwerkingen van MTX met als einddoel een voorspelmodel ontwikkelen. Een simpele bloedtest en het beantwoorden van een aantal vragen zou de reumatoloog van een advies kunnen voorzien voor de te kiezen therapie. Een tweede doel van dit proefschrift was het beter begrijpen van het mechanisme tussen de risicofactoren van non-response en bijwerkingen. Dit mechanisme is onderzocht door te kijken naar intracellulaire MTX-PG concentraties als tussenliggende factor die de werking en bijwerkingen van MTX beïnvloeden. Een derde doel van dit proefschrift was het onderzoeken of er een relatie was tussen intracellulaire MTX-PG concentraties, non-response en bijwerkingen van MTX. Een vierde doel was om eventuele positieve metabole neveneffecten van MTX vast te stellen. zoals een positief effect op de glucose homeostase, gemeten door een afname in HbA_{1c}. Voordat alle doelen van dit proefschrift werden onderzocht is eerst gekeken naar de voor en nadelen van longitudinale studies ten opzichte van cross-sectionele studieontwerpen in farmacogenetisch onderzoek bij artritis.

Figuur 1 laat de relaties zien die werden onderzocht in dit proefschrift en de belangrijkste bevindingen. Relatie A is de relatie tussen determinanten en MTX non-response en bijwerkingen. Relatie B zijn de relaties tussen determinanten en

intracellulaire MTX-PG concentraties. Relatie C zijn de relaties tussen MTX-PG concentraties en MTX werking en bijwerkingen.



Figuur 1 De relaties A,B,C die werden onderzocht in dit proefschrift en de belangrijkste bevindingen. A) Bepaalde SNPs, een lager folaat in rode bloedcellen, een hogere HAQ, een hoger BMI en roken zijn geassocieerd met een hogere DAS28. Bepaalde SNPs zijn geassocieerd met bijwerkingen. B) Een hogere leeftijd, hogere MTX dosering, hoger folaat in erytrocyten en een SNP zijn geassocieerd met hogere concentraties MTX-PG. C) Hogere concentraties MTX-PG zijn geassocieerd met een lagere DAS28 en lagere concentratie HbA_{1c} en niet geassocieerd met bijwerkingen. SNP, single nucleotide polymorphism (veel voorkomende puntmutaties in genen); HAQ, Health assessment questionnaire (vragenlijst over gezondheidstoestand); BMI, bodymass index (gewicht-lengte index); DAS28, disease activity score in 28 joints (ziekteactiviteit score in 28 gewrichten); MTX, methotrexaat; PG, polyglutamaat; HbA_{1c}, geglycolyseerd hemoglobine.

In **hoofdstuk 1** worden de hoofddoelen samengevat en wordt er teruggeblikt op de literatuur. In **hoofdstuk 2** worden de voordelen van longitudinale studieontwerpen ten opzichte van cross-sectionele studieontwerpen onderzocht. Dit hoofdstuk laat zien dat in jeugdreuma het voorspellen van de werking van MTX op 1 tijdspunt minder informatief is dan het voorspellen van de werking op meerdere tijdspunten. Dit komt doordat een redelijk aantal patiënten non-responders zijn en bij een volgende keer responders en andersom. Dit probleem is opgelost bij een longitudinaal studieontwerp omdat de resultaten over de tijd van elk individu worden gebruikt.

In **hoofdstuk 3A** zijn de relaties tussen veel voorkomende puntmutaties (single nucleotide polymorphisms, SNP) in genen die betrokken zijn bij het cellulaire transport en polyglutamylering van MTX aan de ene kant en non-response van MTX bij jeugdreuma aan de andere kant onderzocht. Het hebben van een adenosine triphosphate binding cassette transporter (*ABC*) B*1* rs1045642 of *ABCC3* rs4793665 variant genotype verhoogde de kans op het goed werken van MTX. Het hebben van solute carrier (*SLC*) *19A1* rs1051266 variant genotype verlaagde de kans op het goed werken van MTX.

In **hoofdstuk 3B** wordt beschreven dat de *ABCB1* rs1045642 en *SLC19A1* rs1051266 SNPs geassocieerd waren met moeheid. Dezelfde SNPs zijn ook

onderzocht in relatie tot bijwerkingen van MTX therapie bij 387 volwassen patiënten met reumatoïde artritis. In deze groep patiënten was de *ABCB1* rs1045642 SNP geassocieerd met algehele malaise. Om goed uit te zoeken of genetische verschillen een relatie hebben met MTX bijwerkingen zijn grote internationale samenwerkingen nodig met gestandaardiseerde maten voor bijwerkingen.

In **hoofdstuk 4** is onderzocht of de folaatmetabolisme bio merker concentraties: homocysteïne in plasma, vitamine B12 in serum, folaat in serum, vitamine B6 in rode bloedcellen, en folaat in rode bloedcellen, bij start van MTX therapie geassocieerd waren met non-response en bijwerkingen van MTX therapie bij RA in het 'behandeling van het Rotterdam vroege artritis cohort' (treatment in Rotterdam Early Arthritis Cohort, tREACH) van 285 RA patiënten en in het 'methotrexaat in Rotterdam' (MTX-R) cohort van 102 patiënten. Een lagere folaat concentratie in rode bloedcellen voor de start van MTX was geassocieerd met een hogere ziekteactiviteit 3 maanden na de start van MTX. Geen van de onderzochte bio merkers was geassocieerd met bijwerkingen.

De associatie tussen folaat polyglutamaat (folaat-PG) concentraties voor de start van MTX en MTX-PG concentraties in rode bloedcellen 3 maanden na de start van MTX is onderzocht in **hoofdstuk 5**. Hiervoor zijn 67 RA patiënten uit het tREACH en MTX-R cohort gebruikt. Korte keten folaat-PG5-7 voor de MTX start waren geassocieerd met korte keten MTX-PG1. Medium/lange keten folaat-PG6-9 voor de start van MTX waren geassocieerd met medium/lange keten MTX-PG3-5. Deze bevindingen zijn consistent met de hypothese dat folaat concentratie in rode bloedcellen voor de start van MTX een reflectie is van de capaciteit van het lichaam om MTX concentraties cellulair op te bouwen en vast te houden.

Het doel van **hoofdstuk 6** is het definiëren van factoren die MTX-PG concentraties in rode bloedcellen 3 maanden na de start van MTX in RA patiënten beïnvloeden. Drieënnegentig patiënten van het MTX-R cohort en 247 patiënten uit de tREACH werden voor deze studie gebruikt. Een hogere leeftijd, een hogere MTX dosering, hogere folaat concentraties in rode bloedcellen en het hebben van een *FPGS* rs4451422 wildtype genotype waren geassocieerd met hogere MTX-PG concentraties in rode bloedcellen.

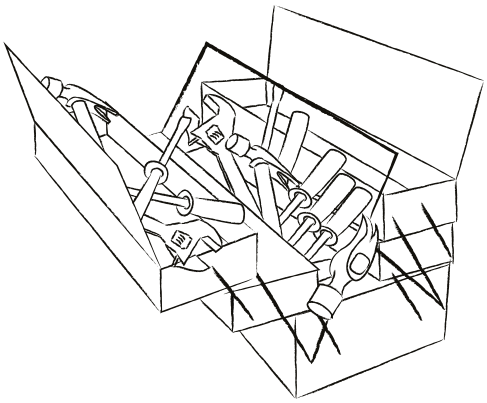
In **hoofdstuk 7** is onderzocht of MTX-PG concentraties in rode bloedcellen in RA patiënten waren geassocieerd met ziekteactiviteit of bijwerkingen van MTX. Honderdtwee patiënten uit het MTX-R cohort en 285 patiënten uit de tREACH werden voor deze studie gebruikt. Hogere MTX-PG concentraties in rode bloedcellen waren geassocieerd met een lagere ziekteactiviteit over 9 maanden MTX gebruik. Geen van de MTX-PG concentraties was geassocieerd met bijwerkingen van MTX.

Het doel van **hoofdstuk 8** is het onderzoeken of MTX gebruik en MTX-PG concentraties in rode bloedcellen waren geassocieerd met veranderingen in de concentraties van geglycoliseerd hemoglobine (HbA_{1c}) vergeleken met andere therapieën bij RA patiënten. In de tREACH werden patiënten gerandomiseerd in 6 behandelarmen. In het MTX-R cohort werd de therapie gekozen door de reumatoloog. MTX gebruik en hogere MTX-PG concentraties in rode bloedcellen waren geassocieerd

met een afname van HbA_{1c} concentraties na 3 maanden therapie in beide cohorten. Therapie met 3 DMARDs en therapie met alleen hydroxychloroquine verlaagden HbA_{1c} concentraties. Therapie met glucocorticosteroïden verhoogde HbA_{1c} concentraties.

In **hoofdstuk 9** werden klinische, genetische en metabole determinanten bepaald voor de start van MTX in de tREACH (n=285) en het MTX-R cohort (n=102) voor het maken van een voorspelmodel voor MTX non-respons en bijwerkingen. DAS28>5.1, vragenlijst over gezondheidstoestand (health assessment questionnaire, HAQ) score groter dan 0.6, roken, gewicht-lengte index (bodymass index, BMI) groter dan 25 kg/m^2, *ABCB1* rs1045642 genotype, *ABCC3* rs4793665 genotype en folaat in rode bloedcellen concentratie lager dan 750 nmol/L werden geïncludeerd in het uiteindelijke voorspelmodel voor DAS28<3.2 na 3 maanden MTX therapie. Dit voorspelmodel voorspelde goed welke patiënten een hoge kans hadden om een hoge ziekteactiviteit te ontwikkelen na 3 maanden MTX therapie. Geen van de onderzochte variabelen was significant geassocieerd met bijwerkingen 3 maanden na het starten met MTX. Daarom kon er voor bijwerkingen geen voorspelmodel ontwikkeld worden.

In dit proefschrift zijn pogingen ondernomen om alle 5 de doelen te realiseren: 1) *ABCB1* rs1045642, *ABCC3* rs4793665 en *SLC19A1* rs1051266 SNPs zijn geïdentificeerd als mogelijke genetische risicofactoren voor het niet werken van MTX bij artritis. *ABCC3* rs4793665 en *SLC19A1* rs1051266 SNPs zijn geïdentificeerd als mogelijke risicofactoren voor moeheid bij jeugdreuma patiënten na MTX therapie. De *ABCB1* rs1045642 SNP was geïdentificeerd als mogelijke risicofactor voor algehele malaise bij RA patiënten met MTX therapie. Lage folaat concentratie in rode bloedcellen is geïdentificeerd als metabole risicofactor voor het niet werken van MTX therapie bij RA; 2) Intracellulaire MTX-PG concentraties zouden een tussenliggende factor kunnen zijn in de associatie tussen lage folaat concentraties in rode bloedcellen voor MTX start en het minder goed werken van MTX 3 maanden na de MTX start; 3) Hogere MTX-PG concentraties in rode bloedcellen zijn geassocieerd met een lagere ziekteactiviteit; 4) MTX gebruik en hogere intracellulaire MTX-PG concentraties zijn geassocieerd met een afname van HbA_{1c} na 3 maanden MTX therapie; 5) DAS28>5.1, HAQ>0.6, *ABCB1* rs1045642 genotype, *ABCC3* rs4793665 genotype, folaat in rode bloedcellen<750 nmol/L, roken en een BMI>25 Kg/m^2 zijn geïncludeerd in een voorspelmodel voor een DAS28>3.2 op 3 maanden na de start van de MTX therapie.



ADDENDUM

List of Abbreviations

PhD Portfolio

Dankwoord

LIST OF ABBRIVIATIONS

ABCB1	Adenosine triphosphate-binding cassette transporter B1
ACRped30	American College of Rheumatology 30% criteria for response
ADA	Adenosine deaminase
ADORA2A	Adenosine A2A receptor
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
ALAT	Alanine-aminotransferase
AMPD1	Adenosine monophosphate deaminase 1
ASAT	Aspartate-aminotransferase
ATIC	5-aminoimidazole-4-carboxamide ribonucleotide transformylase
ATP	Adenosine triphosphate
AUC	Area under the curve
BCRP	Breast cancer resistance protein
BMI	Mody mass index
CCP	cyclic citrullinated peptide
CHAQ	Child health assessment questionnaire
CRP	C-reactive protein
DAS28	Disease activity score in 28 joints
DHF	Dehydrofolate
DHFR	Dihydrofolate reductase
DM	Diabetes mellitus
DMARD	Disease-modifying antirheumatic drug
eGFR	Estimated glomerular filtration rate
ESI	Electrospray ionizaion
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate
EULAR	European league against rheumatism
FOLR1	Folate receptor 1
FPGS	Folyl-polyglutamate synthetase
FPRP	False-positive report probability
GC	Glucocorticoid
GGH	Gamma-glutamyl hydrolase
GI	Gastrointestinal
HAQ	Health assessment questionnaire
HbA_{1c}	Glycosylated hemoglobin
HCQ	Hydroxychloroquine
HWE	Hardy-Weinberg equilibrium
ILAR	International league of associations for rheumatology
IMPDH	Inosine-5'-monophosphate dehydrogenase
IR	Interquartile range

ITPA	Inosine triphosphatase
JIA	Juvenile idiopathic arthritis
LC-MS/MS	Liquid chromatochraphy-tandem mass spectrometry
MAF	Minor allele frequency
MCAR	Missing completely at random
MDR	Multifactor dimensionality reduction
MDRD	Modification of diet in renal disease
MRP1	multidrug resistance protein 1
MTHFD	Methylenetetrahydrofolate-dehydrogenase
MTHFR	5,10-methylenetetrahydrofolate reductase
MTRR	Methionine synthase reductase
MTX	Methotrexate
MTX-R	Methotrexate in Rotterdam cohort
NCBI	National center for biotechnology information
NPV	Negative predictive value
NSAID	Non-steroidal anti-inflammatory drug
PCFT	Protein coupled folate transporter
PG	Polyglutamate
PGA	Physician global assessment of disease acivity
P-gp	P-glycoprotein
PPV	Positive predictive value
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RFC	Reduced folate carrier
ROC	Receiver operating characteristics
rs	Reverence single nucleotide polymorphism number
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SHMT	Serine-hydroxymethyltransferase
SJC	Swollen joint count
SLC19A1	Solute carrier 19A1
SNP	Single nucleotide polymorphism
TDM	Therapeutic drug monitoring
TDT	Triple DMARD therapy
THF	Tetrahydrofolate
TJC	Tender joint count
TNF	Tumor necrosis factor
tREACH	Treatment in Rotterdam early arthritis cohort
TS	Thymidylate synthase
VAS	Visual analogue scale

PHD PORTFOLIO

PhD candidate:	Maurits Calixtus Franciscus Johannes de Rotte
Department:	Erasmus MC, Clinical chemistry
Research schools:	Netherlands institute for Health sciences (NIHES)
	Erasmus Postgraduate school Molecular Medicine (MolMed)
PhD period:	April 2008 – september 2014
Promotores:	Prof.dr. J. Lindemans
	Prof.dr. J.M.W. Hazes
Copromotor:	dr. R. de Jonge

PhD education

Basic and translational endocrinology, MolMed 2013
English biomedical writing and communication, MolMed 2012
Courses for the Quantitative Researcher, NIHES 2009
Modern Statistical Methods, NIHES 2008
LC-MS introduction course, Avans-plus 2008
Genome wide association analysis, NIHES 2008
Large-scale multicenter studies, NIHES 2008
Genomics in molecular medicine, NIHES 2008
Principles of Genetic Epidemiology, NIHES 2008
Principles of research in medicine and epidemiology, NIHES 2008

Scientific meetings

Nederlandse Vereniging voor Klinische Chemie, poster, Veldhoven Netherlands 2014
European League Against Rheumatism, poster, Madrid,Spain 2013
Nederlandse Vereniging voor Klinische Chemie, 2 posters, Veldhoven 2013
Molecular Medicine Day, poster, Rotterdam Netherlands 2013
American Congress of Rheumatology, 2 posters, Washington USA 2012
European League Against Rheumatism, poster, Rome Italy 2010
Molecular Medicine Day, poster, Rotterdam 2010
European League Against Rheumatism Congress, poster, Copenhagen Denmark 2009
Nederlandse Vereniging voor Klinische Chemie oral (abstract-price), Veldhoven 2009

Foreign visit

Determination of folate-polyglutamates at Tufts University Boston USA 2008

Teaching

Supervising graduation, laboratory technician student, Hogeschool, Rotterdam 2013
Supervising graduation, laboratory technician student, Avans Hogeschool, Breda 2012
classes clinical chemistry, students, technicians, physicians, Erasmus MC 2010-2014

About the author

Maurits de Rotte was born on December 11, 1980 in Utrecht, the Netherlands. After graduating high school in 1999 at the Rijnlands Lyceum in Oegstgeest, he worked and traveled for a year in Australia, New Zeeland and the United States of America.
In 2000 he started his study in pharmacy at the faculty of mathematics and natural sciences of the Rijksuniversiteit Groningen. He received his bachelor degree in pharmacy in 2004. He performed his graduate thesis at the Cincinnati Children's Hospital Medical Center, Ohio USA in 2006 on the subject: Determination of inosine 5'-monophosphate dehydrogenase activity in isolated mononuclear cells of pediatric renal transplant recipients following administration of mycophenolate mofetil. Additionally he followed a pharmaceutical training at the public pharmacy in Assen and at the hospital pharmacy of the Erasmus MC in Rotterdam. Subsequently he received his master degree in pharmacy (PharmD) in 2007. In 2008 he started to work on the research project which is described in this thesis at both the departments of clinical chemistry and rheumatology of the Erasmus MC, under the supervision of prof.dr. J. Lindemans, prof.dr. J.M.W. Hazes and dr. R. de Jonge. In 2010 he started his residency in clinical chemistry at the Erasmus MC which included an internship at the Reinier de Graaf hospital in Delft. He will finish his training in clinical chemistry in 2015 and thus become a registered laboratory specialist in clinical chemistry. Maurits lives in Rotterdam and is married to Florentien de Steenwinkel.

Over de auteur

Maurits de Rotte werd op 11 december 1980 in Utrecht geboren. In 1999 deed hij eindexamen op het Rijnlands Lyceum in Oegstgeest. Aansluitend ging hij een jaar werken en reizen in Australië, New Zeeland en de Verenigde Staten van Amerika.
Hij startte met de studie farmacie aan de faculteit der wiskunde en natuurwetenschappen aan de Rijksuniversiteit Groningen in 2000. In 2004 ontving hij zijn bachelor diploma in de farmacie. Zijn master onderzoek deed hij op het Cincinnati children's hospital medical center, Ohio VS in 2006 op het onderwerp: Determination of inosine 5'-monophosphate dehydrogenase activity in isolated mononuclear cells of pediatric renal transplant recipients following administration of mycophenolate mofetil. Na de opleiding tot apotheker bij een openbare apotheek in Assen en de ziekenhuisapotheek van het Erasmus MC in Rotterdam ontving hij zijn master in de farmacie en werd apotheker in 2007. In 2008 startte hij het onderzoeksproject dat is beschreven in dit proefschrift bij de afdelingen klinische chemie en reumatologie van het Erasmus MC, onder begeleiding van prof.dr. J. Lindemans, prof.dr. J.M.W. Hazes en dr. R. de Jonge. In 2010 startte hij met de opleiding tot klinisch chemicus bij het Erasmus MC met modules bij het Reinier de Graaf ziekenhuis in Delft. Hij zal deze opleiding afronden in 2015 en dan laboratoriumspecialist klinische chemie worden. Maurits woont in Rotterdam en is getrouwd met Florentien de Steenwinkel.

List of publications

This thesis

E. den Boer, **M.C.F.J. de Rotte,** S.M.F. Pluijm, S.G. Heil, J.M.W. Hazes, R. de Jonge. Clinical, metabolic and genetic determinants of erythrocyte methotrexate-polyglutamate concentrations at 3 months of treatment in rheumatoid arthritis. *Journal of Rheumatology, (2014) In press.*

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Other research
M. Bulatović Ćalasan, E. den Boer, **M.C.F.J. de Rotte**, S.J. Vastert, S.S.M. Kamphuis, R. de Jonge, N.M. Wulffraat. Methotrexate polyglutamates in erythrocytes are associated with lower disease activity in juvenile idiopathic arthritis patients. *Annals of the Rheumatic Diseases (2013) Epub ahead of print.*

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We become what we behold. We shape our tools, and thereafter our tools shape us.
(Marshall McLuhan)