TAILOR-MADE METHOTREXATE TREATMENT IN JUVENILE IDIOPATHIC ARTHRITIS: AN ESTABLISHED DRUG REVISITED

Maja Bulatović Ćalasan

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METHOTREXAAT BEHANDELING OP MAAT BIJ JEUGDREUMA:
EEN NIEUWE KIJK OP EEN BEKEND MEDICIJN

(met een samenvatting in het Nederlands)

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Maja Bulatović Ćalasan

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Prof.dr. N.M. Wulffraat

Co-promotoren: Dr. R. de Jonge
Dr. F. van Wijk

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Za moje roditelje

For my parents

CONTENTS

1

GENERAL INTRODUCTION

"Two roads diverged in a wood, and I –
I took the one less travelled by,
And that has made all the difference"
Robert Frost, The Road not Taken

Juvenile idiopathic arthritis

Juvenile idiopathic arthritis is one of the most common autoimmune diseases in childhood with a reported prevalence between 16 and 150 per 100.000.[1] JIA is a heterogeneous disease, characterized by chronic inflammation of one or more joints, which begins before the age of 16, persists for more than 6 weeks and is of unknown origin.[1,2] It encompasses various subtypes, defined by the International League of Associations for Rheumatology (ILAR) criteria, whose severity and clinical course differ.[2,3] In persistent oligoarticular JIA, a maximum of 4 joints is affected in the first 6 months of disease, usually involving the larger joints of the legs, with the knee being the most affected followed by the ankles. This JIA subtype can have a mild, even self-limiting course, whereas extended oligoarticular JIA, affecting more than 4 joints after the first 6 months of disease, can have a more severe outcome, thus resembling polyarticular JIA. Polyarticular JIA, which is subdivided into rheumatoid factor positive and negative, affects 5 or more joints within the first 6 months of disease, involving not only the large joints, but also the small joints of hands and feet. This subtype displays a severe and progressive course of disease. Furthermore, psoriatic JIA is characterized by psoriatic rash and arthritis of both small and large joints, sometimes progressing to involve the sacroiliac joint. Similarly to psoriatic JIA, enthesitis-related JIA also affects the sacroiliac joint next to joints of lower extremities, with its main feature being enthesitis. Finally, systemic JIA is characterized by arthritis in one or more joints accompanied by extra-articular systemic features, such as fever, rash and serositis; this subtype can have a very severe disease course leading to substantial joint destruction.[1] Although heterogeneous, the common denominator in JIA is chronic arthritis, which can lead to joint destruction and long-term disabilities[1,2,4] and in turn a heavy toll on children, their parents and society.[5,6] Such serious consequences put the aim of attaining tight disease control in JIA to the forefront.

Treatment

In the past 15 years, a myriad of therapeutic options has made it possible to realize the aim of complete disease control in JIA, thus probably improving the long-term prognosis of the disease.[1] The therapeutic arsenal includes the initial treatment with non-steroidal inflammatory drugs followed by intra-articular corticosteroid joint injections, which is sometimes sufficient to gain disease control in oligoarticular JIA patients. In polyarticular and systemic JIA, a new class of drugs, the so-called biologicals, which target the cytokine mediators (Tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and IL-6, reviewed further in Chapter 2) of the

inflammatory process, have gained an important place in their treatment.[7-15]Although new effective treatments are emerging, the well-established disease modifying anti-rheumatic drug Methotrexate (MTX) still remains the anchor drug playing a central role in the treatment of almost all JIA subtypes.[16,17]

MTX as cornerstone treatment in JIA

Efficacy and safety of MTX has first been established in RA.[18-21] In JIA, 4 and 10 years after FDA's approval of MTX in RA, the efficacy of low-dose MTX was confirmed in polyarticular, extended oligoarticular and systemic JIA patients, and the dose of MTX with greatest effectiveness (15 mg/m^2/week) in polyarticular JIA patients was established in three randomized controlled trials (RCT).[22-24] No trials have been conducted in persistent oligoarticular JIA, even though this subtype is also treated with MTX. MTX leads to significant disease control or even disease remission in up to 70% of JIA patients.[16,22,23,25,26] Furthermore, 50% remains in drug-free remission for more than 2 years upon MTX discontinuation.[25] Interestingly, oral MTX has never officially been approved by the Food and Drug administration (FDA) or European medicines agency (EMA) as a therapy for JIA. On October 14th 2013 the FDA finally approved subcutaneous MTX for polyarticular JIA. Nevertheless, MTX became the cornerstone treatment in the therapeutic management of JIA.[16] What is even more, MTX remained the cornerstone treatment in JIA, in spite of the emergence of biologicals.[16] In fact, MTX is taken by many more patients than biologicals, including those on biologicals, since the combination treatment with MTX increases effectiveness of biologicals.[27-30] Furthermore, in head-to-head comparisons between biologicals and MTX in new-onset untreated RA patients, MTX was similarly efficacious as TNFα inhibitors.[27-30] In JIA, however, studies comparing biologicals and MTX head-to-head have not been conducted.

Step-up treatment approach and paradigm shift

The central role of MTX in the treatment in JIA is mirrored in the recent American College of Rheumatology recommendations.[16,17] The ACR recommendations put forward treatment algorithms for 5 treatment groups, where each treatment group includes several ILAR JIA subtypes: a) patients with a history of arthritis of 4 or fewer joints, b) 5 or more joints, c) active sacroiliac arthritis, d) systemic arthritis without systemic features and varying degrees of active arthritis, and e) systemic arthritis with active systemic features and varying degrees of synovitis (Table 1). In all, except the last treatment group, MTX is initiated either after NSAIDs and glucorticoid injections (in group 1) or at the time of diagnosis in conjuction with NSAIDs and joint injections (in groups 2 and 4), followed by addition of biologicals (TNFα inhibitor for all groups and anakinra/canakinumab (IL-1 (receptor) antagonist), tocilizumab (IL-6 antagonist) or abatacept (T cell co-stimulation modulator) for systemic JIA) after on average 3 to 6 months of MTX monotherapy in those with an insufficient response to MTX (Table 1). Indeed, in 30-40% of

JIA patients, MTX is not sufficiently effective.[23-25,31] These recommendations follow the accepted treatment philosophy in JIA, in which therapy is escalated in a step-wise fashion, starting with MTX and adding biologicals only in those patients unresponsive to MTX. Over the past years, however, changes in the treatment mentality of paediatric rheumatologists ensued, prompted by the need to establish early disease control and prevent irreversible joint damage[32-34], thus resulting in a lower threshold to start early combination treatment with biologicals.

This novel paradigm led to a new generation of clinical trials of early aggressive therapy in JIA comparing MTX with combination therapy of MTX and TNFα inhibitors (but not with TNFα inhibitors only) in MTX-naïve polyarticular JIA patients, rather than in patients with a poor MTX response.[35,36] Although the aggressive combination therapy was more effective in reaching clinically inactive disease (without statistical significance in one trial) than MTX alone, MTX monotherapy was still very efficacious in attaining clinically inactive disease or low disease activity in the fist 6 months of therapy.[35,36] Therefore, in order to establish fast disease control, early treatment with biologicals is not always obligatory and for many patients MTX monotherapy does suffice. In fact, the use of biologicals in all patients (in combination with MTX), no matter how effective, is not plausible nor desirable due to their high costs[6] (reviewed in Chapter 2), potentially serious adverse effects, which could include development of inflammatory bowel disease and malignancies such as leukaemia and lymphoma[37-39] and inevitable over-treatment of patients who would have benefited from MTX monotherapy. At the same time, some patients receiving MTX monotherapy could be under-treated and may indeed necessitate early treatment with biologicals. The first step towards such tailor-made treatment decisions is optimization of the use of anchor drug MTX.

Table 1. American College of Rheumatology treatment recommendations

Treatment group	JIA subtype	Methotrexate	Biological at 3 months	Biological at 6 months	Poor prognostic factors (PPF)
Arthritis of 4 or fewer joints*	Persistent oligoarthritis Psoriatic Enthesitis-related Undifferentiated	DA moderate PPF present DA high PPF irrespective	*TNFα inhibitor:* DA moderate/high PPF present	*TNFα inhibitor:* DA high PPF irrespective	Arthritis of hip/cervical spine Arthritis of wrist/ankle or inflammatory marker elevation Radiographic damage
Arthritis of 5 or more joints†	Persistent oligoarthritis Polyarthritis (RF^+ and RF^-) Psoriatic Enthesitis-related Undifferentiated	DA high PPF irrespective DA moderate PPF present	*TNFα inhibitor:* DA high PPF irrespective	*TNFα inhibitor:* DA low PPF irrespective *2nd TNFα inhibitor (4 months after 1st):* DA moderate/high PPF irrespective	Arthritis of hip/cervical spine Positive RF Radiographic damage
Active sacroiliac arthritis‡	Psoriatic Enthesitis-related		*TNFα inhibitor:* DA high PPF present (upon NSAID) DA high/moderate PPF irrespective/present (upon MTX)	*TNFα inhibitor:* DA moderate PPF absent	Radiographic damage of any joint
Systemic arthritis with active systemic features and varying degrees of synovitis	Systemic JIA		Initial treatment: Anakinra (NSAIDs/systemic glucocorticoids)	Continued treatment: Anakinra Canakinumab Tocilizumab MTX TNFα inhibitor	Choice of therapy depending on number of active joints and physician global assessment

Systemic arthritis without systemic features in varying degrees of synovitis[&]	Systemic JIA	Active joints >4	Abatacept Anakinra TNFα inhibitor Tocilizumab	Abatacept Anakinra TNFα inhibitor Tocilizumab	Choice of therapy depending on number of active joints and physician global assessment

*Start with NSAID and/or glucocorticoid injections. NSAID monotherapy may be sufficient for patients with low DA and no PPF.
[†]Start with NSAID for 1 or 2 months, if DA low and PPF present or DA moderate or PPF irrespective. Otherwise, MTX is started immediately.
[‡]Initiation of TNFα inhibitors is more readily recommended in these patients.
[&]In case of >4 active joints, NSAID treatment can also be initiated. In case of <4 active joints, joint injections can be initiated. The continued treatment can then be done with MTX as well.
Disease activity levels are defined as low, moderate and high using combinations of erythrocyte sedimentation rate or C-reactive protein levels, physician global assessment of disease activity (0-10 scale) and patient/parent global assessment of well-being (0-10 scale). For more details see ACR recommendations.
Abbreviations: JIA: juvenile idiopathic arthritis; TNFα: tumor-necrosis factor α; DA: disease activity; PPF: poor prognostic factor; NSAID: non-steroidal anti-inflammatory drug; MTX: methotrexate

Methotrexate revisited – addressing the unmet needs

Optimization of MTX use can be accomplished if the unmet needs of this well-established drug are addressed. A crucial unmet need is the lack of tools to identify patients who will be responsive to MTX and will therefore benefit from MTX monotherapy, and those who will be partially responsive or unresponsive to MTX, thus requiring fast MTX dose escalation or addition of biologicals, early in the disease course. Recognizing the need for tailor-made treatment, namely giving the most appropriate treatment to individual patients based on prognostic factors (Table 1), the ACR recommendations defined levels of disease activity (low, moderate and high) and prognostic features, which allow for more rapid escalation or skipping of certain treatment levels in patients with high disease activity and poor prognostic features[16] (Table 1). In line with tailor-made disease control, ACR recommendations were updated specifically for systemic JIA – a disease that is poorly sensitive to anti-TNF drugs, to include new anti-IL-1 and anti-IL-6 treatments.[17] The abovementioned prognostic features do not necessarily reflect MTX response-specific prognostic factors. Moreover, features of poor prognosis included involvement of ankle, wrist, hip or cervical spine and radiographic changes for active arthritis, which occur late in the disease course.[40] Tailor-made therapy, however, should be based on objective predictors, such as gene polymorphisms in transporters and enzymes of the MTX metabolic pathway (reviewed in Chapter 2, Figure 1), effectors of MTX response like MTX polyglutamates or more general immunological biomarkers such as cytokine profiles, which are present before MTX start or early in the disease course thus enabling prediction of MTX response. In order to optimize treatment with MTX, not only MTX's efficacy, but also MTX's adverse effects should be addressed. Although MTX is considered to be a safe drug in JIA, with serious adverse effects such as bone marrow suppression and hepatotoxicity being rare, the use of MTX may be compromised by the most common MTX-related adverse effect, namely gastrointestinal intolerance.[4] Therefore, identifying the scope as well as effective treatment and preventive strategies of gastrointestinal intolerance is crucial to attain disease control in a tailor-made fashion. In spite of MTX's firmly established clinical efficacy in JIA, MTX's suppressive or modulatory effects on the immune system (reviewed in Chapter 2), specifically on T cells *in vivo*, remain poorly elucidated. Addressing this unmet need could provide not only unique insights on MTX's mechanism of action, but also on immunological predictors of (non)-response. Taken together, addressing the abovementioned unmet needs has the potential not only to tailor MTX treatment, but also to tailor JIA treatment in general, thus enabling early disease control and preventing joint damage and long-term disabilities.

SCOPE AND OUTLINE OF THE THESIS

In this thesis, we study MTX treatment response, gastrointestinal intolerance, as the most common MTX-induced adverse effect in JIA, and MTX's effects on T cells, and we investigate novel tools for steering tailor-made therapeutic decisions in JIA. **Chapter 2** addresses the history of MTX, establishment of clinical efficacy and use in JIA and RA in the era of biologicals as well as MTX's mechanism of action. This chapter also discusses the unmet needs of this well-established drug and how their resolution could lead to optimization of MTX treatment in JIA, which is further scrutinized in the rest of this thesis. In **Chapter 3**, genetic determinants, namely single nucleotide polymorphisms (SNPs) in MTX efflux and influx transporters, associated with MTX response are identified. **Chapter 4** goes a step further and transforms associations of SNPs with MTX efficacy into as prediction model for MTX (non)-response, as a potential tool for optimizing MTX treatment in JIA. **Chapter 5** investigates association of disease activity in JIA patients on MTX, determined with the Juvenile Arthritis Disease Activity Score (**Chapter 6**), with intracellular MTX polyglutamates (MTX-PGs), and discusses the applicability of MTX-PGs as a therapeutic drug monitoring tool. The second part of this thesis focuses on pre- and post-treatment gastrointestinal adverse effects, the so-called MTX intolerance. **Chapter 7** and **Chapter 8** explore the nature and the prevalence of these adverse effects in JIA and RA and discuss differences with respect to MTX intolerance between the two populations. **Chapter 9** explores the treatment options for MTX intolerance in a randomized controlled trial, whereas **Chapter 10** provides a prediction tool for MTX intolerance. **Chapter 11** investigates quantitative and qualitative effects of MTX on regulatory and effector T cells in JIA patients during MTX treatment. Finally, **Chapter 12** discusses the future prospective and possible consequences of findings described in this thesis for tailor-made (MTX) treatment in JIA.

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2

TRANSLATIONAL MEDICINE FROM BEDSIDE TO BENCH AND BACK AGAIN: METHOTREXATE REVISITED

Maja Bulatović Ćalasan[1,2], Rogier M. Thurlings[2,3], Nico M. Wulffraat[1] and Berent J. Prakken[1,2]

[1] Center for Molecular & Cellular Intervention (CMCI), Department of Pediatric Immunology, University Medical Centre Utrecht, Wilhelmina Children's Hospital, Utrecht, The Netherlands; [2]Eureka Institute for Translational Medicine, Siracusa, Italy; [3]Academic Medical Centre Amsterdam, Department of Clinical Immunology and Rheumatology, Amsterdam, The Netherlands

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ABSTRACT

Translational medicine efforts, geared towards the development of new drugs, have brought numerous biologicals from the bedside of rheumatoid and juvenile idiopathic arthritis patients to bench. Biologicals took the attention of the pharmaceutical industry, as well as rheumatologists, away from the first drug that advanced the treatment of rheumatic diseases – methotrexate (MTX). As a consequence, crucial unmet needs surrounding MTX still remain poorly elucidated, even though this anchor drug is taken by many more patients than biologicals and its efficacy has not consistently been surpassed by biologicals in MTX-naive patients. These unmet needs include an incomplete understanding of anti-inflammatory actions of MTX and the inability to predict MTX response. Addressing these needs will result not only in optimization of MTX use, but also of biological use, leading to personalized tailor-made therapy. This review will discuss the place of MTX in juvenile idiopathic arthritis, illustrated by examples from rheumatoid arthritis, and translational efforts to address its unmet needs.

INTRODUCTION

Translational medicine can be defined as a continuum of activities going from the conception of an idea to advanced clinical testing and, ultimately, the development of a new medical technology or drug.[1] Translational medicine also comprises the reverse route, notably translation of observations in patients (i.e., using an already existing drug), to further investigations in the laboratory. Nevertheless, most attention is focused on the translational route leading to the development of a new drug. A successful example of such translational medicine efforts in rheumatic diseases was the introduction of new biologic drugs, which represented a major advancement in the treatment of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) patients. Since the development of biologicals, the well-established disease-modifying antirheumatic drug (DMARD) methotrexate (MTX) was no longer the only treatment of RA and JIA. Moreover, biologicals were placed in the spotlight by both pharmaceutical companies that developed them and by adult and pediatric rheumatology communities. More recently, rheumatologists started to advocate early treatment with biologicals, with the aim of achieving tight disease control very early on during the disease course.[2–5] In addition, much research and funding is directed towards investigating their mechanism of action, efficacy and (long-term) safety in large patient registries.[6] The focus on biologicals is, therefore, diverting research and funding away from MTX, which is undesirable for several reasons. MTX, which once advanced the treatment of rheumatic diseases, is still an anchor drug, taken by many more patients than biologicals, including those on biologicals. Moreover, in head-to-head comparisons in RA, MTX is similarly efficacious to TNF-α inhibitors and it increases the effectiveness of most biologicals.[7–12] In spite of these merits and the use of MTX for decades, several crucial aspects of this drug have remained poorly elucidated. Although efficacious, the mecha nisms that govern the anti-inflammatory func tions of MTX in rheumatic diseases are still not completely understood. Moreover, large patient registries to monitor long-term safety of MTX compared with that of biologicals are lacking. In addition, which patients will respond to MTX and which patients will be unresponsive cannot be reliably predicted before starting the therapy. Consequently, it is unclear which patients warrant the addition of biologicals and what the right time would be to add them to MTX. Therefore, MTX needs to be revisited using the reverse route of translational medicine to bring MTX from the patient's bedside back to bench in order to address the abovementioned unmet needs. This review will focus on the current place of MTX in the treatment of JIA, supported by illustrative examples from RA, as well as the current and future translational efforts to address the abovementioned unmet needs of MTX.

The road from MTX to biologicals

MTX represented the first major advancement in the treatment of rheumatic diseases. As such MTX gained the leading role in the management of both RA and JIA. This section will discuss: the establishment of MTX as an anchor drug; the use of MTX in the era of biologicals and its efficacy compared with biologicals; and the paradigm shift towards early aggressive therapy with biologicals and in this context, the importance of addressing the unmet need of MTX, namely prediction of MTX response.

Introduction & establishment of MTX as an anchor drug

Aminopterin, a folic acid antagonist similar to MTX, was developed at the beginning of 1950s as an antineoplastic drug for the treatment of malignancies, in particular childhood leukemia. Aminopterin's ability to inhibit connective tissue proliferation led to its first effective administration in psoriasis, psoriatic arthritis (PsA) and RA in 1951.[13] Modifications of aminopterin struc ture, allowing easier production, led to development of amethopterin, better known as MTX.[13] In 1962, the positive effects of MTX on RA, and in particular on PsA, were first reported.[14] Although MTX was extensively studied and used in the treatment of psoriasis, where it became the standard-of-care, the rheumatologists' interest in MTX lagged behind. Finally, in 1972 a beneficial effect of low-dose intramuscular MTX was shown in 29 RA patients, followed by various open-label studies.[13,14]

Encouraging evidence generated from these uncontrolled studies prompted the performance of four placebo-controlled trials in RA (Table 1).[15–18] These randomized trials, conducted in 1984 and 1985, were instrumental for the approval of MTX as a therapy for RA by the US FDA in 1988. Subsequent randomized placebo-controlled trials, which compared the short and long-term efficacy of MTX with older DMARDs, such as azathioprine and gold compounds, showed that MTX was more efficacious and less toxic.[14] MTX was a big step forward for the treatment of RA, and is now recommended and used as the first-choice DMARD in the management of RA.[19] The introduction of MTX in pediatric diseases lagged far behind. The first report on the use of MTX in JIA appeared in 1986 [20]. Two randomized controlled trials (RCTs), 4 and 10 years after the FDA's approval of MTX in RA, established the efficacy of low-dose MTX in polyarticular, extended oligoarticular and systemic JIA patients (Table 1).[21,22] In 2004, a multicenter RCT in polyarticular JIA, unresponsive to standard doses of oral MTX (8–12.5 mg/m^2 per week), compared efficacy and safety of the intermediate parenteral MTX dose (15 mg/m^2 /week) with a higher dose (30 mg/m 2 /week) and found that MTX exerted its maximum therapeutic effect at 15 mg/m 2 /week, whereas a further increase to 30 mg/m 2 /week did not improve its efficacy.[23] In spite of MTX's efficacy in JIA, MTX has never officially been FDA or EMA approved as a therapy for JIA. Nevertheless, MTX is now the cornerstone treatment in the therapeutic management of JIA.[24]

Table 1. Landmark randomized controlled trials of methotrexate in rheumatoid arthritis and juvenile idiopathic arthritis

Study (year)	Study design	Study duration (months)	Patients	Sample size (n)	MTX dose (mg/week)[†]	Outcome	Ref.
RA							
Thompson *et al.* (1984)	MTX vs. placebo (parallel)	6	Established[‡] RA	48	Group1: 10 Group 2: 25	Significant improvement in joint pain/tenderness/ swelling indices in both groups compared to placebo	15
Weinblatt *et al.* (1985)	MTX vs. placebo (cross-over)	24	Established RA	28	7.5-15	50% improvement in joint pain/tenderness index and swelling index in 54% and 39% patients, respectively	16
Williams *et al.* (1985)	MTX vs. placebo (parallel)	18	Established RA	189	7.5-15	50% improvement in joint pain/tenderness index and swelling index in 32% and 21% patients, respectively	17
Andersen *et al.* (1985)	MTX vs. placebo (cross-over)	13	Established RA	12	-	Significant improvement in joint tenderness/swelling indices compared to placebo	18
JIA							
Giannini *et al.* (1992)	MTX vs. placebo (parallel)	6	Established polyarticular JIA (including systemic JIA)	127	Group 1: 5 Group 2: 10	Significant improvement in joint severity score and physician/parent GA only with higher MTX dose in 63% compared to 32% in the placebo group	22
Woo *et al.* (2000)	MTX vs. placebo (cross-over)	4	Established systemic and extended oligoarticular JIA	88	15-20	Significant improvement with MTX compared to placebo – ACR30 reached in 73% of patients on MTX vs. 34% of patients on placebo. In systemic JIA, poor effect on systemic symptoms	21

[†] In JIA patients, MTX dose is expressed in mg/m^2/week.
[‡] Established disease is refractory to NSAIDs and/or other disease modifying anti-rheumatic drugs and/or steroids.
ACR30: 30% improvement from baseline; GA: Global assessment; JIA: Juvenile idiopathic arthritis; MTX: Methotrexate; RA: Rheumatoid arthritis.

MTX in the era of biologicals

The appearance and rise of MTX as the cornerstone drug was followed by a new era in the treatment of rheumatic diseases, marked by the emergence of the first biological – a TNF-α inhibitor – more than a decade ago. TNF-α inhibitors were a novel therapeutic option for patients who did not respond sufficiently to MTX. Since the introduction of the first TNF-α inhibitor, translational medicine efforts pushed a multitude of biologicals onto the market. The following biologicals are now either approved or used 'off label' in the treatment of RA and/or JIA: TNF-α -blocking agents (etanercept, adalimumab and infliximab; and only in RA: certolizumab and golimumab), T-cell costimulation modulator (abatacept), IL-1-blocking agents (anakinra), IL-6-blocking agents (tocilizumab) and B-cell depletion agent (only in RA: rituximab). The current standard-of-care in RA and JIA, prescribes the use of biologicals together with MTX in patients who respond insufficiently to MTX treatment.[19,24]

In RA, the efficacy of many biologicals (infliximab, certolizumab, anakinra and abatacept) was demonstrated in RTCs, by comparing the combination treatment of MTX and the biological versus MTX alone, in patients with an insufficient response to MTX.[25–28] These trials did not provide a true comparison of MTX and the biological, as they failed to compare MTX monotherapy with the biological monotherapy in MTX-naive patients. Nevertheless, six trials in RA did provide head-to-head comparisons of MTX with adalimumab, golimumab in MTX-naive patients,[9,11] and with etanercept and tocilizumab in MTX-naive patients[7,10,29] and patients previously treated with MTX, who did not have an insufficient response (Table 2).[10,29] Of note is that previous MTX use of patients included in the latter trials[10,29] did not affect the response rates to MTX, etanercept and tocilizumab. Strikingly, MTX was (nearly) as effective as the highly acclaimed TNF-α inhibitors in reducing disease activity or even inducing clinical remission after 6–24 months of treatment (Table 2).

Moreover, the combination therapy with biologicals and MTX was more efficacious than either therapy alone.[9–11,30] By contrast, MTX was less effective than tociluzimab (Table 2) , and did not increase its efficacy.[29,31] In JIA, etanercept, adalimumab, abatacept and tocilizumab are approved by the FDA and EMA in patients refractory to MTX, whereas infliximab and anakinra and are still used off label. The relatively fast approval in JIA was owing to the implementation of the pediatric rule by the FDA and EMA, which demands companies wishing to register a new treatment in adults to test their product in children if there is a pediatric equivalent of the condition at stake.[32] The pediatric rheumatology community, led by Pediatric Rheumatology International Trials Organization (PRINTO) in Europe and Pediatric Rheumatology Collaborative Study Group (PRCSG) in North America, took advantage of this rule, thus facilitating trials with biologicals.[33] Short- and long-term efficacy and safety of etanercept, adalimumab and abatacept have been established in randomized controlled withdrawal trials in polyarticular JIA patients who were nonresponsive to MTX.[34–38] Moreover, the efficacy of anakinra and tocilizumab has been demonstrated in systemic JIA, a disease poorly sensitive to TNF-α

blockade.[39–41] According to the withdrawal trial design, patients with inadequate response to MTX, start with the biological (with or without MTX) in the initial open-label part of the trial, whereupon responders are randomly assigned to receive either the biological (with or without MTX) or the placebo (with or without MTX) until the disease flares. Unlike in RA, no trials in JIA contain head-to-head comparisons of MTX with a biological in MTX-naive JIA patients.

Table 2. Trials in rheumatoid arthritis comparing methotrexate with biologicals head-to-head and trials in juvenile idiopathic arthritis comparing methotrexate with biologicals plus methotrexate in methotrexate-naive patients

Study	Study design	Study duration (months)	ACR50 (%) MTX vs. biological	ACR70 (%) MTX vs. biological	Clinical remission†	Ref.
RA‡						
Bathon *et al.* 2000	MTX vs. etanercept	12	~42/~48	~20/~25	-	7
Klareskog *et al.* (2004)	MTX vs. etanercept	12	43/48	19/24	17/17	10§
Van der Heijde *et al.* (2006)	MTX vs. etanercept	24	42/54	21/27	19/22	12§
Breedveld *et al.* (2006)	MTX vs. adalimumab	12 24	46/41 43/37	28/28 28/26	21/23 25/25	11
Emery *et al.* 2009	MTX vs. golimumab	6	29/33	16/14	28/25	9
Jones *et al.* 2010	MTX vs. tocilizumab	6	34/45¶	14/27¶	12/37¶	29§
JIA						
Wallace *et al.* (2012)	MTX vs. Etanercept+MTX+ prednisolone	4 6	- -	44/71 -	- 23/40	4
Tynjälä *et al.* (2011)	MTX vs. MTX+infliximab	12	60/100¶	50/71¶	25/68¶	5

† In RA, remission was defined as Disease Activity Score (DAS-28) < 2.6; in JIA, remission was defined as no joints with active arthritis, no fever, no rash, no serositis, no splenomegaly, no active uveitis, erythrocyte sedimentation rate in the normal range and Physician's Global Assessment of Disease Activity of 0.
‡ Trials [9-12] compared MTX and biological alone with MTX plus biological, which was superior to either therapy alone (results not shown).
§These trials also included patients with previous MTX use that did not have a lack of response.
¶Statistically significant differences in favor of biologicals in RA or combination therapy in JIA
ACR50: 50% improvement from baseline; ACR70: 70% improvement from baseline; MTX: Methotrexate.

Early aggressive therapy in MTX-naive patients

In early RA, initial treatment consists of MTX in combination with prednisone in order to achieve early remission induction. By contrast, in JIA the role of prednisone in achieving early disease remission is controversial. However, achievement of early tight disease control remained as important in JIA as in RA, which led to a change in the treatment philosophy of JIA towards early aggressive treatment with biologicals in MTX-naive patients. Recently, this novel paradigm led to a new generation of clinical trials comparing MTX with combination therapy of MTX and TNF-α inhibitors in MTX-naive patients, rather than in patients with a poor MTX response. The TREAT study compared the efficacy of subcutaneous MTX monotherapy with a combination therapy of etanercept, subcutaneous MTX and low-dose prednisolone in MTX-naive polyarticular JIA patients.[4] Its primary end point was the attainment of clinically inactive disease at 6 months after treatment start. In the combination treatment (MTX plus etanercept plus prednisolone) arm, 40% of patients reached the end point, whereas in the MTX monotherapy arm 23% of patients reached clinically inactive disease by 6 months (Table 2). The difference between the groups was not statistically significant, as the percentage of patients in the combination treatment group reaching clinical remission was lower than the expected 60% and the efficacy of subcutaneous MTX monotherapy was higher than the expected 20%. It is noteworthy that after 4 months of treatment, as many as 44% of patients in the MTX monotherapy arm versus 71% of patients in the combination treatment arm met the ACRPedi 70 criteria, thus reaching low disease activity (70% improvement from baseline in a minimum of three to six ACR core-set criteria with no more than one of the remaining variables worsening by >30%; Table 2).[42] Another trial of early aggressive therapy in polyarticular MTX-naive JIA patients demonstrated similar efficacy of MTX monotherapy, which induced clinical remission in 25% of patients, and higher efficacy of combination therapy with MTX plus infliximab, which induced clinical remission in 68% of patients (Table 2).[5] MTX monotherapy was, therefore, effective and exceeded expectations in establishing low disease activity and clinical remission when compared with very aggressive combination therapy in MTX-naive polyarticular JIA patients.

Taken together, trials in RA and JIA show that MTX efficacy has not been consistently surpassed by biologicals (TNF-α inhibitors), thus underlining MTX efficacy in MTX-naive patients. Moreover, in order to establish fast tight disease control, early treatment with biologicals is not always obligatory and for many patients MTX monotherapy does suffice. In fact, some patients receiving the combination therapy with biologicals are inevitably overtreated, whereas at the same time others receiving MTX monotherapy can be undertreated. In order to avoid under- or over-treatment, tailor-made treatment appropriate for each individual patient should be achieved. The first step towards such tailor-made therapy is optimization of the use of the anchor drug, MTX.

Optimization of MTX treatment & prediction of MTX response

Tight disease control is of utmost importance in the treatment of rheumatic diseases in order to achieve low disease activity and even clinical remission. Tight disease control should be achieved in a tailor-made fashion by predicting MTX response before its start in individual patients.[43] The identification of MTX responders when starting MTX will enable treatment with an effective, safe and cheap drug and spare these patients expensive biologicals with a poorly elucidated safety profile, which could include the development of malignancies.[6] Of note is that the annual costs of a biological – that is, etanercept – can exceed the annual cost of MTX by 15-fold (~US$1000 for MTX vs ~US$15.000 for etanercept). At the same time, timely identified MTX nonresponders could then quickly receive additional biologicals, protecting them from progressive joint damage and long-term disability. This section will address examples of tight disease control through the optimization of MTX therapy and current and future efforts geared towards tailor-made therapy through prediction of MTX (non)response.

Tight disease control through optimization of MTX use

In RA, various studies showed that tight disease control, aimed at low disease activity and remission, is possible with the anchor drug MTX. A double-blind placebo-controlled RCT, comparing different dosages of oral MTX in longstanding RA patients who failed to respond to other DMARDs, showed that higher starting doses of 12.5–20 mg/week had a larger effect than placebo on tender joint count, pain and global status, whereas lower doses of 5–10 mg/week had a significantly higher effect than placebo only on pain and global status.[44,45] Higher MTX dose was more effective than lower MTX dose, although not significantly.[44,45] Another RCT, in DMARD-naive early RA patients – the CAMERA study – compared an intensive treatment strategy, in which MTX was escalated monthly up to a mean maximum dose of 25 mg/week within 4 months according to a predefined protocol, with a conventional treatment strategy, in which MTX was escalated every 3 months up to a mean maximum dose of 18 mg/week.[43] Within 1 year, 35% of patients in the intensive treatment strategy group versus 14% in the conventional strategy group achieved at least one period of remission; within 2 years the difference between the two groups was 50 versus 37% in favor of the intensive treatment strategy group.[43] This tight disease control in the patients in the intensive treatment strategy arm was accomplished by the immediate start of MTX therapy in early DMARD-naive RA and with an optimal monitoring protocol, resulting in optimal MTX dose and escalation scheme of MTX treatment. European League Against Rheumatism recommendations for the therapeutic management of RA corroborate the central role of MTX in establishing tight disease control.[19]

In JIA, the TREAT trial showed that MTX use could be optimized leading to tight disease control in polyarticular JIA patients. In the MTX monotherapy arm, patients received subcutaneous MTX at a maximally effective dosage of 0.5 mg/kg/week (a maximum of 40 mg) from the very start, thus deviating from the standard-of-care in polyarticular JIA patients,

which comprises a start with oral MTX at lower dosages followed by the dosage increase and/or switch to subcutaneous MTX.[4] Using this regimen, 23% of patients reached clinical remission within 6 months. The excellent response to subcutaneous MTX at a maximum dose from the very start suggests that a new standard dose and route of administration for optimal effectiveness of MTX in treating polyarticular JIA may be warranted. However, in JIA, no RCTs comparing the efficacy of either different dosages of oral MTX or the efficacy of oral versus parenteral MTX have been performed. An open study did show that parenteral MTX was not superior to oral MTX during the first year of MTX treatment,[46] which is in contrast to RA, where patients starting parenteral MTX did achieve better clinical response than patients starting oral MTX.[47]

Accomplishing tight disease control through therapy optimization is also clearly mirrored in the recently issued ACR recommendations for the treatment of JIA.[24] On the one hand, these recommendations follow the accepted treatment philosophy in JIA, which includes a step-wise escalation of therapy: NSAIDs followed by MTX, and then biologicals in patients nonresponsive to MTX after 3–6 months of MTX monotherapy. On the other hand, the new recommendations incorporate a novel aspect, which mirrors the need for tailor-made tight disease control, in which predefined levels of disease activity (low, moderate or high) and prognostic features, identified from the literature, allow for more rapid escalation or skipping of certain treatment levels in patients with high disease activity and poor prognostic features.[24,48,49]

In spite of this forward thinking, the prognostic features used do not necessarily reflect MTX response-specific prognostic factors. Moreover, features of poor prognosis included involvement of ankle, wrist, hip or cervical spine and radiographic changes for active arthritis, which occur late in the disease course.[49] Targeted tailor-made treatment for individual patients should be based on objective predictors present early in the disease course that could predict MTX response before starting MTX treatment. Such early objective predictors could include intelligent-design biomarkers for MTX efficacy, such as gene polymorphisms in transporters and enzymes of the MTX metabolic pathway, or more general immunological biomarkers, such as cytokine profiles.

Tailor-made therapy through prediction of MTX response

MTX is a folic acid analog that inhibits essential enzymes of the folate, purine and pyrimidine pathways, which are crucial for cellular growth and proliferation (Figure 1). Briefly, MTX enters the cell through several transporters: reduced folate carrier, proton-coupled folate transporter and folate receptors. Once inside the cell, MTX receives up to six glutamate residues by folylpolyglutamate synthase to form MTX polyglutamates (MTX-PG2–7). In low-dose MTX treatment in RA and JIA, the pentaglutamate MTX (MTX-PG5) is the highest order of glutamation detected.[50] Polyglutamation with more than three residues increases intracellular retention of MTX – MTX-PG is impermeable to cell membranes and can no longer be expelled

from the cell by efflux transporters such as ATP-binding cassette (ABC) transporters (ABCC1–4 or MRP) and (ABCG2 or BCRP). MTX-PG is a potent inhibitor of dihydrofolate reductase (DHFR), consequently inhibiting folic acid conversion into tetrahydrofolate and subsequent formation of different tetrahydrofolates; the lack of these one-carbon donors and cofactors leads to DNA methylation inhibition (through reduced methionine production) and purine/pyrimidine synthesis inhibition. Besides DHFR inhibition, MTX-PG potently inhibits enzymes involved directly in the formation of purines – 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) formyltransferase, adenosine deaminase and AMP deaminase, as well as the formation of pyrimidines – thymidylate synthase (Figure 1) .

Alterations in transporters and/or enzymes in the abovementioned metabolic pathways, such as single nucleotide polymorphisms (SNPs) in genes encoding for these proteins, could influence their expression level and/or enzymatic activity and in turn the efficacy of MTX. Indeed, SNPs in genes encoding a folate pathway enzyme methylenetetrahydrofolate reductase and purine/pyrimidine pathway enzymes AICAR formyltransferase, AMP deaminase 1 and thymidylate synthase demonstrated associations with MTX (non)response in RA and JIA.[51–56] Moreover, SNPs in genes encoding MTX influx and efflux transporters – reduced folate carrier, ABCB1 and ABCC3 – have also been associated with MTX (non)response in RA and JIA.[51,57–60] Momentarily, a large international genome-wide association study to identify genomic loci associated with MTX response in JIA is underway.

However, the abovementioned associations of SNPs with MTX response cannot be directly used as tools for tailored clinical decision-making in individual patients. Nevertheless, they are objective biomarkers that can be easily measured for a relatively affordable price before MTX start. Therefore, we have taken a step forward in the direction of tailor-made therapy and transformed associations of SNP with MTX efficacy into a prediction model for MTX nonresponse. The prediction model was developed in a JIA cohort of 183 patients and subsequently validated in 104 JIA patients.[61] It consisted of erythrocyte sedimentation rate and four SNPs in genes coding for enzymes and transporters of the MTX metabolic pathway – methionine synthase reductase, proton-coupled folate transporter, ABCB1/MDR-1 and ABCC1/MRP1. The prediction model classified 72% of patients correctly in the derivation cohort and 65% in the validation cohort as either responders or nonresponders to MTX treatment. SNPs were essential for the adequate prediction of MTX nonresponse because clinical parameters or the laboratory parameter erythrocyte sedimentation rate alone were not able to predict MTX nonresponse.[61] The same was shown for a clinical–genetic prediction model in RA patients.[62] The prediction model was subsequently converted into a risk score system, ranging from 0 to 11 points, whereby each risk score carried a certain probability of being an MTX nonresponder. In an ideal situation, the physicians would use the risk score to tailor-make their therapeutic strategies to individual patients according to the corresponding probability of MTX (non) response.

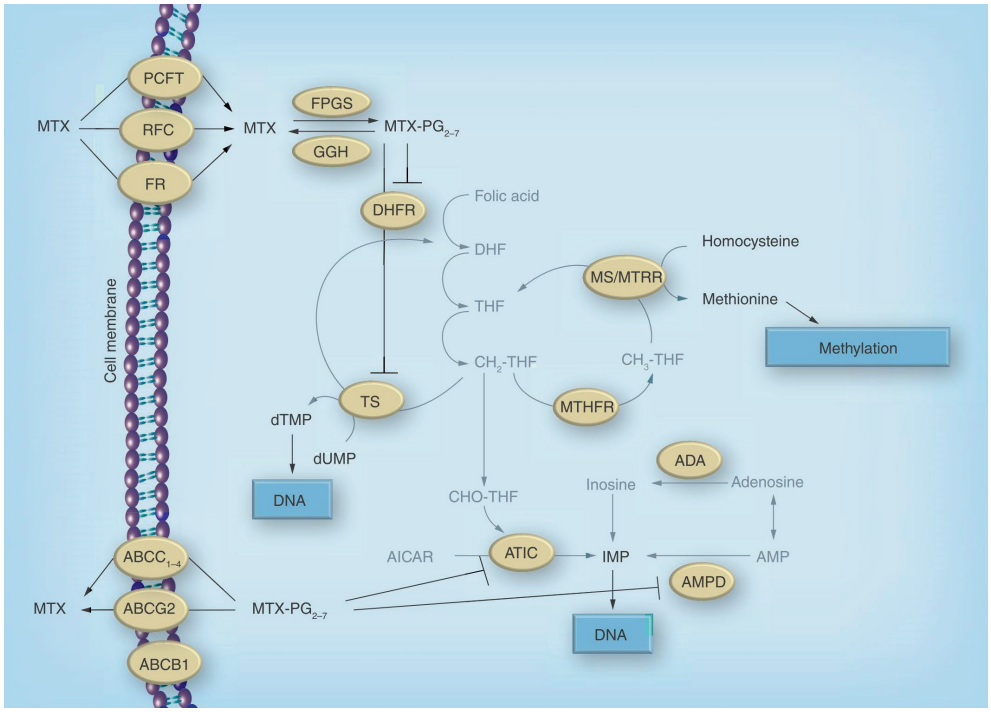

Figure 1. Cellular pathway of methotrexate – influx, conversion to polyglutamates and downstream effects. Cellular uptake of MTX occurs through several transporters. Efflux of MTX-PG2–3 is mediated through ABC transporters. Within the cell, FPGS converts MTX into MTX-PGs, whereas GGH removes the glutamate residues. MTX-PGs impede the generation of THF cofactors in the folate pathway through DHFR inhibition, hindering methylation; inhibit purine synthesis (dTMP) through TS inhibition; and inhibit pyrimidine synthesis (IMP) through ATIC, AMPD and ADA inhibition. ATIC inhibition leads to AICAR accumulation. AICAR also inhibits AMPD and ADA, resulting in accumulation of adenosine, which has anti-inflammatory activity. Polymorphisms in genes encoding for many of these enzymes and transporters can modulate the efficacy of MTX. Further details are presented in the text. ABCB1/ABCC1–4/ABCG2: ATP-binding cassette transporter subfamily B/C/G; ADA: Adenosine deaminase; AICAR: Aminoimidazole carboxamide ribonucleotide; AMPD: AMP deaminase; ATIC: Aminoimidazole carboxamide ribonucleotide formyltransferase; CH2-THF: Methylene tetrahydrofolate; CH3-THF: Methyl tetrahydrofolate; CHO-THF: Formyl tetrahydrofolate; DHF: Dihydrofolate; DHFR: Dihydrofolate reductase; dTMP: Deoxythymidine monophosphate; dUMP: Deoxyuridine reductase; FPGS: Folylpolyglutamate synthase; FR: Folate receptor; GGH: g-glutamyl hydrolase; IMP: Inosine monophosphate; MS/MTRR: Methionine synthase/methionine synthase reductase; MTHFR: Methylenetetrahydrofolate reductase; MTX: Methotrexate; MTX-PG: Methotrexate polyglutamate; PCFT: Proton-coupled folate transporter; RFC: Reduced folate carrier; THF: Tetrahydrofolate; TS: Thymidylate synthase.

Although this prediction model offers promise of target tailor-made therapy, additional efforts have to be made in order to bring the model from the bench to bedside of all JIA patients and use it in daily clinical practice. First, the impact of the prediction rule on physician's behavior in the clinical practice and on the clinical outcomes needs to be assessed.[63,64] Such an impact analysis is the only manner to determine whether the prediction model is better than clinical judgment and whether the model can be used in a broader context.[64] The impact study should be conducted as a randomized trial in which physicians are randomized to an intervention group, exposed to the prediction model or the control group having no knowledge of it.[64] Furthermore, the predictive power of the model is not perfect, which warrants its validation in a larger international JIA cohort and/or improvement with new biomarkers, for example, cytokines (discussed in the following section). Optimization of MTX treatment through prediction of MTX (non)response can also lead to the optimization of biological use, since patients responding inadequately to MTX will be identified early, leading to targeted tailor-made therapy and as such towards tight disease control and disease remission in all patients.

Patient registries for rare & long-term adverse effects

MTX is generally considered to be a safe drug. However, its use is often hindered by a very frequent adverse effect, namely gastrointestinal intolerance.[65,66] Despite its safety in the short term, patients with prolonged MTX use could be exposed to rare adverse effects, which can only be monitored using large patient registries. Unlike for MTX, registries for biologicals were established to monitor their long-term efficacy and adverse effects soon after their approval for the treatment of JIA.[6] In 2009, the FDA issued a black-box warning about a possible association between the use of TNF-α inhibitors (infliximab and etanercept) and the development of malignancies, lymphoma in particular, in JIA patients.[6] Recently it was reported that similarly to RA, JIA is also associated with an elevated risk of malignancies, whereas treatment with TNF-α inhibitors did not seem to be associated with the development of malignancies.[67] Nevertheless, in order to reliably establish whether the use of biologicals in JIA is associated with an increased risk of malignancies, we do not only need large registries of patients on biologicals, but also large registries of patients on MTX to serve as a control group for biological users. This registry would be able to determine whether the incidence of malignancies is increased in JIA and the relative contribution of JIA, MTX and biologicals in cancer development. Indeed, the EU FP7 project Pharmachild is currently setting up such a comprehensive registry.

Mechanisms of action of MTX

MTX is the cornerstone in the treatment of rheumatic diseases; however, a sound understanding of MTX anti-inflammatory effects on the immune system is lacking. In JIA as well as in RA, T cells are important for the regulation of inflammation in autoimmune disease.[33] A higher frequency of Treg in peripheral blood and synovial fluid were shown in JIA patients with remitting disease, thus correlating with a favorable disease course and lower disease activity.[68–70] Moreover, synovial fluid of JIA patients has an increased number of effector T cells (Teff) expressing the RORγt transcription factor (Th17 cells), which have a reciprocal relationship with Treg in the joint.[69,70] Although in RA, reduced suppressive function of peripheral blood Treg has been reported, in JIA Treg in peripheral blood and in synovial fluid seem to be functional.[71–73] Instead, synovial fluid Teff in JIA patients are resistant to Treg-mediated suppression.[73] The importance of Treg and Teff in chronic inflammation of rheumatic diseases can, therefore, not be denied.

MTX induces low disease activity or even disease remission in more than 70% of JIA patients using MTX.[74] Moreover, JIA patients in remission, as opposed to RA, are taken off MTX and 50% of these patients remain in disease remission after discontinuing the medication.[74] Keeping this in mind together with the role of T cells in the pathophysiology of JIA, it seems possible that MTX exerts its anti-inflammatory action on Treg and Teff. Nevertheless, surprisingly little is known about the effect of MTX on T cells during MTX treatment. Therefore, MTX deserves to be brought from the bedside back to the bench to determine its anti-inflammatory effects on the patient's immune system, and in particular on T cells, during MTX treatment. This line of research could provide insights into immunological differences between MTX responders and nonresponders, as well as biomarkers of MTX response, which could eventually facilitate tailor-made therapy in JIA. This section will discuss antiproliferative and anti-inflammatory effects of MTX in model systems followed by the current translational efforts to address the effects of MTX *in vivo* in JIA patients during MTX treatment.

Mechanism of action of MTX in model systems

The antiproliferative effects of MTX, through inhibition of folate metabolism and de novo purine and pyrimidine synthesis, are responsible for the efficacy of MTX in malignant diseases. Historically, the antiproliferative effects of MTX prompted the use of this drug in rheumatic diseases as well. However, MTX is used in considerably lower dosages in rheumatic diseases (up to 40 mg/week) than in malignant diseases (up to 5000 mg/week). Although MTX-related adverse effects such as leucopenia, anemia, stomatitis, gastrointestinal ulcerations and hepatic toxicity could indeed arise due to antiproliferative effects of MTX, they rarely occur in both RA and, particularly, JIA. Moreover, although MTX inhibits the folate pathway enzyme DHFR by competing with folic acid, folic acid supplementation does not diminish MTX efficacy; folic acid (5 mg, 24–48 h after MTX) is in fact a standard-of-care treatment in RA and JIA patients on MTX.[75–79] Taken together, antiproliferative mechanisms of MTX may not play the most prominent role in rheumatic diseases.

Therefore, adenosine-mediated anti-inflammatory function has been ascribed to MTX.[80] MTX-PG potently inhibits AICAR formyltransferase, which results in intracellular accumulation of AICAR, inhibition of the AMP deaminase enzyme and intracellular accumulation of AMP (Figure 1).[81,82] AMP is either transported extracellularly and converted by an ecto-5´nucleotidase CD73 into anti-inflammatory adenosine or first converted into ADP and/or ATP, which are subsequently transported extracellularly and converted into adenosine by a concerted action of CD39 and CD73.[83] Consequently, adenosine mediates its anti-inflammatory effects by binding to one or more adenosine receptors (A1, A2A, A2B and A3).[84] Research in an animal model of acute inflammation (air-pouch animal model) has shown that low-dose MTX treatment resulted in intracellular accumulation of AICAR in splenocytes, increased adenosine, decreased leukocyte (mainly neutrophil) accumulation and lower TNF-α levels in inflammatory exudates of air pouches; all of which did not occur in either CD73 or A2A and A3 adenosine receptor knock-out mice, suggesting that adenosine produced extracellularly and its signaling through receptors were instrumental for anti-inflammatory effects of low-dose MTX.[83,85–87] In a single chronic inflammation model of rat adjuvant arthritis, 4-week administration of low-dose MTX inhibited the development of adjuvant arthritis, whereas blockade of adenosine receptors by two nonselective adenosine receptor antagonists, theophylline and caffeine, abrogated the anti-inflammatory effect of MTX.[88] This study did not further explore the effects of low-dose MTX on the immune system of rats with adjuvant arthritis.

In RA and JIA patients, evidence of MTX-induced adenosine is indirect and conflicting: two studies in RA and psoriasis patients on low-dose MTX reported increased plasma and urine adenosine levels after MTX administration; whereas others demonstrated no changes in blood adenosine levels after MTX administration in RA and JIA.[89–92] It is, therefore, difficult to prove that MTX leads to adenosine release in patients on low-dose MTX, since adenosine cannot be measured reliably due to its very short half-life of approximately 1 min.[92] If adenosine is released, it is quickly taken up locally, at the site of inflammation and cannot be detected systemically. Therefore, functional consequences of MTX-induced adenosine on chronic inflammation in RA and JIA have not been shown, and would be exceedingly difficult to show. It could be speculated that MTX-induced adenosine affects Treg and Teff, since it has been shown that adenosine, induced by Tregs, through abundantly present CD39 and CD73, increased Treg frequency and their suppressive function towards Teff proliferation and cytokine production.[93–97]

Besides the possible contribution of adenosine to anti-inflammatory actions of the MTX, much research has focused on MTX-induced apoptosis of T cells *in vitro*, since deletion of (autoreactive) T cells may play a role in resolving chronic auto-immune inflammation in rheumatic diseases. Several studies showed that T cells activated with phytohemagglutinin (PHA) or anti-CD3 (but not resting T cells), are susceptible to apoptosis induced by both preincubation and concomitant incubation with MTX at doses between 100 and 10 μM.[98–101] T cells were rescued from apoptosis by the addition of folic acid and thymidine, which

suggested that MTX-induced apoptosis resulted from DHFR and thymidylate synthase inhibition.[98] MTX-induced apoptosis was at least in part mediated by MTX-induced production of reactive oxygen species (ROS), as both apoptosis and ROS production were abrogated by a ROS scavenger.[101] However, MTX was also found to be cytostatic rather than cytotoxic, halting proliferation at the G1 phase of the cell cycle.[102] Two recent studies showed that rather than directly inducing apoptosis, MTX increased sensitivity to apoptosis of Jurkat T-cell lines and anti-CD3-activated peripheral blood mononuclear cells (PBMC) upon their exposure to apoptosis-inducing hydrogen peroxide, anti-Fas receptor and to secondary anti-CD3 stimulation.[99,100] The latter suggests that MTX may specifically stimulate apoptosis of autoreactive T cells upon secondary stimulation with an antigen. Briefly, MTX increased sensitivity to apoptosis by inducing expression and activity of JNK and its target genes, which play key roles in promoting apoptosis. JNK-dependent MTX-induced apoptosis was mediated in part by ROS and abrogated by a ROS scavenger.[100]

Other research focused on MTX-induced inhibition of cytokines, whose importance in RA and JIA pathophysiology has been high-lighted by the success of biological therapies, directed against cytokines and their receptors. MTX *in vitro* inhibited TNF-α, IL-4, IL-13 and IFN-γ production by anti-CD3-activated T cells and PBMC of RA patients, whereas IL-1, IL-6, IL-8 and IL-1β production by lipopolysaccharide-stimulated PBMC was not affected by MTX.[103–105] Moreover, MTX in culture with PHA-stimulated PBMC of RA patients increased IL4 and IL10 gene expression and protein production.[106] Although MTX may employ the above-mentioned antiproliferative mechanisms on T cells, the experimental approaches used failed to reflect the clinical reality of patients treated with MTX. As opposed to animal models, in which the effects of MTX are observed in a matter of days or weeks, and to cell culture systems, in which MTX effects are observed within hours or days, the full blown effects of MTX in patients are delayed and can only be reliably evaluated after 3–6 months of treatment. Second, MTX concentrations used in the majority of *in vitro* experiments are high, in micromolar ranges, compared with concentrations used in low-dose MTX treatment; concentrations of 10–50 nM more accurately correspond to low-dose MTX.[85,107,108] Last but not least, MTX exerts the anti-inflammatory functions through its polyglutamate forms.[81] RA patients with higher MTX-PG were shown to be more likely to achieve a therapeutic response to MTX.[109,110] Polyglutamation during incubation with MTX does not reflect that of the *in vivo* situation.

Although MTX *in vitro* rapidly enters the cells and is readily polyglutamated with one to four glutamate residues within 24 h, much of the polyglutamated drug is transported outside the cell through efflux transporters, since polyglutamates with up to three glutamate residues remain substrate to efflux transporters.[81,111–113] Moreover, even at high concentrations of MTX (1 μM) *in vitro*, only a small amount of longer chain MTX-PG (four glutamate residues) are present after a 24-h incubation.[111] In RA patients, on the other hand, MTX-PG take between 10 and 140 weeks to reach the steady state in red blood cells after the last final MTX dose.[114] Moreover,

longer-chain polyglutamates (three to five) take longer to become detectable (3–8 weeks) and even longer to reach the steady state. As it takes 3–6 months to evaluate the full-blown effect of MTX, longer chain polyglutamates, which are barely present in cells incubated with high concentrations of MTX, may have a more important role in the clinical response compared with shorter chain polyglutamates.[114] Taken together, *in vitro* experiments do not reflect the clinical *in vivo* setting. Thus, anti-inflammatory effects of MTX on the immune system should not only be assessed *in vitro* and in models, but also *in vivo* in patients during MTX treatment.

Mechanism of action of MTX in vivo

Much research on the anti-inflammatory effects of MTX *in vivo* has focused primarily on the effects of MTX on cytokines during MTX treatment. In RA patients, the assessment of cytokine production after 6–9 months of MTX treatment showed a decrease in TNF-α-producing CD4 + T cells and an increase in IL-10-producing CD4 + T cells upon phorbol 12-myristate 13-acetate (PMA) stimulation of ex vivo isolated PBMC, although no increase in IL-10 measured in the supernatants of PHA-stimulated PBMC after 3–6 months of MTX treatment was detected.[115,116] Another study reported no changes in serum levels of IL-10, IL-4, IFN-γ, IL-17, IL-23, TNF-α, IL-1β and IL-6 in RA patients after 3 months of therapy, whereas others did demonstrate a decrease in serum IFN-γ and IL-6 after 4 months and 1 year of MTX treatment.[117–119] In the synovial tissue of RA patients receiving MTX for 1–4 months, TNF-α and IL-1β expression was reduced compared with their expression before MTX start.[120,121] Regrettably, the abovementioned studies do not delineate differences in cytokine production between responders and nonresponders nor do they examine the effects of MTX treatment on other phenotypic and effector functions of T cells (or other immune cells) of responders and nonresponders.

Nevertheless, in order to gain insights into the anti-inflammatory effects of MTX on the immune system of patients with rheumatic diseases, both aspects need to be addressed in a longitudinal study. Presently, we are evaluating Treg frequency, phenotype and suppressive capacity on Teff proliferation and cytokine production, and Teff phenotype, activation status, proliferation and cytokine production, as well as cytokine profiles in the plasma and serum of JIA patients during MTX treatment. JIA is a suitable disease model to study the effects of MTX on T cells during treatment, since JIA patients receive MTX monotherapy without additional DMARDs and often also without steroids, as opposed to RA patients who habitually receive additional DMARDs and prednisone. This allows us to purely determine the MTX-induced anti-inflammatory effects on T cells in the absence of confounding factors.

MTX is not adequately effective in approximately 30% of JIA patients.[74] The existence of both MTX responders and nonresponders can provide us with valuable insights into the possible differential effects of MTX on Treg and Teff in these two groups, which could in turn further elucidate the anti-inflammatory effects of MTX. In addition, differences between the two groups at the start of MTX may reveal immunological predictors of MTX (non)response.

These predictors could be cytokines, as they are objective and can be readily measured. Our group has previously shown that cytokine and chemokine signatures can serve as biomarkers for disease diagnosis, as well as disease prognosis, since they differ between different JIA subtypes.[122–124] Another class of inflammatory molecules – myeloid-related proteins (MRPs) – has proven to be an excellent biomarker in several autoimmune and inflammatory diseases, particularly systemic onset JIA.[125,126] MRP-8 (S100A8) and MRP-14 (S100A14) have been shown to predict disease relapse after stopping MTX, and these biomarkers could be used to guide the discontinuation of MTX in patients with clinically inactive disease.[74]

Therefore, bringing MTX from the bedside to the bench by scrutinizing its anti-inflammatory effects in JIA patients during MTX treatment has the potential to provide unique insights not only on the mechanism of action of MTX *in vivo*, but also on immunological predictors of response, which could eventually contribute to the advancement of tailor-made therapy in JIA.

CONCLUSION

Several decades ago MTX advanced the treatment of RA and JIA in a major way. In the past decade, a new era in the treatment of RA and JIA was brought about by biologicals. Despite MTX's status as a cornerstone treatment in these rheumatic diseases, biologicals have increasingly been diverting attention from this well-established drug. The new biologicals, which remain patented and therefore expensive, have redirected the interest of pharmaceutical companies and research funding bodies away from an 'off patent' old drug such as MTX. Rheumatologists and researchers have been losing interest in this cheap, safe and effective drug, whose efficacy has not consistently been surpassed by biologicals in MTX-naive patients. The waned interest from the clinical and scientific community could lead to a failure to resolve several crucial unmet needs that surround the decade-long use of MTX. These unmet needs include: the incomplete understanding of anti-inflammatory actions of MTX on the patient's immune system; lack of registries to monitor long-term adverse effects of MTX compared with biologicals; and prediction of MTX (non)response with objective genetic and immunological parameters. In particular in JIA, translational efforts are made to adequately address these unmet needs by bringing MTX from the patient's bedside back to the bench. Addressing and resolving these issues will lead not only to optimization of MTX use, but also to the optimal use of biologicals. This in turn will lead to tailor-made therapy for individual patients, resulting in tight disease control, clinical remission and good long-term functional outcomes for patients with JIA.

FUTURE PERSPECTIVE

Presently, the ultimate therapeutic goal in rheumatic diseases is the achievement of tight disease control and clinical remission early on in the disease course. In the future, the ultimate therapeutic goal is to achieve clinical remission in a tailor-made fashion for individual patients. In the past decade, translational medicine efforts brought biologicals to the market, which made tight disease control and clinical remission possible for RA and JIA patients with an inadequate response to MTX. With the therapeutic paradigm shifting towards the early application of biologicals, even before knowing the patient's response to MTX, biologicals have overshadowed the well-established and efficacious MTX. Nevertheless, the use of biologicals from the very start in all patients is not plausible owing to their high costs, potentially serious adverse effects and inevitable overtreatment of patients who would have benefited from MTX only. Moreover, such use of biologicals does not satisfy the future goal of tailor-made treatment for individual patients. Tailor-made treatment will have to be achieved by optimizing the use of MTX, which is still the cornerstone treatment in RA and JIA. Optimization of MTX use will be accomplished if the unmet needs of this well-established drug are addressed by bringing MTX from the patients' bedside back to the bench. Employing this other aspect of translational medicine will shed light on the unmet needs surrounding MTX use, namely its anti-inflammatory mechanism of action, which may lead to the development of other new medicines. In addition, it will be important to better predict MTX (non)response using risk profiles based on objective genetic (SNPs) and immunological (cytokines) biomarkers. Resolving these issues, which is currently the case in JIA, will tell us not only which patients will reach tight disease control with MTX only, but also which patients will need more aggressive treatment, such as biologicals, to reach this therapeutic goal. Therefore, optimization of MTX use will also lead to optimization of biological use in patients with rheumatic diseases, thus satisfying the ultimate future goal of personalized tailor-made therapy.

EXECUTIVE SUMMARY

- Translational medicine efforts, focused on the development of new drugs, have brought biologicals from the bench to the bedside of rheumatoid arthritis and juvenile idiopathic arthritis patients.
- Biologicals have captured the attention of the pharmaceutical industry, research funding bodies and adult and pediatric rheumatologists, thus diverting interest from the anchor drug in the treatment of these rheumatic diseases – methotrexate (MTX).
- The waned interest is regrettable since many rheumatic disease patients use MTX as its efficacy is similar to that of biologicals, which are more effective when used in combination with MTX.

- While MTX is losing its well-deserved attention from clinical, scientific and pharmaceutical audiences, the crucial unmet needs remain: the incomplete understanding of its anti-inflammatory mechanism of action, lack of large patient registries to monitor adverse effects and inability to predict MTX response.
- To address these unmet needs, translational medicine efforts need to be made to bring MTX from the bedside back to bench.
- In juvenile idiopathic arthritis, these unmet needs are presently addressed through: a prediction model for MTX nonresponse and its future implementation in daily clinical practice; construction of large patient registries to monitor adverse effects of juvenile idiopathic arthritis patients on biologicals and MTX; and investigation of anti-inflammatory effect of MTX on the immune system in patients with good and poor MTX response.
- Addressing and resolving these unmet needs could lead to optimization not only of MTX treatment, but also of biologicals, resulting in appropriate tailor-made treatment and tight disease control in all patients.

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PART I

METHOTREXATE TREATMENT RESPONSE

3

ABCB1 AND ABCC3 GENE POLYMORPHISMS ARE ASSOCIATED WITH FIRST-YEAR RESPONSE TO METHOTREXATE IN JUVENILE IDIOPATHIC ARTHRITIS

Maurits C.F.J. de Rotte[1], Maja Bulatović[2], Marloes W. Heijstek[2], Gerrit Jansen[3], Sandra G. Heil[1], Ron H.N. van Schaik[1], Nico M. Wulffraat[2] and Robert de Jonge[1]

[1]Department of Clinical Chemistry, Erasmus MC, University Medical Center, Rotterdam; [2]Department of Pediatric Immunology, University Medical Center Utrecht, Wilhelmina Children's Hospital, Utrecht; and Department of Rheumatology, [3]VU University Medical Center, Amsterdam, The Netherlands.

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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. Patient 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.

In weekly low-dose MTX treatment, MTX polyglutamates accumulate intracellularly and thus 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)[5,6,7,8]. Nonresponders 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 through 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 through folylpolyglutamate synthetase (*FPGS*) and MTX polyglutamate breakdown through gamma-glutamyl hydrolase (*GGH*)[3].

In contrast to RA[10], studies in JIA examining associations of SNP in genes involved in MTX transport (uptake/efflux) and polyglutamylation are scarce[11,12,13,14,15,16,17]. 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 SNP in genes involved in cellular MTX transport and polyglutamylation in relation to MTX response in a longitudinal JIA cohort. We hypothesize that SNP in genes involved in MTX transport and polyglutamylation affect response to MTX in JIA.

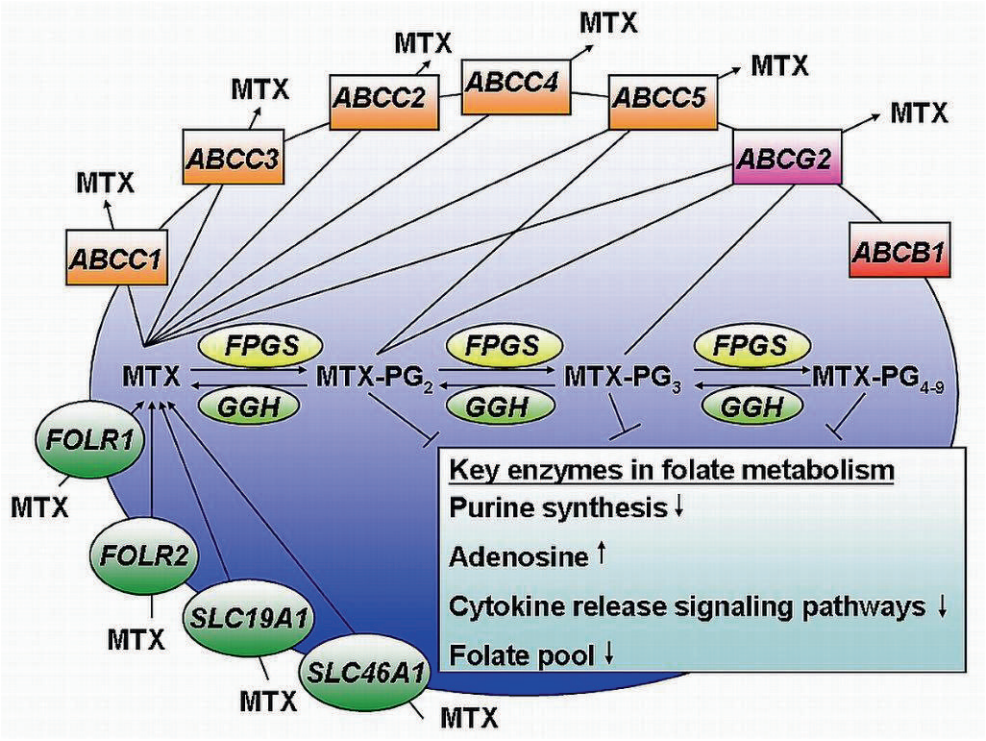

Figure 1. Cellular MTX transport routes for MTX influx and efflux in relation to polyglutamylation and mechanisms for arthritis suppression. MTX polyglutamates (MTX-PG) can inhibit several key enzymes in folate metabolism and may cause a decreased de novo purine biosynthesis, increased adenosine release, direct or indirect effects on cytokine release signaling pathways, and folate depletion, all of which may lead to suppression of arthritis. ABCB1, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCG2: adenosine triphosphate-binding cassette transporter subfamily B/C/G member 1/2/3/4/5; FPGS: folylpolyglutamate synthetase; FOLR1/2: folate receptor 1/2; GGH: gamma-glutamyl hydrolase; SLC46A1/19A1: solute carrier 46A1/19A1.

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. 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 well-being using the Childhood Health Assessment Questionnaire; (3) functional ability, measured using the Childhood Health Assessment Questionnaire 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 (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-α (TNF-α) was a criterion for nonresponse.

SNP selection

SNP in genes involved in MTX transport and polyglutamylation were selected based on the following criteria: minor allele frequency (MAF) > 0.10 in the Hapmap and National Center for Biotechnology Information (NCBI) database[20,21] or a proven functionality in relation to MTX, JIA, RA, or folate metabolism[22,23,24,25,26,27,28,29,30]. If no information was known for a particular gene, we selected tagging SNP 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 expected that SNP with an MAF < 0.10 would not have sufficient data distribution for statistical analysis. Preferably, 2 SNP were selected per gene, which were located in different haplotype blocks. The following 21 SNP in 13 genes were chosen: *ABCB1* rs1128503, rs2032582, rs1045642; *ABCC1* rs35592, rs3784862; *ABCC2* rs4148396, rs717620; *ABCC3* rs4793665, rs3785911; *ABCC4* rs868853, rs2274407; *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] to assess the percentage of genetic variation that was covered by the investigated SNP of all the genetic variation possible within each gene.

We standardized our SNP nomenclature based on the probes labeled with fluorescent dyes VIC and FAM, 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.

A haplotype is a combination of alleles at adjacent locations on the chromosome that are transmitted together. We included haplotype analysis in our study to test whether the effect of the haplotypes on MTX response was larger than that of the corresponding SNP alone. Lewontin's D prime (D') and correlation coefficient (R^2) were calculated by Haploview to assess linkage disequilibrium of SNP within each gene. SNP that were in linkage disequilibrium ($D' \neq 0$) 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 Biochemicals, 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 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 in which 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 SNP. 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 SNP followed the recessive mode of inheritance. We therefore chose a recessive inheritance model to increase the statistical power. Consequently, genotypes and haplotypes were divided 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 for 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.

SNP or haplotypes with sufficient distribution of data for statistical analysis (at least 1 responder and 1 nonresponder 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 OR and CI over the whole treatment period[33]. Generalized linear mixed models were fitted using SAS v. 9.2 (SAS Institute Inc., Cary, NC, USA). A random intercept logistical 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 had multiple testing problems, we tested the significant SNP 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 because 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%) 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 nonresponders on those visits. Patients taking sulfasalazine were not considered nonresponders. Despite the heterogeneity of the study population, we did observe equal MTX response rates among different JIA subtypes.

Table 1. Characteristics of patients with juvenile idiopathic arthritis (JIA) at the time of starting methotrexate (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, yrs, median (range)	9.0 (1.4-18.8)
Disease duration at MTX start, yrs, 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 wellbeing (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)
Intraarticular steroids, n (%)	41 (14.3)

* CHAQ was assessed for 280 patients, included after 1994, when the C-HAQ was introduced in our clinic. ** RF was assessed for 234 patients. PGA: physician global assessment of disease activity; CHAQ: Child Health Assessment Questionnaire; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; NSAID: nonsteroidal antiinflammatory drugs.

SNP analysis

Only the *ABCC2* rs717620 SNP deviated from HWE ($p = 0.038$). 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 SNP investigated, the sequencing analysis confirmed the expected SNP. There were < 5% discrepancies between duplicate runs.

Of the 21 genotyped SNP, statistical analyses, for the univariate association between genotype and MTX response in JIA, could be performed on 17 SNP (Table 2). For the other 4 SNP investigated, there was insufficient distribution of data for statistical analysis (not at least 1 responder and 1 nonresponder for each genotype on every visit). A p value < 0.2 for ACRped70 after univariate analysis was observed for the following 6 SNP: *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 SNP 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 SNP 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 SNP remained the same in the oligoarticular and polyarticular JIA subtypes only. We found similar effects sizes as those 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 taking oral MTX and patients receiving parenteral MTX. We also checked the prevalence of SNP between routes of administration. The MAF for the patients taking oral MTX at baseline (n = 270) were comparable with the MAF for the patients receiving parenteral MTX at baseline (n = 17). Table 3 shows the reconstructed haplotypes. None of the haplotypes remained significant after multivariate analysis.

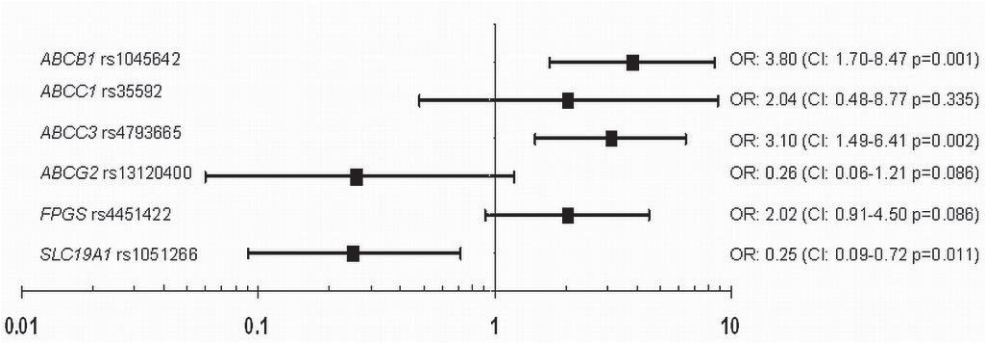

Figure 2. Multivariate analysis of relation between ABCB1 rs1045642, ABCC1 rs35592, ABCC3 rs4793665, ABCG2 rs13120400, FPGS rs4451422, and SLC19A1 rs1051266, and American College of Rheumatology 70% pediatric criteria with OR, 95% CI, and p values. Covariates in multivariate analysis: disease duration prior to start of MTX treatment, physician's global assessment of disease activity at baseline, and MTX dose. ABCB1, ABCC1, ABCC3, ABCG2: adenosine triphosphate-binding cassette transporter subfamily B/C/G member 1/2/3; FPGS: folylpolyglutamate synthetase; SLC46A1/19A1: solute carrier 46A1/19A1.

Table 2. Genes and 21 single-nucleotide polymorphisms (SNP) within cellular methotrexate (MTX) transport routes and polyglutamylation in relation to response (ACRped70) over the first year of MTX therapy in 287 patients with juvenile idiopathic arthritis. Analyses were performed according to a recessive inheritance model.

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	**		27
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

*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 nonresponder for each genotype on every visit). †No tagging SNP were available and an SNP with minor allele frequency > 0.10 was chosen. ‡Tagging SNP were selected by Hapmap database and Haploview. ACRped70: American College of Rheumatology 70% pediatric criteria; GC: gene coverage; HWE: Hardy-Weinberg equilibrium; WT: wild type; het: heterozygous; var: variant; 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; rs: reference SNP number.

DISCUSSION

In our longitudinal study, we identified 2 SNP 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 potentially permanent disability[34]. Therefore, identifying determinants of MTX response would be a major development in JIA therapy.

The SNP 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 our study, the authors did not find a significant association for SNP 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 SNP in *FPGS* and *GGH* and response to MTX[14].

To our knowledge, our 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 statistically significant association[38,39,40] between *ABCB1* polymorphisms and response to MTX. *ABCB1* belongs to the efflux transporters of the *ABC* superfamily, 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 our study; if the *ABCB1* rs1045642 polymorphism is associated with a diminished extrusion of inflammatory mediators, it 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[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 toward 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 SNP 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]. SNP 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 SNP tested = 0.003); 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* SNP has no additional value, and that determination of the rs1045642 SNP alone may suffice.

Some limitations of our study should be considered. Because of the large number of SNP tested, the observed positive associations may be spurious. However, when we analyzed all SNP in relation to ACRped50, similar results were obtained. Multivariate analysis yielded OR 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 SNP 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 SNP may be in linkage disequilibrium with the true causal variant. For the other genes investigated in our 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 over-estimation 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 so our results should be replicated. For that, multicenter studies with large patient numbers are needed, which for rare diseases such 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.

Table 3. Haplotypes of SNP in genes within cellular MTX transport routes and polyglutamylation in relation to response (ACRped70) over the first year of MTX therapy in JIA. Haplotype analysis was performed according to a recessive inheritance model and therefore only homozygous haplotypes were analyzed.

Gene	rs numbers	Haplotypes	Frequency	OR (95%CI) Univariate	p Univariate
ABCB1	rs1128503/rs32032582/rs1045642	GCA	0.10	*	
ABCB1	rs1128503/rs32032582/rs1045642	AAA	0.37	2.44 (0.80-7.46)	0.117
ABCB1	rs1128503/rs32032582/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 Multivariate**
ABCB1	rs1128503/rs32032582/rs1045642	AAA	0.37	3.01 (0.72-5.65)	0.184
ABCB1	rs1128503/rs32032582/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

* Insufficient distribution of data for statistical analysis (not at least 1 responder and 1 nonresponder for each haplotype on every visit). MTX: methotrexate; SNP: single-nucleotide polymorphism; ACRped70: American College of Rheumatology 70% pediatric criteria; ABCB1/ABCC2/ABCG2: adenosine triphosphate-binding cassette transporter subfamily B/C/G member 1/2; GGH: gamma glutamyl hydrolase.

Unlike other studies that examined the associations of SNP within genes in the MTX metabolic pathway with MTX response in JIA[11,12,13,15], we analyzed our data longitudinally. A study in patients with RA 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 have 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 SNP significantly associated with MTX response compared to the general linear mixed model.

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

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4

PREDICTION OF CLINICAL NON-RESPONSE TO METHOTREXATE TREATMENT IN JUVENILE IDIOPATHIC ARTHRITIS

Maja Bulatović[1*], Marloes W. Heijstek[1*], E. H. Pieter Van Dijkhuizen[1], Nico M. Wulffraat[1], Saskia M. F. Pluijm[2] and Robert de Jonge[3]

*contributed equally

[1]Department of Pediatric Immunology, University Medical Center Utrecht, Wilhelmina Children's Hospital, Utrecht, The Netherlands; [2]Department of Public Health, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands; [3]Department of Clinical Chemistry, Erasmus University Medical Center, Rotterdam, The Netherlands

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ABSTRACT

Objectives

Methotrexate (MTX) is a cheap and efficacious drug in juvenile idiopathic arthritis (JIA) treatment. If JIA patients are unresponsive to MTX, early and effective combination treatment with biologicals is required to prevent joint damage. The authors developed a prediction model to identify JIA patients not responding to MTX.

Methods

In a cohort of 183 JIA patients, clinical variables and single nucleotide polymorphisms (SNPs) in genes involved in the mechanism of action of MTX were determined at the start of MTX treatment. These variables were used to construct a prediction model for non-response to MTX treatment during the first year of treatment. Non-response to MTX was defined according the American College of Rheumatology paediatric 70 criteria. The prediction model was validated in a cohort of 104 JIA patients.

Results

The prediction model included: erythrocyte sedimentation rate and SNPs in genes coding for methionine synthase reductase, multidrug resistance 1 (MDR-1/ABCB1), multidrug resistance protein 1 (MRP-1/ABCC1) and proton-coupled folate transporter (PCFT). The area under the receiver operating characteristics curve (AUC) was 0.72 (95% CI: 0.63 to 0.81). In the validation cohort, the AUC was 0.65 (95% CI: 0.54 to 0.77). The prediction model was transformed into a total risk score (range 0–11). At a cut-off of ≥3, sensitivity was 78%, specificity 49%, positive predictive value was 83% and negative predictive value 41%.

Conclusions

The prediction model that we developed and validated combines clinical and genetic variables to identify JIA patients not responding to MTX treatment. This model could assist clinicians in making individualised treatment decisions.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is one of the most common chronic rheumatic diseases in childhood with a reported prevalence between 16 and 159 per 100,000.[1] In the treatment of JIA, methotrexate (MTX) is the cornerstone disease-modifying antirheumatic drug. MTX is efficacious in 30%–70% of patients, depending on the JIA subtype.[2,3] Patients who do not respond or partially respond to MTX are given biologicals such as tumour necrosis factor α (TNFα) inhibitors, interleukin 1 (IL-1) receptor blockers or IL-6 blockers alone or in combination with MTX. The high efficacy of these combination therapies[4–8] is leading to a tendency to apply biologicals early in the treatment of JIA, even before knowing the patient's response to MTX monotherapy.[9–11] This is consistent with the need for early effective treatment of JIA, crucial for preventing irreversible joint destruction and long-term disabilities.[1,4,12] However, combination therapy is unnecessary in those patients who could respond to MTX monotherapy, given that the long-term adverse effects of biologicals, particularly TNFα blockers, are largely unknown and could include development of autoimmune phenomena such as inflammatory bowel disease and malignancies such as leukaemia and lymphoma.[12–16] To ensure that only patients unresponsive to MTX receive early additional treatment with biologicals and those responsive to MTX are spared costly drugs with potentially serious adverse effects, it is crucial to predict those patients who will be unresponsive to MTX monotherapy.

A prediction model for MTX efficacy was successfully constructed in rheumatoid arthritis (RA).[17] However, to date no model has been constructed to predict MTX non-response in JIA. The aim of this study was to develop and validate such a prediction model, using clinical and genetic predictors.

METHODS

Study design and patients

Two observational cohort studies were performed at the Wilhelmina Children's Hospital, University Medical Center Utrecht. The derivation cohort, consisting of retrospectively collected patients who had started MTX monotherapy between 1990 and 2006, was used to develop the prediction model. The validation cohort, consisting of prospectively collected patients who had started MTX monotherapy between January 2007 and June 2010, was used to test the external validity of the model.

Patients, aged 1–18 years, with a confirmed JIA according to the International League of Associations for Rheumatology criteria[18] and an available blood sample were eligible for inclusion. Patients were excluded if longitudinal data after start of MTX treatment could not be retrieved and blood samples could not be used to determine the SNPs. Their clinical data on disease characteristics, disease activity and medication use were collected from medical charts

at the moment of MTX start and at 3, 6 and 12 months after MTX start. This study was approved by the University Medical Center Utrecht Medical Ethics Committee.

Assessment of MTX clinical response

Clinical response to MTX in the first year of treatment was determined using the American College of Rheumatology paediatric 70 (ACR70) criteria for disease activity.[19] The validated core-set criteria[20] for disease activity were: (1) Physician's global assessment of disease activity on a 10 cm visual analogue scale; (2) Number of active joints, defined by joint swelling or limitation of movement accompanied by pain and tenderness; (3) Number of joints with limitation of movement; (4) Physical functional ability, measured with the Childhood Health Assessment questionnaire (CHAQ) disability on a 0–3 scale;[21] (5) Parent or patient assessment of patient's well-being on a 10 cm visual analogue scale; and 6) Erythrocyte sedimentation rate (ESR). Good clinical response to MTX according to ACR70 criteria means at least 70% improvement in at least three of the six core-set criteria, with no more than 30% worsening in more than one of the remaining criteria.

MTX non-responders were defined as patients who did not satisfy the ACR70 criteria in at least two out of three visits during the first year of MTX treatment. This definition was used since clinical response to MTX is known to fluctuate in a large proportion of patients between different time points in the first year of treatment.[22] MTX non-responders also included patients discontinuing MTX and/or switching to anti-TNFα therapy or other biologicals due to insufficient effect of MTX.

Clinical and genetic variables

At baseline, JIA was divided into three subtype categories: oligoarticular JIA, polyarticular JIA and other subtypes including systemic, psoriatic and enthesitis-related JIA (table 1). Other disease characteristics, core-set criteria and information on medication use are shown in table 1.

The genetic variables, single nucleotide polymorphism (SNPs), were selected based on their involvement in the MTX metabolic pathways, their high polymorphic allele frequency and documented functional effects. DNA for SNP analysis was obtained from whole blood or isolated peripheral blood mononuclear cells. Genomic DNA was isolated using the QIAmp DNA Mini Blood Kit (Qiagen, Venlo, The Netherlands). The following SNPs were determined using real-time PCR with Taqman technique according to protocols provided by the manufacturer (Taqman, Applied Biosystems, Foster City, California, USA): methylenetetrahydrofolate reductase (MTHFR rs1801133 and rs1801131), reduced folate carrier (RFC/SLC19A1 rs1051266), methionine synthase reductase (MTRR rs1801394), inosine triphosphatase (ITPA rs1127354), adenosine monophosphate deaminase (AMPD1 rs17602729), 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC rs2372536), adenosine-deaminase (ADA

rs73598374), adenosine A2A receptor (ADORA2A rs5751876), multidrug resistance 1 (MDR-1/ABCB1 rs1128503, rs1045642, rs2032582), multidrug resistance protein 1-5 (MRP-1/ABCC1 rs35592, rs3784862; MRP-2/ABCC2 rs4148396, rs717620; MRP-3/ABCC3 rs4793665, rs3785911; MRP-4/ABCC4 rs868853, rs2274407; MRP-5/ABCC5 rs2139560), breast cancer resistance protein (BCRP/ABCG2 rs13120400, rs2231142), γ glutamyl hydrolase (GGH rs10106587, rs3758149) and proton-coupled folate transporter (PCFT rs2239907).

Statistical analysis

To construct a risk model to predict non-responders to MTX, backward logistic regression analysis was performed in several stages. First, all continuous clinical variables were dichotomised to facilitate the use of the model in daily clinical practice. Second, univariate ORs with 95% CI were calculated (table 1). If two potential predictors correlated (Spearman's r≥0.40), the clinically more relevant or the more significant variable in the univariate analysis was given preference. Third, to obtain the final prediction model, clinical and genetic variables with a p value of ≤0.20 on the log-likelihood test were combined in the multivariate logistic regression analysis.

To calculate predicted probabilities of being an MTX non-responder, we used the following formula:

$$P_{MTXoutcome} = \frac{e^{(\beta_0+\beta_1 \cdot x_1+\beta_2 \cdot x_2+.....+\beta_n \cdot x_n)}}{1+e^{(\beta_0+\beta_1 \cdot x_1+\beta_2 \cdot x_2+.....+\beta_n \cdot x_n)}},$$

where P is the predicted probability of being an MTX non-responder, β0 is the constant and β1, β2 and βp represent the regression coefficients for each of the predictors x1, x2 and xp.

To evaluate the predictive power of the model, we used the predicted probabilities for MTX non-response to construct a receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) measures the concordance of predictive 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 employed 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 (table 3). Within the total risk score, sensitivity, specificity, positive predictive value and negative predictive value were calculated for several cut-off scores.

The prediction model was externally validated in the validation cohort. To do this, we entered the regression coefficients of the predictors obtained from the derivation cohort into the abovementioned formula. This was used to construct a ROC curve for the validation cohort. All statistical analyses were carried out with SPSS V.15.0.0 (SPSS, Chicago, Illinois, USA).

Table 1. Prevalence, univariate OR (95%CI) for potential predictors of ACR70 MTX non-response for derivation and validation cohorts at baseline

Variables		Derivation cohort (n=183)		Validation cohort (n=104)	
		Frequency (%)	OR (95%CI)	Frequency (%)	OR (95%CI)
Female		122 (66.7)	1.10 (0.53-2.30)	61 (58.7)	1.72 (0.76-3.90)
JIA subtype					
Oligoarticular (persistent/extended)		66 (36.1)	Reference	45 (43.3)	Reference
Polyarticular (RF negative/positive)		68 (37.2)	0.81 (0.36-1.81)	39 (37.5)	2.92 (1.13-7.52)
Other (systemic-onset/psoriatic/enthesitis-related)		49 (26.8)	1.20 (0.47-3.04)	20 (19.2)	2.04 (0.66-6.27)
Disease characteristics					
ANA positive		91 (49.7)	0.99 (0.49-1.99)	55 (52.9)	0.25 (0.10-0.65)
Age at onset	>5 years	85 (46.4)	0.95 (0.47-1.91)	59 (56.7)	0.76 (0.33-1.73)
Age at MTX start	>6 years	129 (70.5)	0.75 (0.34-1.67)	74 (74.0)	1.43 (0.58-3.53)
Disease duration*	>1 year	113 (61.7)	1.86 (0.92-3.78)	44 (42.3)	1.77 (0.77-4.12)
Core set criteria					
Active joints	>2	110 (60.1)	0.77 (0.37-1.59)	55 (52.9)	0.93 (0.41-2.12)
Limited joints*	>1	126 (68.9)	0.59 (0.26-1.33)	59 (56.7)	0.73 (0.31-1.69)
PGA	>3 cm	150 (82.0)	0.59 (0.21-1.63)	55 (52.9)	1.32 (0.58-2.99)
ESR[a]	>12 mm/hr	114 (62.3)	0.38 (0.16-0.93)	66 (63.5)	0.70 (0.28-1.77)
Medication†					
MTX dose	>10 mg/week	64 (35.0)	0.66 (0.32-1.36)	45 (43.3)	1.13 (0.50-2.57)
Folic acid		101 (55.2)	0.78 (0.38-1.59)	96 (92.3)	0.61 (0.12-3.18)
Single nucleotide polymorphisms					
MTHFR rs1801133 C>T	CT/TT vs. CC	94 (51.3)	1.45 (0.72-2.93)	49 (47.2)	1.67 (0.73-3.80)
MTHFR rs1801131A>C	AC/CC vs. AA	97 (53.0)	0.79 (0.39-1.61)	56 (53.9)	0.37 (0.16-0.87)
MTRR rs1801394 A>G[a]	AG/GG vs. AA	147 (80.3)	0.28 (0.08-0.96)	78 (75.0)	0.62 (0.23-1.66)
AMPD1 rs17602729 G>A	GA/AA vs. GG	42 (22.9)	1.03 (0.45-2.39)	24 (24.0)	0.74 (0.29-1.86)
ATIC rs2372536 C>G	CG/GG vs. CC	105 (57.4)	0.97 (0.48-1.96)	72 (69.2)	0.65 (0.26-1.61)
ADORA2A rs5751876 C>T	CT/TT vs. CC	109 (59.6)	0.87 (0.42-1.79)	73 (70.2)	1.29 (0.54-3.08)
MDR-1/ABCB1 rs1128503 G>A	AA vs. GA/GG	30 (16.4)	0.57 (0.24-1.38)	17 (16.3)	0.71 (0.25-2.07)
MDR-1/ABCB1 rs1045642 G>A*	AA vs. GA/GG	43 (23.5)	0.39 (0.18-0.84)	32 (30.8)	0.47 (0.20-1.10)
MRP-1/ABCC1 rs35592 T>C*	TC/CC vs. TT	76 (41.6)	0.50 (0.24-1.01)	36 (34.6)	0.75 (0.32-1.72)
MRP-1/ABCC1 rs3784862 A>G	AG/GG vs. AA	83 (47.0)	0.97 (0.48-1.97)	47 (45.2)	1.28 (0.56-2.90)

MRP-2/ABCC2 rs717620 C>T	CT/TT vs. CC	66 (36.1)	0.92 (0.45-1.91)	38 (36.6)	0.84 (0.36-1.93)
MRP-3/ABCC3 rs4793665 T>C	TC/CC vs. TT	132 (72.1)	1.54 (0.73-3.27)	65 (62.5)	2.24 (0.97-5.14)
MRP-3/ABCC3 rs3785911 A>C	AC/CC vs. AA	101 (55.2)	1.01 (0.50-2.04)	49 (47.1)	1.18 (0.52-2.65)
MRP-5/ABCC5 rs2139560 G>A	GA/AA vs. GG	127 (69.4)	1.49 (0.72-3.12)	61 (58.7)	1.57 (0.69-3.57)
BCRP/ABCG2 rs13120400 T>C	TC/CC vs. TT	85 (46.5)	1.23 (0.61-2.49)	48 (46.1)	1.85 (0.80-4.27)
GGH rs10106587A>C	AC/CC vs. AA	90 (49.1)	0.65 (0.32-1.32)	58 (53.9)	1.14 (0.50-2.58)
GGH rs3758149 G>A	GA/AA vs. GG	84 (45.9)	1.38 (0.68-2.81)	58 (53.9)	1.36 (0.60-3.07)
PCFT rs2239907 C>T*	CT/TT vs. CC	125 (68.3)	0.56 (0.25-1.26)	69 (66.4)	1.44 (0.51-4.11)

Variables with >10% missing values and/or observed frequencies of <5 with respect to MTX clinical response in the derivation or the validation cohort were excluded from the univariate logistic analysis: RF, CHAQ disability, CHAQ well-being, *RFC/SLC19A1* rs1051266 *C>T, ITPA* rs1127354 *G>T, ADA* rs73598374 *C>T, MDR-1/ABCB1* rs2032582 *C>A/T, MRP-4/ABCC4* rs868853 *T>C, MRP4/ABCC4* rs2274407 *C>A, BCRP/ABCG2* rs2231142 *G>T.* Variable *MRP2/ABCC2* rs4148396 *C>T* was excluded from the univariate logistic analysis because it correlated with *MRP-2/ABCC2* rs717620 *C>T.*

*Variables significantly associated with MTX non-response (p≤0.20) were included in the multivariate logistic regression analysis. p values – disease duration >1 year: p=0.09; limited joints >1: p=0.20; ESR: p=0.03; *MTRR* rs1801394 *A>G*: p=0.04; *MDR-1/ABCB1* rs1128503 *C>T*; p=0.02; *MRP-1/ABCC1* rs35592 *T>C*: p=0.05; *PCFT* rs2239907*C>T*: p=0.16.

†In the derivation cohort, 170 (92.9%) patients were on non-steroidal anti-inflammatory drugs (NSAIDs) and 2 (1.1%) patients were on sulphasalazine at MTX start. In the validation cohort, 78 (75.0%) patients were on NSAIDs and six (5.8%) patients were on sulphasalazine at MTX start.

OR, odds ratios; CI, confidence interval; ACR70, American College of Rheumatology paediatric 70; ANA, antinuclear antibody; CHAQ, Childhood Health Assessment Questionnaire; ESR, erythrocyte sedimentation rate; JIA, Juvenile Idiopathic Arthritis; MTX, Methotrexate; PGA, physician global assessment; RF, rheumatoid factor.

RESULTS

Patient characteristics

183 patients were included in the derivation cohort after removal of five patients due to missing longitudinal data. Upon eliminating three patients receiving IL-1 receptor blockers at MTX start, 104 patients were included in the validation cohort.

Baseline characteristics (table 1) did not differ significantly between the cohorts, besides disease duration before MTX start, which was longer in the derivation (median: 1.9 years, IQR: 0.3–7.6) than in the validation cohort (median: 0.8 years, IQR: 0.3–4.5) (p=0.001). MTX starting dose was comparable between the two cohorts, namely 9.4 mg/m^2/week in the derivation and 9.8 mg/m^2/week in the validation cohort. Within the cohorts, MTX starting dose was equivalent in future responders and non-responders. However, in the derivation, but not in the validation cohort, MTX dose was significantly higher in non-responders compared with responders at 6 months (12.3 mg/m^2/week vs 9.3 mg/m^2/week) and at 12 months (10.9 mg/m^2/week vs 7.1 mg/m^2/week) after MTX start.

In the derivation cohort, after 1 year of treatment, 149 patients (81.4%) were still on MTX, and 27 patients (14.8%) had stopped MTX due to insufficient effect (n=5), disease remission (n=18), gastrointestinal intolerance (n=3) or hepatotoxicity (n=1). In the validation cohort, 99 (92.5%) patients were still receiving MTX after 1 year and eight patients (7.7%) had stopped MTX due to insufficient effect (n=3), disease remission (n=2) and gastrointestinal intolerance (n=3).

During the first year of treatment, 143 patients (78.1%) in the derivation and 68 patients (65.4%) in the validation cohort were ACR70 non-responders (table 2), while 114 (62.3%) patients in the derivation and 52 (50%) patients in validation cohort were ACR50 non-responders (data not shown). These frequencies corresponded to the frequencies at 6 months after MTX start, which is a commonly used time point to establish MTX efficacy. The ACR70 non-responder frequencies in the validation cohort were similar to those found earlier;[3] however, ACR70 frequencies in the derivation cohort were higher, possibly due to significantly longer disease duration before MTX start[23] in this cohort.

Table 2. ACR70 MTX non-response frequency (%)

Time point	Derivation cohort (n=183)	Validation cohort (n=104)
3 months	175 (95.6)	83 (79.8)
6 months	140 (76.5)	64 (61.5)
12 months	103 (56.3)	52 (50.0)
1st year of treatment*	143 (78.1)	68 (65.4)

*According to definition: ACR70 non-responder in at least two out of three time points during the first year of treatment.
ACR70, American College of Rheumatology paediatric 70; MTX, methotrexate.

Prediction model for MTX non-responders according to ACR70

The following variables, univariately associated (p≤0.20) with MTX non-response, were included in the multivariate logistic regression: disease duration, limited joints, ESR, MTRR rs1801394, MDR-1/ABCB1 rs1045642, MRP-1/ABCC1 rs35592 and PCFT rs2239907 (table 1). Variables of the final prediction model consisted of ESR and MTRR rs1801394, MDR-1/ABCB1 rs1045642, MRP-1/ABCC1 rs35592 and PCFT rs2239907 (table 2). The AUC of the prediction model was 0.72 (95% CI: 0.63 to 0.81), indicating that it classified 72% of patients correctly (table 3). The Hosmer–Lemeshow goodness-of-fit test was not statistically significant (p=0.91), indicating that the model fit the data well.

These predictors were used to test the model in a validation cohort. The AUC of the validation cohort was 0.65 (95% CI: 0.54 to 0.77), indicating that 65% of patients were classified correctly (table 3).

To enable healthcare professionals to easily use the model, the regression coefficients (β) of the model's predictors, transformed into simple scores, were used to compute an individual risk score for being an MTX non-responder (table 3). This score ranged from 0 to 11 points with a higher score reflecting a higher probability of non-response. The risk score of a patient that has all predictors of the final model is calculated by adding up the constant to the simple scores, assigned to individual predictors: 11 (the constant)+(−2)+(−3)+(−2)+(−2)+(−2), which results in a risk score of 0. If all predictors are present, the probability of non-response is 0.42. On the other hand, the risk score of a patient having no predictors would be equal to the constant of 11. If no predictors are present, the probability of non-response is 0.98. Within the 0–11 range, the diagnostic accuracy of different cut-offs for predicting the risk of being an MTX non-responder was evaluated by computing the corresponding sensitivity, specificity, positive predictive value and negative predictive value (table 4).

Our goal was to correctly identify as many future MTX non-responders as possible (high sensitivity), while attempting to avoid misidentification of MTX responders as MTX non-responders as much as possible (reasonable specificity). In the derivation cohort, this was reached at the cut-off ≥3, where 98 of 125 (78%) MTX non-responders and 19 of 39 (49%) MTX responders were identified correctly; 27 non-responders were classified as responders (false negatives) and 20 responders were classified as non-responders (false positives) (table 4). Similarly, in the validation cohort, at the cut-off ≥3, 48 (79%) of 61 MTX non-responders were identified correctly, whereas nine (26%) of 34 MTX responders were identified correctly; 13 non-responders were classified as responders (false negatives) and 25 responders were classified as non-responders (false positives).

Table 3. Prediction model and scores for ACR70 MTX non-response

Predictors		β	Score	OR (95%CI)	p value
Clinical					
ESR	>12 mm/hr	-0.820	-2	0.44 (0.17-1.12)	0.09
Genetic					
MTRR rs1801394 A>G	AG/GG	-1.172	-3	0.31 (0.09-1.11)	0.07
MDR-1/ABCB1 rs1045642 G>A	AA	-0.714	-2	0.49 (0.22-1.11)	0.09
MRP-1/ABCC1 rs35592 T>C	TC/CC	-0.793	-2	0.45 (0.21-0.98)	0.04
PCFT rs2239907 C>T	CT/TT	-0.569	-2	0.57 (0.24-1.36)	0.20
Constant		3.758	11		
AUC derivation cohort (95% CI)		0.72 (0.63-0.81)			
AUC validation cohort (95% CI)		0.65 (0.54-0.77)			
Hosmer-Lemeshow test (p value)		0.91			

Example: Risk score of a JIA patient having all predictors is calculated as follows: Add up the constant (11) to scores of individual predictors, namely 11+(-2)+(-3)+(-2)+(-2)+(-2), which equals 0 points.
ACR70, American College of Rheumatology paediatric 70; ESR, erythrocyte sedimentation rate; MTX, methotrexate.

Table 4. Diagnostic parameters for various risk score cut-offs predicting ACR70 MTX non-response

Cohort	Cut-off	Probability of MTX non-response	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Derivation	≥1	0.42	97	13	78	55
	≥3	0.60	78	49	83	41
	≥5	0.77	50	82	90	34
	≥6	0.88	44	87	92	33
	≥7	0.90	24	95	94	28
	≥8	0.93	16	97	95	27
Validation	≥1	0.62	97	9	66	60
	≥3	0.77	79	26	66	41
	≥5	0.88	46	68	72	41
	≥6	0.91	40	71	71	40
	≥7	0.91	21	88	76	38
	≥8	0.91	11	91	70	36

Risk scores were calculated in 164 patients in de derivation cohort (n=19 baseline erythrocyte sedimentation rate (ESR) values were missing) and in 95 patients in the validation cohort (n=8 ESR values were missing and n=1 failed genotyping of *PCFT* gene).
ACR70, American College of Rheumatology paediatric 70; MTX, methotrexate; NPV, negative predictive value; PPV, positive predictive value.

DISCUSSION

We developed and validated a prediction model for clinical non-response in two large JIA cohorts consisting of ESR and four SNPs in the MTRR, MDR-1/ABCB1, MRP-1/ABCC1 and PCFT genes. The model classified 72% of patients correctly in the derivation and 65% in the validation cohort.

To our knowledge, no previous studies have constructed a model to predict MTX non-response in JIA. Several studies did report associations of MTX non-response in JIA with polyarticular disease, longer disease duration, ANA negativity and a higher level of disability.[24,25] In our study, longer disease duration and ANA negativity were univariately associated with MTX non-response, although not significantly. Moreover, extended oligoarticular JIA subtype was associated with MTX response.[25] However, we and others[26,27] observed equal MTX response rates among different JIA subtypes. Therefore, in the present study JIA subtype was not a predictor of MTX non-response. Furthermore, no effect modification was detected upon restricting the analysis to the more prevalent oligo and polyarthritis subtypes since the prediction model preserved its predictive power (AUC: 0.72, 95% CI: 0.61 to 0.82).

An MTX efficacy prediction model was constructed in RA, classifying 85% of patients correctly.[17] This model contained four clinical variables and four SNPs encoding AMPD1, ATIC, ITPA and MTHFD1 (methylenetetrahydrofolate dehydrogenase) enzymes. Despite differences in definitions of response and in demographics of RA and JIA patients, inclusion of SNPs was essential for adequate prediction of MTX non-response in both models. Our prediction model with ESR only yielded a poor AUC of 0.59 (95% CI: 0.49 to 0.69), whereas the addition of SNPs raised the AUC to 0.72. Therefore, SNPs were crucial for a good prediction of MTX non-responders in JIA.

The goal of our model is to correctly identify future non-responders who can be given early additional treatment with biologicals, and simultaneously to keep misidentification of future responders as non-responders to a minimum. This goal stems from the following important changes in treatment mentality of paediatric rheumatologists over the past years, prompted by the need to establish early disease control to prevent irreversible joint damage. First, paediatric rheumatologists no longer consider MTX response according to ACR30 or ACR50 sufficient, but judge it to be good only if patients satisfy the more stringent ACR70 criteria.[28–30] Furthermore, they consider patients MTX responders if they satisfy these criteria already within 3 months after MTX start. These changes in treatment mentality have resulted in a lower threshold to start early combination treatment with biologicals. Although very effective, biologicals potentially carry a heightened risk of malignancies and inflammatory bowel disease.[12–16] To address these risks, while considering it crucial to adequately treat MTX non-responders as early as possible with biologicals and at same time restrict their use to those patients who really need them, we selected a cut-off ≥3 as the optimal score. Using

this cut-off in the derivation cohort would allow 98 (78%) of 125 non-responders to receive early additional treatment with biologicals, and spare 19 (49%) of 39 patients, identified as responders, from receiving them. In the validation cohort, 79% of non-responders would be given timely biological treatment, whereas 26% of patients identified as responders would be spared from receiving them (table 4). Although the sensitivity at this cut-off was the same for both cohorts, the specificity was considerably lower in the validation cohort (49% vs 26%), which is due to its relatively small size.

The choice of a cut-off, however, depends on the clinical goal. A cut-off ≥6 could be chosen, if clinicians use the prediction model primarily to select as many responders as possible, while avoiding misidentification of non-responders as responders. At this cut-off, 34 of 39 (87%) MTX responders were identified correctly, while 55 of 125 (44%) MTX non-responders were identified correctly. Similar diagnostic parameters were obtained in the validation cohort (Table 4).

Our model was constructed for ACR70 non-responders in at least two of three visits during the first year of treatment, due to known fluctuations in MTX (non-)response during the first year.[22] Nevertheless, the model had an equally strong predictive power for ACR70 non-responders (AUC=0.71, 95% CI: 0.62 to 0.80) at 6 months after MTX start. Depending on the clinician's preference, the model could also be applied for a less stringent ACR50 non-response, since its predictive power was strong both in the first year of treatment (AUC=0.70, 95% CI: 0.61 to 0.77) and at 6 months after MTX start (AUC=0.72, 95% CI: 0.63 to 0.80).

Further studies are needed to evaluate the effect of these SNPs on enzyme activity and transporter function. As we and others have shown, the non-synonymous rs1045642 SNP in the MDR-1/ABCB1 efflux transporter gene was associated with a higher probability of good clinical response to MTX.[31] The synonymous rs35592 SNP in another MRP-1/ABCC1 efflux transporter gene has been associated with higher risk of MTX non-response in psoriasis patients,[32] whereas here this SNP was associated with a lower risk of non-response. The synonymous PCFT rs2239907 SNP, whose protein is an influx transporter, has not been described earlier in relation to MTX efficacy in arthritis. Finally, the non-synonymous MTRR rs1801394 SNP was associated with decreased MTX sensitivity in acute lymphoblastic leukaemia,[33] whereas in our JIA cohorts it conferred a decreased risk of MTX non-response.

A limitation of the model is its moderate predictive power of 65% in the relatively small validation cohort. This can impede its direct clinical use, indicating the need for further refinement. Therefore, to confirm the model's clinical applicability, validation will be performed in a large international cohort prior to its implementation in daily clinical practice. Pharmacogenetic testing may also challenge the model's application in daily clinical practice. Nevertheless, we show that SNPs are indispensable to adequately predict MTX non-responders in our JIA cohorts. Furthermore, such testing is becoming routinely available and less expensive.

Our model predicted and validated MTX non-response in two JIA cohorts by combining clinical and genetic variables. The model offers the promise of personalised treatment in JIA where patients unresponsive to MTX monotherapy will promptly receive additional treatment with biologicals and those destined to be MTX responders will not. Therefore, we will implement the model in daily clinical practice to establish whether its use will result in reduction of disease activity and better disease control in JIA patients.

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5

METHOTREXATE POLYGLUTAMATES IN ERYTHROCYTES ARE ASSOCIATED WITH LOW DISEASE ACTIVITY IN JUVENILE IDIOPATHIC ARTHRITIS PATIENTS

Maja Bulatović Ćalasan[1], Ethan den Boer[2], Maurits C.F.J de Rotte[2], Sebastiaan J. Vastert[1], Sylvia Kamphuis[3], Robert de Jonge[2] and Nico M. Wulffraat[1]

[1]Department of Paediatric Immunology, University Medical Centre Utrecht, Wilhelmina Children's Hospital, Utrecht, The Netherlands; [2]Department of Clinical Chemistry, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands; [3]Department of Paediatric Rheumatology, Erasmus University Medical Centre Rotterdam, Sophia Children's Hospital, Rotterdam, The Netherlands

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ABSTRACT

Objective

To determine association of erythrocyte methotrexate polyglutamates (MTX-PG) with disease activity and adverse effects in a prospective juvenile idiopathic arthritis (JIA) cohort.

Methods

One hundred and thirteen JIA patients were followed from MTX start until 12 months. Erythrocyte MTX-PGs with 1–5 glutamate residues were measured at 3 months with tandem mass spectrometry. The outcomes were Juvenile Arthritis Disease Activity Score (JADAS)-27 and adverse effects. To determine associations of MTX-PGs with JADAS-27 at 3 months and during 1 year of MTX treatment, linear regression and linear mixed-model analyses were used. To determine associations of MTX-PGs with adverse effects during 1 year of MTX treatment, logistic regression was used. Analyses were corrected for JADAS-27 at baseline and co-medication.

Results

Median JADAS-27 decreased from 12.7 (IQR: 7.8–18.2) at baseline to 2.9 (IQR: 0.1–6.5) at 12 months. Higher concentrations of MTX-PG3 (β: −0.006, p=0.005), MTX-PG4 (β: −0.015, p=0.004), MTX-PG5 (β: −0.051, p=0.011) and MTX-PG3–5 (β: −0.004, p=0.003) were associated with lower disease activity at 3 months. 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. None of the MTX-PGs was associated with adverse effects.

Conclusions

In the first prospective study in JIA, long-chain MTX-PGs were associated with lower JADAS-27 at 3 months and during 1 year of MTX treatment. Erythrocyte MTX-PG could be a plausible candidate for therapeutic drug monitoring of MTX in JIA.

INTRODUCTION

In the treatment of juvenile idiopathic arthritis (JIA), methotrexate (MTX) is the anchor disease-modifying antirheumatic drug, due to its safety and efficacy.[1] However, in 30–40% of JIA patients, MTX is not sufficiently effective.[1–5] Moreover, gastrointestinal adverse effects occur in as many as half of all JIA patients on MTX, potentially leading to decreased MTX efficacy and premature discontinuation of MTX.[6–10] Nevertheless, early effective treatment remains crucial in order to prevent joint destruction and long-term disabilities.[10,11] It is therefore important to provide clinicians with tools that could guide tailor-made treatment decisions early in disease course, for example, to give MTX monotherapy to MTX-responsive patients, or to adjust the MTX dose or give biologicals (in combination with MTX) to those poorly responsive or having adverse effects.[12] To date, we and others identified clinical[13–15] and genetic[16–21] factors associated with MTX efficacy, and constructed a clinical-genetic model for MTX non-response,[12] which could assist clinicians in making individualised treatment decisions.

Besides the aforementioned, measurement of MTX concentrations in blood, the so-called therapeutic drug monitoring (TDM) of MTX, could be a powerful tool in steering tailor-made therapeutic decisions directly. TDM of plasma MTX concentrations is not possible, as MTX in plasma is eliminated within 24 h,[22] and is not correlated with disease activity.[23] However, intracellularly retained MTX could be a reliable TDM tool. MTX, which contains one glutamate residue, is polyglutamated with up to four glutamate chains (MTX-PG1–5)[24] intracellularly, which prevents MTX's efflux by various transporters. MTX-PGs in erythrocytes are representative of polyglutamation in bone marrow progenitors[25] and could therefore be representative of MTX-PG levels in other cell types such as lymphocytes.[26] Polyglutamation enhances MTX's affinity for target enzymes in the folate, purine and pyrimidine pathways,[27] thus promoting MTX's anti-inflammatory effects. Therefore, MTX-PGs could be biomarkers of response to MTX and could thus be used as a TDM tool.

Several groups investigated the association of erythrocyte MTX-PGs with disease activity in rheumatoid arthritis (RA) and JIA. The results were conflicting, showing association of MTX-PGs with lower, but also with higher disease activity,[22,28–34] and no association with disease activity.[17,35] The majority of these studies17,[28–30,32,35] faced two drawbacks which could have influenced their conclusions. First, patients were not prospectively followed from MTX start, which makes a fair comparison of disease activity status between patients difficult. Second, patients used MTX from few months to 22 years, which, given that MTX-PG accumulation is a function of time,[23] complicates comparison of MTX-PG concentrations between patients. Moreover, MTX-PGs have to be measured early after MTX start, if they are to be used as biomarkers of patient's response to MTX and as a TDM tool.

The aim of this study was to determine whether erythrocyte MTX-PGs, measured at 3 months after MTX start, were associated with disease activity and adverse effects in a large prospective JIA cohort, followed for 1 year after MTX start.

PATIENTS AND METHODS

Study design and patients

A prospective investigator-initiated clinical trial on efficacy and adverse effects of MTX (ISRCTN13524271) was performed at University Medical Center, Utrecht, and Erasmus University Medical Center, Rotterdam, The Netherlands, between August 2007 and February 2013. It was approved by the ethics committees of the participating centres, and conducted according to good clinical practice guidelines.

Patients aged 2–18 years, with a confirmed JIA diagnosis36, starting MTX for treatment of arthritis (not uveitis) without concomitant biological treatment, were included. Those who had stopped MTX for more than 6 months, but restarted MTX due to a relapse, were also included. At MTX start and 3, 6 and 12 months after MTX start, patients' clinical data (table 1) were documented. At the 3-month visit, patients provided a blood sample for MTX-PG quantification. This time-point was chosen because: (A) this is the first occasion for clinicians to evaluate MTX response and make subsequent therapeutic decisions after start; (B) detection of MTX-PGs was expected, given rapid accumulation (7 or 10–20 weeks) of high MTX-PG concentrations.[23,37]

Disease activity

Disease activity, as primary outcome, was assessed during the 12-month follow-up with the composite Juvenile Arthritis Disease Activity Score (JADAS)-27, measured in 27 joints (range 0–57 points).[38]

Adverse effects

Adverse effects (MTX intolerance, hepatotoxicity and bone marrow suppression) were assessed as secondary outcomes during 12 months. MTX intolerance prevalence was determined using the validated MTX Intolerance Severity Score (MISS).[6] It included abdominal pain, nausea and vomiting occurring after, as well as before (anticipatory), and when thinking (associative) MTX administration, accompanied by behavioural symptoms (restlessness, irritability, crying and refusal of MTX).[6] MTX intolerance was defined as a score of ≥6 on the MISS, including at least one anticipatory, associative, or behavioural symptom.[6] Hepatotoxicity was defined as increase in liver enzymes (ALAT and/or ASAT), two times the upper limit of normal, and bone marrow suppression as lymphocyte count <0.9×109/L, granulocyte count <1.5×109/L and/or thrombocyte count <20×109/L.[39]

Quantification of MTX-PGs in erythrocytes

To determine MTX-PG concentrations, EDTA whole-blood samples on ice were obtained at the median of 3 months (range 1.5–4.5 months) after MTX start, and were centrifuged at 1400 g for 10 min to pellet the erythrocytes. The pellets were stored at −80°C until used.

MTX-PG1–5 (nmol/L of packed erythrocytes), were measured separately with a novel liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) method, which uses stable isotopes for quantification.[24] Individually measured MTX-PGs were summed up to obtain the total MTX-PG concentration.

Statistical analysis

Prior to determining associations of MTX-PGs with JADAS-27 using linear regression analysis, its assumptions pertaining to the outcome (JADAS-27) were checked. Normality of residuals was checked with Normal Probability Plot for JADAS-27 standardised residuals, and homogeneity of variance and linearity of the model were checked by plotting JADAS-27 standardised residuals on the Y axis against predicted standardised values on the X axis. Since linear regression assumptions were violated, JADAS-27 was logarithmically transformed using the common logarithm (log10). First, multivariate linear regression analysis was performed to determine associations of MTX-PGs, measured at 3 months, with the 3-month JADAS-27 (cross-sectional analysis). The analyses included covariates, potentially affecting disease activity at 3 months: baseline JADAS-27, JIA subtype and baseline non-steroidal anti-inflammatory drug (NSAID) use. Second, analysis of covariance (ANCOVA) was performed to compare the 3-month JADAS-27 (geometric mean) between three groups (tertiles) of MTX-PG concentrations, while correcting for baseline JADAS-27. The 1st tertile reflected the lowest MTX-PG concentrations, whereas the 3rd tertile reflected the highest MTX-PG concentrations. The Bonferroni adjustment was applied for multiple comparisons.

Finally, linear mixed-model analysis was performed to determine associations of MTX-PGs with JADAS-27 during the entire follow-up (longitudinal analysis). This model enables a repeated measurement analysis with unequal periods of time between the visits. Moreover, this model considers each patient to have his own pattern of JADAS-27 over time.

To determine associations of MTX-PGs with adverse effects in the first year of MTX use, multivariate logistic regression was performed, including NSAID use (potentially affecting adverse effect occurrence) as a covariate.

Statistical analyses were carried out with SPSS V.20.0.0 (SPSS, Chicago, Illinois, USA). All comparisons were two-sided at α=5%. Linear regression and mixed model were represented as regression coefficients (β), and logistic regression as OR, all with 95% CI.

RESULTS

Patients and disease activity

Of 161 eligible patients, 48 patients were excluded due to a missing blood sample (n=29) and a blood sample at 6 instead of 3 months after MTX start (n=19), resulting in 113 patients included in the analyses (table 1). The starting median MTX dose was 9.9 mg/m2/week (IQR: 9.0–11.4)

(table 1). The median dose was 10.0 (9.1–11.5) at 3 months, 10.5 (9.2–12.7) at 6 months, and 10.8 mg/m2/week (9.4–13.0) at 12 months upon MTX start. After 3 months of MTX use, 7 (6.2%) of 113 patients discontinued MTX due to insufficient effect (n=4), gastrointestinal intolerance (n=2) or toxicity (elevated liver enzymes: n=1) (table 1). Seven patients (6.2%) received additional medication (hydroxychloroquine (n=1) and anti-TNFα (n=6)). Median JADAS-27 decreased from 12.7 points (IQR: 7.8–18.2) at MTX start to 2.9 points (IQR: 0.1–6.5) after 12 months (table 2).

Table 1. Baseline characteristics of 113 patients

Female, N (%)	68 (60.2)
Age at MTX start, years, median (IQR)	12.1 (7.5-14.5)
Age at onset, years, median (IQR)	8.8 (3.8-12.3)
JIA subtype, N (%)	
Persistent oligoarticular	26 (23.0)
Extended oligoarticular	16 (14.2)
Polyarticular*	49 (43.4)
Psoriatic	10 (8.8)
Enthesitis-related	10 (8.8)
Systemic-onset	2 (1.8)
Core-Set Criteria, median (IQR)	
Physician global assessment disease activity (0-10)	3 (2.0-4.0)
Joints with limited range of motion	2 (1-5)
Joints with active arthritis	4 (1-9)
CHAQ disability (0-3)	0.9 (0.4-1.6)
Parent/patient global assessment of well-being (0-10) †	4.1 (2.0-7.1)
Parent/patient global assessment of pain (0-10) ‡	3.9 (1.6-7.0)
ESR (mm/hour)	15.0 (7.0-40.0)
Medication	
Methotrexate dose, mg/m^2/wk, median (IQR)§	9.9 (9.0-11.4)
Folic acid, N (%)¶	113 (100)
NSAIDs, N (%)**	91 (80.5)

*Rheumatoid Factor (RF) positive n=8 (16.3% of all polyarticular JIA patients).
†Available in 111 patients.
‡Available in 112 patients.
§Parenteral MTX (n=2 (1.8%)), concomitant treatment with sulfasalazine (n=4 (3.5%)), oral steroids (n=4 (3.5%)), local steroids (n=11 (9.7%)).
¶Dosis: 5 mg/week, 24h after MTX intake.
**Ibuprofen (n=36), Naproxen (n=22), indomethacin (n=17), diclofenac (n=14), etoricoxib (n=1), meloxicam (n=1).
CHAQ, Childhood Health Assessment Questionnaire; ESR, erythrocyte sedimentation rate; JADAS, Juvenile Arthritis Disease Activity Score; JIA, juvenile idiopathic arthritis; MTX, methotrexate; NSAIDs, Non-steroidal anti-inflammatory drugs.

Table 2. JADAS-27

Time point	Median (IQR)
MTX start	12.7 (7.8-18.2)*
3 months	5.9 (2.8-11.5) †
6 months	4.9 (1.4-9.4) ‡
12 months	2.9 (0.1-6.5)§

*Determined in 111 patients.
†Determined in 110 patients.
‡Determined in 104 patients.
§Determined in 101 patients.
JADAS, Juvenile Arthritis Disease Activity Score; MTX, methotrexate.

MTX-PG concentrations in erythrocytes

Concentrations of short-chain polyglutamates MTX-PG1 and MTX-PG2, long-chain polyglutamates MTX-PG3, MTX-PG4, MTX-PG5 and total MTX-PG are shown in table 3. After 3 months of MTX use, the concentration of MTX-PG1, the native form of MTX, was the highest with the median of 25.3 nmol/L, followed by MTX-PG3 (23.0) and MTX-PG2 (18.7). Concentrations of MTX-PGs with 4 and 5 glutamate residues were considerably lower, with medians of 4.2 and 0.7 nmol/L, respectively. The predominant MTX-PGs were MTX-PG1, which accounted for 32.9% and MTX-PG3, which accounted for 32.7% of total MTX-PG.

Table 3. Concentrations of MTX-PGs and proportion of individual MTX-PGs in relation to total MTX-PG

	Concentration, nmol/l*, median (IQR)	Proportion, %, median (IQR)
MTX-PG1	25.3 (17.9-33.8)	32.9 (25.5-44.4)
MTX-PG2	18.7 (14.8-24.3)	25.7 (21.5-29.5)
MTX-PG3	23.0 (13.9-33.9)	32.7 (24.0-38.5)
MTX-PG4	4.2 (2.0-8.7)	6.4 (3.6-9.5)
MTX-PG5	0.7 (0.3-1.8)	0.9 (1.5-0.9)
Total MTX-PG	79.0 (53.4-103.2)	Reference

*Expressed as nmol/liter of packed erythrocytes.
MTX-PG, methotrexate polyglutamate.treood.in nmol/liter of bloof rther information.mpliance, and knowledge of both EU and eastern-european markets and companies.

Long-chain MTX-PGs are associated with lower disease activity 3 months after MTX start

In cross-sectional analysis, higher concentrations of long-chain MTX-PG3, MTX-PG4, MTX-PG5 and their sum MTX-PG3–5 at 3 months were associated with lower JADAS-27 and hence lower disease activity at 3 months after MTX start (table 4). Moreover, these MTX-PGs were also associated with improvement in JADAS-27 between 3 months and the baseline (MTX-PG3: β=0.006 (−0.010 to −0.001), p=0.010; MTX-PG4: β=−0.015 (−0.025 to −0.005), p=0.005; MTX-PG5: β=−0.048 (−0.087 to −0.009), p=0.017 and MTX-PG3–5: β=−0.004 (−0.007 to −0.001), p=0.006). Conversely, short-chain MTX-PG1, MTX-PG2 and total MTX-PG were not significantly associated with JADAS-27 (table 4) and JADAS-27 improvement (data not shown).

Table 4. Associations of MTX-PGs with JADAS-27

	Cross-sectional analysis* (β (95%-CI))	Longitudinal analysis† (β (95%-CI))
MTX-PG1	0.000 (-0.004 to 0.003)	0.001 (-0.003 to 0.005)
MTX-PG2	-0.002 (-0.010 to 0.006)	-0.001 (-0.007 to 0.011)
MTX-PG3	-0.006 (-0.010 to -0.002) ‡	-0.005 (-0.010 to -0.001) §
MTX-PG4	-0.015 (-0.026 to -0.005) ‡	-0.014 (-0.026 to -0.003) §
MTX-PG5	-0.051 (-0.090 to -0.012) ‡	-0.049 (-0.092 to -0.007) §
MTX-PG3-5	-0.004 (-0.007 to -0.001) ‡	-0.004 (-0.007 to -0.001) §
Total MTX-PG	-0.002 (-0.003 to 0.000)	-0.001 (-0.003 to 0.001)

*Cross-sectional: multivariate linear regression for association of MTX-PGs with JADAS-27 at 3 months.
†Longitudinal: linear mixed model for association of MTX-PGs with JADAS-27 during the first year of MTX treatment.
‡Significant p values (<0.05) specified from top to bottom: p=0.005, p=0.004, p=0.011, p=0.003.
§Significant p values (<0.05) specified from top to bottom: p=0.028, p=0.014, p=0.023, p=0.018.
Example: The significant associations of MTX-PGs with JADAS-27 represent the following: with 1 nM increase in MTX-PG, the logarithmic JADAS-27 decreases by the respective β.
Abbreviations: JADAS, Juvenile Arthritis Disease Activity Score; MTX-PG, methotrexate polyglutamate.

The inverse relationship between MTX-PGs and JADAS-27 is also shown in figure 1. JADAS-27 showed the same pattern for all long-chain MTX-PGs and total MTX-PG, notably the higher the MTX-PG concentrations, the lower the JADAS-27. This pattern was not observed for JADAS-27 corresponding to short-chain MTX-PGs. The JADAS-27 geometric means (95% CI) corresponding to the 3rd MTX-PG tertile (highest concentration) were significantly lower than those of the 1st tertile for: MTX-PG3 (4.9 (3.9 to 6.2) vs 8.6 (6.8 to 11.0), p=0.004); MTX-PG4 (5.3 (4.1 to 6.7) vs 8.2 (6.4 to 10.4), p=0.037), MTX-PG5 (5.1 (4.0 to 6.6) vs 8.3 (6.5 to 10.7), p=0.021), MTX-PG3–5 (5.1 (4.0 to 6.5) vs 8.8 (6.9 to 11.2), p=0.006), and total MTX-PG (5.1 (4.0 to 6.6) vs 7.9 (6.2 to 10.2), p=0.042) (figure 1).

Additionally, compared to patients who continued MTX, long-chain MTX-PG concentrations (median, IQR) were significantly lower in patients who stopped MTX due to insufficient response (n=4) (MTX-PG3: 7.6 (1.8 to 14.3) vs 23.3 (14.9 to 34.3), p=0.009; MTX-PG4: 0.8 (0.1 to 3.1) vs 4.6 (2.1 to 8.8), p=0.020; MTX-PG5: 0.2 (0.1 to 0.7) vs 0.7 (0.3 to 1.8), p=0.046). In this group, MTX dose at start (median, IQR) and during follow-up (data not shown) were also significantly lower (6.3 (5.3 to 7.8) vs 9.9 (9.1 to 11.5)). Conversely, MTX-PG levels and MTX dose (data not shown) were comparable between patients who received concomitant medication due to insufficient response to MTX (n=7) and those who received MTX monotherapy (MTX-PG3: 32.4 (21.0 to 35.8) vs 23.7 (13.5 to 33.4), p=0.165; MTX-PG4: 6.8 (3.5 to 8.6) vs 4.0 (1.9 to 8.4), p=0.278; MTX-PG5: 1.0 (0.6 to 1.8) vs 0.7 (0.3 to 1.7), p=0.345).

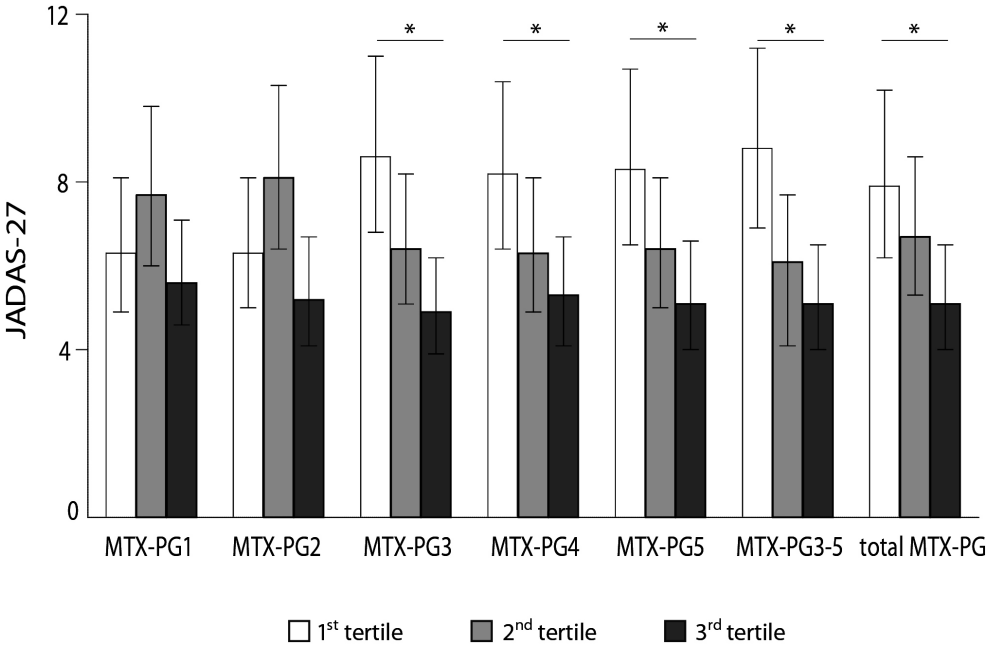

Figure 1. Juvenile Arthritis Disease Activity Score (JADAS)-27 at 3 months corresponding to methotrexate polyglutamates (MTX-PGs) divided into tertiles. Bars show geometric means (back-transformed logarithmic JADAS-27) and 95% CIs. The 1st tertile represents the lowest and the 3rd tertile the highest MTX-PG concentrations. MTX-PG concentrations (nmol/L) within tertiles are: MTX-PG1—1st: lowest to 20.8, 2nd: 20.9 to 29.5, 3rd: 29.6 to highest; MTX-PG2—1st: lowest to 15.6, 2nd: 15.7 to 22.0, 3rd: 22.1 to highest; MTX-PG3—1st: lowest to 17.2, 2nd: 17.3 to 29.4, 3rd: 29.5 to highest; MTXPG4—1st: lowest to 2.8, 2nd: 2.9 to 6.5, 3rd: 6.6 to highest; MTX-PG5—1st: lowest to 0.3, 2nd: 0.4 to 1.1, 3rd: 1.2 to highest; MTX-PG3–5—1st: lowest to 20.6, 2nd: 20.7 to 37.1, 3rd: 37.2 to highest; total MTX-PG—1st: lowest to 61.7, 2nd: 61.8 to 89.5, 3rd: 89.6 to highest concentration. The higher the concentrations of long-chain MTX-PGs and total MTX-PG, the lower the JADAS-27. This pattern was not observed for short-chain MTX-PGs. JADAS-27 corresponding to the 3rd MTX-PG tertile was significantly lower for long-chain MTX-PGs, their sum and total MTX-PG ($p<0.05$). JADAS: Juvenile Arthritis Disease Activity Score; MTX-PGs: methotrexate polyglutamates.

Long-chain MTX-PGs are associated with lower disease activity in the first year of MTX treatment

In the longitudinal analysis, higher concentrations of long-chain MTX-PG3, MTX-PG4, MTX-PG5 and MTX-PG3–5 at 3 months were significantly associated with lower JADAS-27 over time, and hence with lower disease activity in the first year after MTX start (table 2). The effect of abovementioned MTX-PGs on disease activity did not change over time ($p>0.05$).

MTX-PGs are not associated with adverse effects

MTX intolerance prevalence was determined in 89 patients in the first year after MTX start, of whom 45 (50.6%) were intolerant. In the first year after MTX start, hepatotoxicity was observed in 6 (5.3%) of 113, and bone marrow suppression (lymphopenia and granulopenia) in 4 (3.5%)

of 112 patients. MTX-PGs, measured at 3 months, were not associated with MTX intolerance (results shown for total MTX-PG) (OR: 0.99 (1.00 to 1.01), p=0.72), hepatotoxicity (OR: 1.02 (1.00 to 1.04), p=0.08) or bone marrow suppression (OR: 0.98 (0.90 to 1.10), p=0.57) in the first year after MTX start.

DISCUSSION

In a prospective JIA cohort, long-chain MTX-PG3, MTX-PG4, MTX-PG5 and MTX-PG3–5, measured after 3 months of MTX use, were associated with lower JADAS-27 at 3 months. Long-chain MTX-PGs were also associated with lower JADAS-27 during 1 year of MTX treatment. MTX-PGs were not associated with adverse effects.

In line with our findings, higher concentrations of total MTX-PG,[22,31,32] long-chain MTX-PG3[29,37] and MTX-PG5[34] have been associated with response to MTX in RA patients. Conversely, in a single study in RA, MTX-PGs were not associated with lower disease activity.[30] In fact, MTX-PG5 was associated with high rather than low disease activity.[30] In JIA, two studies also failed to show associations of total and individual MTX-PGs with inactive disease and response to MTX.[17,35] In these cross-sectional studies, contrary to our study, disease status was determined retrospectively, which could impact the reliability of the evaluated disease activity status and impede a fair comparison of disease activity status between patients. Furthermore, included patients used MTX for highly variable periods of time, which makes the comparison of MTX-PG concentrations between patients difficult, given that MTX-PG accumulation is dependent on the time of exposure to MTX.[23,37] These issues could have influenced their conclusions on the association of MTX-PGs and disease activity.

Our finding that long-chain MTX-PGs are associated with lower disease activity is consistent with the notion that longer-chain MTX-PGs are more potent inhibitors of target enzymes in the folate, purine and pyrimidine pathways[27] and in turn more potent mediators of MTX's therapeutic efficacy. Nevertheless, a recent study in RA showed that short-chain MTX-PG2 was associated with disease activity score (DAS)28 improvement after 16 weeks of MTX use.[33] In our validated study in RA, short-chain MTX-PG2 and long-chain MTX-PG3 and MTX-PG4, measured longitudinally, were associated with the lower DAS28 during 9 months of MTX treatment.[40]

By contrast with MTX-PG associations with disease activity, no MTX-PGs were associated with adverse effects in our cohort. However, another recent study in JIA did show associations of MTX-PG3–5 with gastrointestinal symptoms and elevated liver enzymes.[17] In the present study, relationship between MTX-PG and adverse effects could have been attenuated by the standard-of-care folic acid use, as this supplement reduces the occurrence of MTX-related adverse effects.[41,42] Moreover, low prevalence of hepatotoxicity (5.3%) and bone marrow suppression (3.5%) in our cohort could have led to spurious conclusions on MTX-PG associations with these adverse effects. In line with our findings in JIA, no associations of

MTX-PGs with adverse effects were found in RA.[28–30,40] Intracellular folate status, rather than MTX-PGs, could be associated with adverse effects. Lower erythrocyte folate polyglutamate concentrations were associated with a history of MTX toxicity in juvenile arthritis patients not currently receiving MTX.[43] Conversely, in our validated study in RA, baseline erythrocyte folate concentrations were not associated with adverse effects.[44]

Similar to earlier studies, long-chain MTX-PG3 was the predominant polyglutamate sybtype.[30,37,45,46] However, MTX-PG concentrations found in our cohort, were lower than previously reported.[30,37,45,46] This could be explained by differing methods used to measure MTX-PGs. We employed MS using stable isotopes to quantify MTX-PGs,[24] whereas others used either MS without stable isotopes46 or liquid chromatography without MS.[30,37] These methods might be less specific and prone to interferences by compounds similar to MTX, such as folates.[24] Nevertheless, compared with our RA study,[40] where MTX-PGs were measured using the same MS method, MTX-PG levels in JIA remained lower, likely due to lower age.[37,45,46] Compared with previous findings in JIA,46 short-chain MTX-PG concentrations were higher (MTX-PG1: 25.3 vs 16.8; MTX-PG2: 18.7 vs 11.8) and long-chain MTX-PG concentrations lower in our cohort (MTX-PG3: 23.0 vs 37.1; MTX-PG4: 4.2 vs 10.3; MTX-PG5: 0.7 vs 2.7). This could be explained by longer MTX use in the abovementioned study (median: 3 years) compared with an average of 3 months in our study, since longer exposure to MTX leads to selective enrichment of long-chain MTX-PGs, at the expense of short-chain MTX-PGs.[37]

The present study is the first step towards the use of MTX-PGs as a TDM tool, as it showed that long-chain MTX-PGs, measured early after MTX start, are related to low disease activity 3 months after MTX start, and also during the first year of MTX treatment. MTX-PGs could be used as a TDM tool to guide clinical decision making, notably to determine whether a patient would benefit from an increase in MTX dose, or whether additional medication, such as biologicals, should be given. In the present study, potential use of MTX-PGs as a TDM tool can be illustrated in patients who stopped MTX and those who received additional medication, due to insufficient effect. Patients who discontinued MTX received lower MTX doses and had lower long-chain MTX-PG concentrations than those who continued MTX. Instead of stopping MTX, these patients may have benefited from MTX dose escalation. On the other hand, patients on additional medication at 6 months had similar MTX doses and MTX-PG concentrations at 3 months, as patients on MTX monotherapy. They remained non-responders, in spite of optimal MTX treatment (reflected by adequate polyglutamation). If timely monitored with TDM, they could have received additional medication earlier than 6 months after MTX start. Taken together, TDM of MTX-PGs could guide clinicians to escalate MTX dose in patients with a low polyglutamation rate, and to offer biologicals in non-responders with adequate polyglutamation.

In order to use MTX-PGs as a TDM tool, MTX-PG pharmacokinetics, in response to MTX dose escalation and/or changes in the route of administration, needs to be determined with

sequential MTX-PG measurements during the first year of MTX treatment. Indeed, higher concentrations of preferentially long-chain MTX-PGs accumulate in patients using higher MTX dose and receiving parenteral MTX.[37,45,46] In our study, higher concentrations of long-chain MTX-PG3,4 and total MTX-PG were associated with higher baseline MTX dose. Knowing how to influence accumulation and concentrations of MTX-PGs, with the aim of maximising response to MTX, could enable optimisation of MTX treatment for individual patients. Pharmacokinetics will therefore be the focus of future research.

In conclusion, this is the first study to show that higher concentrations of long-chain MTX-PG3, MTX-PG4 and MTX-PG5 at 3 months of MTX use are associated with lower disease activity at 3 months and during 1 year of MTX treatment in a prospective JIA cohort. MTX-PGs were, however, not associated with adverse effects. Long-chain MTX-PGs, measured early after MTX start, are a potential TDM tool in JIA.

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6

INTERPRETATION OF THE JUVENILE ARTHRITIS DISEASE ACTIVITY SCORE: RESPONSIVENESS, CLINICALLY IMPORTANT DIFFERENCE AND LEVELS OF DISEASE ACTIVITY IN A PROSPECTIVE COHORT OF PATIENTS WITH JUVENILE IDIOPATHIC ARTHRITIS

Maja Bulatović Ćalasan[1], Lara D. de Vries[1], Sebastiaan J. Vastert[1], Marloes W. Heijstek[1] and Nico M. Wulffraat[1]

[1]Department of Pediatric Rheumatology and Immunology, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht, The Netherlands.

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ABSTRACT

Objectives

The objectives of this study were to assess 27-joint Juvenile Arthritis Disease Activity Score (JADAS-27) responsiveness, JADAS-27 changes corresponding to clinically important differences and cut-off scores for low and high disease activity in a large prospective JIA cohort.

Methods

JADAS-27 responsiveness, using effect size and standardized response mean (SRM), and changes in the JADAS-27 corresponding to clinically important differences were determined for clinical improvement (ACRpedi30) and worsening (flare). To assess whether various degrees of change in the JADAS-27 could be used to demonstrate improvement or worsening in individual patients, diagnostic parameters were computed for cut-off score changes. Finally, cut-off scores for low and high disease activity and their diagnostic parameters were determined.

Results

In 228 patients with 529 consecutive visits, ACRpedi30 was detected in 109 and flare in 111 visits. Regarding responsiveness, the effect size was 0.93 and SRM was 1.26 for clinical improvement, while for clinical worsening the effect size was 0.65 and SRM was 0.60. Changes in the JADAS-27 corresponding to clinically important difference were −5.5 for improvement and +1.7 for worsening. Cut-off score changes in the JADAS-27 had 65–90% sensitivity and 67–86% specificity for improvement, and 31–64% sensitivity and 89–97% specificity for worsening. The JADAS-27 cut-off score for low disease activity was ≤2.7 with 76% sensitivity and 62% specificity, and the cut-off score for high disease activity was ≥6 with 77% sensitivity and 77% specificity.

Conclusion

The JADAS-27 had moderate to good responsiveness and was changed by clinically important differences. The JADAS-27 cut-off scores differentiated between low and high disease activity. These JADAS-27 interpretations could be potentially applicable in clinical care and trials.

INTRODUCTION

JIA is the most common chronic rheumatic disease in childhood.[1] JIA disease activity is variable and appropriate disease control is crucial to prevent irreversible joint destruction and long-term disabilities.[2] To assess and monitor the extent of disease control in JIA, a new composite disease activity score, the 27-joint Juvenile Arthritis Disease Activity Score (JADAS-27), was recently developed and validated.[3] In contrast to relative measures such as the ACR paediatric (ACRpedi) criteria, the JADAS-27 is an absolute disease activity measure that can be used to determine and evaluate disease activity status and course in individual patients.

The ultimate goal is to use this composite score in daily clinical practice and clinical trials, as is done for the DAS in adult RA.[4, 5] In order to use the JADAS-27 in these clinical settings, it is crucial to determine its responsiveness, changes in JADAS-27 that correspond to clinically important differences and the cut-off scores that categorize patients into low and high disease activity. Although these JADAS-27 interpretations are essential for monitoring disease activity in individual patients over time and for comparison of disease activity status between patients, they have not been determined to date. The objectives of this study were to determine the JADAS-27 responsiveness, changes in score corresponding to clinically important difference and cut-off values for low and high disease activity in a large prospective JIA cohort.

PATIENTS AND METHODS

Patients

Clinical data on disease characteristics, disease activity and medication use of JIA patients was prospectively gathered every 3 months for 1 year between August 2007 and April 2011 for three investigator-initiated clinical trials concerning the safety and efficacy of vaccinations (NCT00731965, NCT00815282) and the occurrence of MTX intolerance (ISRCTN13524271). Each patient had up to five outpatient ward visits. The original clinical trials were performed at the University Medical Centre Utrecht (UMCU) and were approved by the Ethics Committee of the UMCU and the Central Committee on Research involving Human Subjects. JIA patients of six subtypes (persistent oligoarticular, extended oligoarticular, polyarticular, psoriatic, enthesitis-related and systemic JIA) with a confirmed diagnosis according to the ILAR criteria were included.[6] Patients having uveitis without joint involvement were excluded. Full ethics approval of the data analysis described in this article was retrospectively obtained from the Central Committee on Research involving Human Subjects.

JADAS-27 computation

The JADAS-27 (range 0–57) was computed by summing the scores of four core-set criteria [3]: physician's global assessment of disease activity (PGA) on a 10 cm visual analogue scale (VAS); parent/patient global assessment of well-being on a 10 cm VAS[7]; active arthritis, defined as joint swelling or limitation of movement accompanied by pain and tenderness, assessed in 27 joints; and ESR (mm/h) normalized to a 0–10 scale, using the formula ESR – 20/10, whereby, before the calculation, ESR values <20 mm/h were converted to 0 and ESR values >120 mm/h were converted to 120. A higher JADAS-27 indicates higher disease activity and a lower JADAS-27 indicates lower disease activity and is favourable to the patient.

Responsiveness

Responsiveness represents the instrument's capacity to detect a change in health status.[8] More specifically, responsiveness represents the capacity of the JADAS-27 to detect a change in disease status. The changes in disease status were classified into disease improvement, defined as an ACRPedi30 [9] response, and disease worsening, defined as a flare.[10] A flare is defined as worsening of ≥40% in two or more core-set criteria (PGA, number of active joints, number of joints with limitation of movement, physical functional ability [measured with the Childhood Health Assessment Questionnaire (CHAQ)], parent/patient assessment of patient's well-being and ESR) without an improvement of ≥30% in two or more of the remaining core-set criteria. To determine the changes in the JADAS-27 and subsequently the changes in disease status, two consecutive visits during the follow-up were compared to each other (3-month visit was compared with the baseline, 6-month compared with 3-month, 9-month compared with 6-month and 12-month compared with 9-month).

To assess the JADAS-27 responsiveness, we computed the effect size and the standardized response mean (SRM) for disease improvement and disease worsening.[11] The effect size was calculated by dividing the mean change in the JADAS-27 between consecutive visits by the S.D. of JADAS-27 scores at baseline (baseline is defined as the visit which the following visit is compared with). The SRM was calculated by dividing the mean change in the JADAS-27 between consecutive visits with the S.D. of that change. The threshold levels for both the effect size and SRM are ≥0.20 = small, ≥0.50 = moderate and ≥0.80 = good.[11]

Clinically important difference

A clinically important difference is a change in score of a construct that would be considered important (meaningful) from the perspective of a patient or a clinician.[8, 12] More specifically, a clinically important difference is a change in the JADAS-27 considered important from the perspective of a clinician. To determine clinically important differences, an anchor-based approach was used.[8, 12] Anchor-based methods require external patient-based or clinical criteria, so-called anchors, to inform whether changes in a construct are important (meaningful).[8] The

external criteria (anchors) used here were ACRpedi30, for disease improvement, and flare, for disease worsening. To determine whether disease improvement or worsening had occurred, two consecutive visits during the follow-up were compared.

First, clinically important differences were calculated as the median changes of the score for consecutive visits in which patients had satisfied the external criterion—ACRpedi30 or flare.[8, 13] We also tested whether the median changes in the JADAS-27, corresponding to clinically important difference, differed between JIA subtypes, using one-way analysis of variance with Bonferroni adjustment for multiple comparisons. Second, to determine how well calculated changes in the JADAS-27 discriminated between visits in which patients had an ACRpedi30 response or flare compared with visits in which they did not, receiver operating characteristic (ROC) curves and the corresponding areas under the ROC curves (AUC) were computed. Third, to examine whether various degrees of change in the JADAS-27 could be used to demonstrate improvement or worsening in individual patients in daily clinical practice, we computed the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for several integer cut-off score changes. These diagnostic parameters provide answers to the following questions: How likely is it that the change in score (i.e. ≤−2) will detect improvement, which has clinically occurred? (sensitivity); How likely is it that the change in score (i.e. >−2) will detect no improvement, which has also occurred clinically? (specificity); Given that a patient has a change in score of e.g. ≤−2, indicating improvement, how likely is this to be clinically correct? (PPV); and Given that a patient has a change in score of e.g. >−2, indicating no improvement, how likely is this to be clinically correct? (NPV).

Cut-off values for low and high disease activity

The JADAS-27 was computed for visits classified into low and high disease activity. Low disease activity was defined as stopping MTX or biologics or having NSAID monotherapy or no medication. Visits in which MTX or biologics were stopped due to adverse effects were excluded. High disease activity was defined as starting MTX, biologics or oral steroids. The JADAS-27 cut-off values were determined using a method previously employed to determine cut-off values for the DAS28 in RA.[14] The cut-off for low disease activity was set at the 75th percentile of the JADAS-27 of the low disease activity group, whereas the cut-off for high disease activity was set at the 25th percentile of the JADAS-27 of the high disease activity group. Determining the cut-off values based on the above-mentioned percentiles pre-defines sensitivity at ~75%. Subsequently the cut-off values' specificity, PPV and NPV were determined. All statistical analyses were performed using IBM SPSS Statistics, version 20.

RESULTS

Of 273 eligible patients, 1 patient with uveitis without joint involvement and 2 patients who failed to visit the outpatient ward were excluded. Two hundred and seventy patients with 1035 visits were included (1 visit, n = 16 patients; 2 visits, n = 22 patients; 3 visits, n = 39 patients; 4 visits, n = 107 patients; 5 visits, n = 86 patients). The JADAS-27 could be calculated in 789 (76.2%) visits (Table 1). The baseline JADAS-27 distribution (median 4.0; range 0–40.5) was skewed to the left (skewness 1.8, kurtosis 4.0). The missing JADAS-27 was due to the absence of the following core-set criteria: active joint count (n = 17), ESR (n = 93), parent/patient global assessment of well-being (n = 53) or a combination of these (n = 83). There were no differences in the remaining core-set criteria between visits with a computable JADAS-27 and those with a missing JADAS-27.

Table 1. Baseline characteristics of 270 JIA patients

Female, *n* (%)	183 (67.8)
Age at inclusion, mean (S.D.), years	9.9 (±4.2)
Age at onset, mean (S.D.), years	6.1 (±4.4)
Subtype JIA, *n* (%)	
Persistent oligoarticular JIA	102 (37.8)
Extended oligoarticular JIA	30 (11.1)
Polyarticular JIA[a]	92 (34.1)
Psoriatic arthritis	14 (5.2)
Enthesitis-related arthritis	8 (3.0)
Systemic onset JIA	24 (8.9)
Core-set Criteria, median (range)	
PGA (0-10)	1.4 (0-8.5)
Number of joints with limited range of motion	1 (0-25)
Number of joints with active arthritis	1 (0-27)
CHAQ disability (0-3)	0.5 (0-2.9)
Parent/patient global assessment of well-being (0-10)	2.0 (0-9.9)
ESR (mm/hour)	10 (2-140)
Medication, *n* (%)	
NSAIDs	165 (60.7)
Methotrexate	164 (60.7)
Dosage, median(range), mg/m^2/wk	10.2 (5.1-28.2)
Biologicals[b]	28 (10.4)
Steroids oral	9 (3.3)
Dosage, median(range), mg/kg/day	3.8 (0.1-15.0)
Steroids local	14 (5.2)
JADAS-27, median (range)	4.0 (0-40.5)

PGA: physician's global assessment of disease activity; CHAQ: Childhood Health Assessment Questionnaire, JADAS: Juvenile Arthritis Disease Activity Score involving 27 joints.
[a]RF positive *n*=14 (5.2%); RF negative *n*=70 (25.9%); RF unknown n=8 (3.0%). [b]Etanercept *n*=20 (7.4%); Anakinra *n*=7 (2.6%); Adalimumab *n*=1 (0.4%).

Responsiveness

To determine responsiveness, the change in the JADAS-27 was calculated for 529 consecutive visits of 228 patients. Of 529 visits, an ACRpedi30 response was detected in 109 (21%) and flare in 111 (21%). For visits in which patients had an ACRpedi30 response, the effect size was 0.93 and SRM was 1.26, whereas for visits in which patients had a flare, the effect size was 0.65 and SRM was 0.60. Therefore the JADAS-27 revealed good responsiveness to change for clinical improvement and moderate responsiveness to change for clinical worsening.

Clinically important difference

The clinically important difference for disease improvement (ACRpedi30) was a median change in the JADAS-27 of −5.5 [interquartile range (IQR) of −9.5 to −2.7]. The clinically important difference for disease worsening (flare) was a median change in the JADAS-27 of +1.7 (IQR +0.3 to +5.0). For disease improvement, the change (decrease) in the JADAS-27 in polyarticular JIA patients was higher than in oligoarticular JIA patients by 1.5 (95% CI 0.2, 2.9; P = 0.02). For disease worsening, the change (increase) in the JADAS-27 was higher in polyarticular JIA patients than in oligoarticular JIA patients by 2.5 (95% CI 1.1, 3.9; P < 0.001).

The AUC under the ROC curve of the above-mentioned changes in the JADAS-27 for clinical improvement was 0.86 (95% CI 0.83, 0.90), indicating that 86% of visits in which patients had an ACRpedi30 response were classified correctly. The AUC under the ROC curve of the above-mentioned changes in the JADAS-27 for clinical worsening was 0.84 (95% CI 0.80, 0.88), indicating that 84% of visits in which patients had a flare were classified correctly.

Table 2 presents the diagnostic parameters of various degrees of change in the JADAS-27 that could be used to monitor and follow individual patients in daily clinical practice. For clinical improvement, cut-off scores showed moderate to (very) good sensitivity of 65–90% (the cut-off score and score changes below it are likely to detect improvement, which has also occurred clinically) and moderate to (very) good specificity of 67–86% (score changes above the cut-off score are likely to detect no improvement, which has also occurred clinically). The best balance between sensitivity (80%) and specificity (78%) was reached at ≤−2. In case of clinical worsening, while cut-off scores had good to very good specificity of 89–97%, they showed relatively poor sensitivity of 31–64%. The best balance between sensitivity (64%) and specificity (89%) was reached at ≥1. PPV, which reveals how likely it is that a patient is clinically improved or worsened if he or she has a certain change in the JADAS-27, is an important parameter since it could be used by clinicians to interpret the score changes in individual patients and to follow them over time. While for clinical worsening the PPV was moderate (62–71%), for clinical improvement the PPV was relatively low (42–54%). On the other hand, the NPV was high for both clinical improvement and worsening (84–96%).

Table 2. Diagnostic parameters of the JADAS-27 cut-off values corresponding to clinical improvement (ACRpedi30) or worsening (flare)

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Clinical improvement				
≤ -1	90	67	42	96
≤ -2	80	78	48	94
≤ -3	72	83	53	92
≤ -4	65	86	54	90
Clinical worsening				
≥ 1	64	89	62	90
≥ 2	47	94	67	87
≥ 3	38	95	67	85
≥ 4	31	97	71	84

JADAS-27: Juvenile Arthritis Disease Activity Score involving 27 joints; ACR, American College of Rheumatology; NPV: negative predictive value; PPV: positive predictive value. JADAS-27 changes were calculated in 529 consecutive visits.

Cut-off values for low and high disease activity

In 316 (30.5%) visits, patients had low disease activity with a median JADAS-27 of 0.5 (IQR 0.0–2.7), whereas in 190 (18.4%) visits, patients had high disease activity with a median JADAS-27 of 11.2 (IQR 6.0–17.6) ($P < 0.001$) (Figure 1). Two cut-off values were selected: at the 75th percentile for low disease activity (JADAS-27 ≤2.7) and at the 25th percentile for high disease activity (JADAS-27 ≥6.0). At the cut-off ≤2.7, sensitivity was 76% and specificity was 62%; in 42% of visits with a JADAS-27 ≤2.7 (PPV), patients were identified correctly as having low disease activity and in 88% of visits with a JADAS-27 >2.7 (NPV), patients were identified correctly as having non-low disease activity. At the cut-off ≥6, sensitivity and specificity were 77%, with a PPV of 41% and an NPV of 94%.

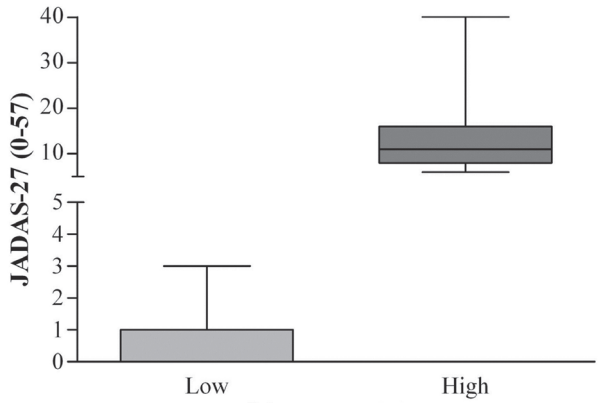

Figure 1. The JADAS-27 cut-off scores for low and high disease activity. The medians (ranges) of the JADAS-27 for low and high disease activity are depicted. These scores are significantly different ($P < 0.001$, Mann–Whitney *U*-test). Low disease activity was defined as visits in which MTX and/or biologics were stopped or in which patients used NSAID monotherapy or no medications. Twenty-four visits in which MTX or biologics were stopped due to adverse effects were excluded. High disease activity was defined as visits in which MTX and/or biologics and/or oral steroids were started.

DISCUSSION

We showed that the JADAS-27 has moderate to good responsiveness to changes in disease activity status. Changes in the JADAS-27 score corresponding to clinically important differences are −5.5 for disease improvement and +1.7 for disease worsening. Moreover, the JADAS-27 cut-off score is ≤2.7 for low disease activity and ≥6 for high disease activity.

The JADAS-27 responsiveness, determined using two measures, i.e. effect size and SRM, was moderate for disease worsening (>0.50) and good for disease improvement (>0.80). Similarly, others have demonstrated good responsiveness of the JADAS-27 in two longitudinal cohorts, with SRM values >0.8 (1.27 and 0.98).[3] Conversely, a clinically important difference for the JADAS-27 has not been previously demonstrated. In our cohort, clinically important differences were a median decrease in score of 5.5 for disease improvement and a median increase in score of 1.7 for disease worsening. Moreover, changes in scores for improvement and worsening were able to discriminate well between visits in which patients had a change in disease activity and visits in which they did not have a change in disease activity (AUCs 0.86 and 0.84, respectively).

In order to use the changes in score in daily clinical practice, clinicians should know how likely it is that patients improved or worsened if they had a certain change in the JADAS-27. Thus diagnostic parameters for various cut-off score changes were computed for disease improvement and worsening (Table 2). While all cut-off scores showed moderate to good sensitivity and specificity for disease improvement, they had relatively low PPVs (<54%), since <54% of visits in which patients had a given JADAS-27 change were not accompanied by ACRpedi30 improvement. Low PPVs could impede the use of cut-off scores for disease improvement in daily clinical practice, as clinicians would not be able to establish with great certainty that a patient with a particular change in score is indeed clinically improved (has reached ACRpedi30) or worsened (had a flare). On the other hand, the cut-off scores for clinical worsening had moderately good PPVs, but relatively low sensitivities, which could be due to an insufficient ability of the JADAS-27 to detect disease worsening or to the definition for disease activity worsening (flare). A flare occurred in an unexpectedly large proportion of visits (21%), suggestive of a lenient definition of flare. Indeed, if a stricter definition of worsening was used, namely a flare with an increase of at least 20% in PGA and 15% in ESR, only 3% of visits fulfilled the requirements for disease activity worsening. Contrary to PPVs, the NPVs for both clinical improvement and worsening were high, indicating that clinicians would be able to establish with great certainty that patients without a particular change in score are indeed clinically not improved or not worsened.

It is noteworthy that clinically important difference is not termed minimal clinically important difference for the following reason. To inform whether changes in the JADAS-27 were clinically important, external clinical criteria (anchors) of ACRpedi30 and flare were

used. Whether the change in the JADAS-27 is minimal depends on the anchor used. Although ACRpedi30 and flare are important from the prospective of a clinician, as they could provoke changes in the therapeutic approach, it is nevertheless possible that ACRpedi30 and flare are more than minimally important from the clinician's prospective. In order to establish minimal clinically importance difference, multiple anchors should be used, e.g. patient's or parent's opinion on the extent of change in disease activity (small vs moderate vs large). Furthermore, as we were unable to calculate the S.E.M.[12] due to a lack of more frequent (i.e. monthly) visits to measure the JADAS-27, we cannot exclude that JADAS-27 changes corresponding to the clinically important difference could be the result of a measurement error rather than true observed changes. However, keeping in mind that changes in the JADAS-27 were able to discriminate well between patients with and without disease improvement and worsening, it is unlikely that the changes in score are the result of a measurement error.

The JADAS-27 is able to discriminate between patients with low and high disease activity. The devised cut-off scores for low and high disease activity can be used to interpret disease activity status and to compare disease activity status between individual patients and patient groups. Recently Consolaro et al.[15] determined cut-off values for minimal, acceptable and inactive disease. Their cut-off for minimal disease activity (2.0–3.8) and inactive disease (1) corresponds to our cut-off for low disease activity of ≤2.7.[15] In addition to the above-mentioned study, we also computed the JADAS-27 cut-off for high disease activity. Although the sensitivity and specificity of the proposed cut-off values for disease activity were satisfactory, their PPVs were relatively low: the PPV of 42% for low disease activity indicates that in 58% of visits in which patients had a JADAS-27 ≤2.7, disease activity was non-low. Similarly, in 59% of visits with a JADAS-27 ≥6, disease activity was non-high. On the contrary, the NPVs for low and high disease activity reached 88% and 94%, respectively. In order to use the cut-off values in daily clinical practice, optimization of diagnostic parameters, and PPV in particular, is warranted. This could be achieved by determining cut-off values for low and high disease activity, defined using a different external criterion, such as parent or patient assessment of disease status, as has been previously done for cut-off values with minimal, acceptable and inactive disease.[15]

In conclusion, we determined the responsiveness of the JADAS-27, changes in the JADAS-27 corresponding to a clinically important difference for disease improvement and worsening and cut-off scores for low and high disease activity in a large prospective JIA cohort. If these results are refined and confirmed using different external criteria to define disease activity changes and states as well as validated in an independent JIA cohort, the above mentioned JADAS-27 interpretations could be potentially applicable in clinical practice and trials for monitoring and comparison of disease activity (changes) in and between individual patients.

KEY MESSAGES

- The JADAS-27 is responsive to change and can be changed by clinically important differences.
- The JADAS-27 differentiates between JIA patients with low and high disease activity.
- These JADAS-27 interpretations could be potentially applicable in clinical practice and trials to assess and monitor (changes in) disease activity.

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PART II

METHOTREXATE INTOLERANCE

7

HIGH PREVALENCE OF METHOTREXATE INTOLERANCE IN JUVENILE IDIOPATHIC ARTHRITIS: DEVELOPMENT AND VALIDATION OF A METHOTREXATE INTOLERANCE SEVERITY SCORE

Maja Bulatović[1], Marloes W. Heijstek[1], Marleen Verkaaik[1], E. H. Pieter van Dijkhuizen[1], Wineke Armbrust[2], Esther P. A. Hoppenreijs[3], Sylvia Kamphuis[4], Wietse Kuis[1], Toine C. G. Egberts[1], Gerben Sinnema[1], Carin M. A. Rademaker[1] and Nico M. Wulffraat[1]

[1]University Medical Centre Utrecht, Utrecht, The Netherlands; [2]University Medical Centre Groningen, Groningen, The Netherlands; [3]University Medical Centre St. Radboud, Nijmegen, The Netherlands; [4]Erasmus Medical Centre Rotterdam, Rotterdam, The Netherlands

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ABSTRACT

Objective

To design and validate a new questionnaire for identifying patients with methotrexate (MTX) intolerance, and to determine the prevalence of MTX intolerance in patients with juvenile idiopathic arthritis (JIA) using this questionnaire.

Methods

The MTX Intolerance Severity Score (MISS) questionnaire was constructed, consisting of 5 domains: stomach ache, nausea, vomiting, sore mouth, and behavioral symptoms. The domains each consisted of 3 questions pertaining to the presence of a symptom upon, prior to (anticipatory), and when thinking of (associative) MTX intake. The MISS questionnaire was validated in 86 patients by determining its discriminative power between patients with and those without MTX intolerance, identified as such by a gold standard (physician's opinion). Using the MISS questionnaire, the prevalence of MTX intolerance was determined in 297 JIA patients.

Results

The MISS questionnaire discriminated well between MTX-intolerant and MTX-tolerant patients. A cutoff score of 6 yielded the best sensitivity (88%) and specificity (80%). MTX intolerance was found in 150 (50.5%) of 297 patients. Of 220 patients receiving oral MTX, 98 (44.5%) experienced MTX intolerance, whereas 67.5% of 77 patients receiving parenteral MTX experienced intolerance to the drug ($P = 0.001$).

Conclusion

Our findings indicate that the MISS questionnaire is a highly sensitive and specific tool for the diagnosis of MTX intolerance, and that there is a high prevalence of MTX intolerance among JIA patients. The prevalence of intolerance in patients receiving parenteral MTX exceeds that in patients receiving oral MTX. The frequent occurrence of anticipatory and associative symptoms suggests that classic conditioning plays an important role in MTX intolerance.

INTRODUCTION

Juvenile idiopathic arthritis (JIA), an autoimmune disease characterized by chronic arthritis in one or more joints, is one of the most common chronic diseases in childhood, with a reported prevalence of between 16 and 159 per 100,000.[1] It encompasses various subtypes whose severity and clinical course differ.[1]

Methotrexate (MTX) is the first-choice disease-modifying antirheumatic drug (DMARD) for the treatment of JIA. It is an effective drug that induces disease remission in >70% of patients.[2, 3] Serious adverse effects, such as hepatotoxicity and bone marrow suppression, are infrequent and usually transient if MTX is stopped.[4] However, gastrointestinal adverse effects, which include nausea, abdominal pain, vomiting, or diarrhea, are common during MTX treatment.[5, 6] Folic acid supplementation is one of the accepted strategies to treat and prevent the development of these adverse effects.[7–10] Despite folic acid use, many JIA patients experience gastrointestinal adverse effects after MTX intake. JIA patients also develop anticipatory and associative gastrointestinal adverse effects occurring before MTX intake and when thinking of MTX as well as behavioral symptoms, such as restlessness and crying, when taking MTX.[6] These adverse effects arise as a conditioned response to the above-mentioned physical symptoms experienced after MTX intake.

Anticipatory nausea and vomiting, related to strongly emetogenic chemotherapy treatments, are well-known conditioned responses in cancer patients, with a reported frequency of 30%.[11, 12] In contrast to cancer patients, this problem has remained largely unrecognized in JIA patients. Although these adverse effects, in concert with behavioral symptoms, could lead to refusal and premature discontinuation of an otherwise efficacious and safe drug, their type and frequency are poorly elucidated.

The objectives of this study were to design and validate a new questionnaire for MTX-related gastrointestinal and behavioral symptoms, termed MTX intolerance, and to use this questionnaire to determine the prevalence of MTX intolerance in patients with JIA.

PATIENTS AND METHODS

Study design, study population and data collection

We performed a cross-sectional descriptive study in 4 University Medical Centres (UMC) in The Netherlands that have pediatric rheumatology departments (Utrecht, Nijmegen, Groningen, and Rotterdam). The MTX intolerance study was approved by the local medical ethics committees, and it was performed under good clinical practice conditions. The study population consisted of patients between 2 and 18 years of age, with a confirmed JIA diagnosis according to the International League of Associations for Rheumatology criteria[13], who were seen in the outpatient clinic between August 2007 and June 2009. All patients had been

receiving either oral or parenteral MTX for at least 3 months at the time of inclusion. Patients with a history of noncompliance to earlier treatments unrelated to MTX were excluded. Written informed consent was obtained from all patients and/or their parents.

Demographic data, such as sex, JIA subtype, and age at disease onset, were obtained from medical records. Information on duration of MTX use, MTX route of administration (oral or parenteral), and MTX dosage (mg/m2/week) was obtained. The physician's global assessment of disease activity (on a 0–10 scale) at the time the questionnaire was completed was documented. We acquired information on the concomitant use of folic acid, nonsteroidal antiinflammatory drugs (NSAIDs), oral steroids (mg/kg), and other DMARDs, such as anti–tumor necrosis factor α therapy and anti–interleukin-1 receptor blockade.

MTX Intolerance Severity Score

To determine the prevalence of MTX intolerance, we designed the MTX Intolerance Severity Score (MISS) questionnaire. MTX intolerance included gastrointestinal adverse effects and behavioral symptoms occurring after MTX intake, before MTX intake (anticipatory symptoms), and/or when thinking of taking the medication (associative symptoms). Behavioral symptoms were included in the MISS questionnaire since they often develop in response to MTX-induced gastrointestinal symptoms or the anticipation thereof. The MISS questionnaire was constructed by 2 physicians (MWH and NMW) and a psychologist (GS), based on their extensive clinical experience with JIA patients with MTX intolerance. The initial MISS questionnaire contained 16 items divided into 5 domains, namely, abdominal pain (stomach ache), nausea, vomiting, oral pain (sore mouth), and behavioral symptoms. The first 4 domains contained 3 items each, pertaining to adverse effects experienced after MTX intake as well as anticipatory and associative adverse effects. The fifth domain, on behavioral symptoms, included restlessness, crying, irritability, and refusal of MTX. On each item a patient could score 0 (no symptoms), 1 point (mild symptoms), 2 points (moderate symptoms), or 3 points (severe symptoms).

Validation of the MISS questionnaire

The initial MISS questionnaire was validated in JIA patients from UMC Utrecht by determining its ability to discriminate between patients with and those without MTX intolerance as established by the gold standard. Since there were no available instruments designed to measure MTX intolerance, a physician's opinion on the presence or absence of MTX intolerance was considered the gold standard. The physician indicated that a patient was intolerant to MTX if the patient or the patient's parents confirmed the following during a short interview at the outpatient clinic: the presence of gastrointestinal symptoms before and after MTX intake, a persistent nature of these symptoms, and/or a negative effect of these symptoms in the days after MTX intake. The physician was blinded with regard to the results of the questionnaire, and the patients were blinded with regard to the physician's opinion.

To analyze whether the MISS questionnaire could significantly ($P \leq 0.05$) discriminate between patients with and those without MTX intolerance, we compared the scores on each item of the questionnaire between the 2 groups, using the Mann-Whitney U test. Next, we removed the items that were not significantly discriminative between patients with and those without MTX intolerance. Additionally, to evaluate the reliability of the MISS questionnaire, its homogeneity was assessed by item-analysis using Cronbach's alpha. Items that lowered the homogeneity of the MISS questionnaire were removed to ensure optimal homogeneity. Subsequently, we determined whether the total score on the modified MISS questionnaire could discriminate between patients with and those without MTX intolerance. To evaluate the discriminant validity of the modified questionnaire, the area under the receiver operating characteristic (ROC) curve and the 95% confidence interval (95% CI) were computed. Next, we determined the optimal cutoff score on the MISS questionnaire for classifying patients as either tolerant or intolerant to MTX. Sensitivity, specificity, the sum of sensitivity and specificity, positive predictive value (PPV), and negative predictive value (NPV) were computed for 10 cutoff values for the MISS questionnaire. The cutoff value with the maximum score for the sum of sensitivity and specificity was considered the optimal cutoff point.

Prevalence of MTX intolerance

We determined the prevalence of MTX intolerance in JIA patients from all 4 UMCs, using the validated MISS questionnaire with the optimal cutoff score. Furthermore, scores on the MISS questionnaire and the prevalence of MTX intolerance in patients receiving oral MTX were compared with those in patients receiving parenteral MTX, by chi-square test. The Mann-Whitney U test was used to determine whether the total MISS and the scores for the separate domains of the MISS, as well as MTX dose, JIA subtype, physician's global assessment of disease activity, disease duration, and duration of MTX use, differed between MTX-intolerant patients who were receiving oral MTX and those who were receiving parenteral MTX.

To evaluate associations of MTX intolerance with clinically relevant covariates, such as the route of MTX administration, MTX dose, comedication (oral steroids, NSAIDs, antiemetics, biologic agents, or other DMARDs), JIA subtype, age, physician's global assessment of disease activity, disease duration, and duration of MTX use, we performed a multivariate logistic regression using a P value of >0.10 as a removal criterion and a P value of ≤ 0.05 as an inclusion criterion. Odds ratios (ORs) and 95% CIs were calculated to express these associations. Statistical analyses were carried out using SPSS, version 15.0.1.

RESULTS

The MISS questionnaire discriminates between patients with and those without MTX intolerance

For the purpose of validation of the MISS questionnaire, 89 patients attending the UMC Utrecht outpatient clinic completed the questionnaire. Three patients did not meet the inclusion criteria and were therefore excluded. According to the gold standard (physician's opinion), 29.1% of the patients experienced MTX intolerance.

All items of the MISS questionnaire were discriminative between patients with and those without MTX intolerance as determined by the gold standard (data not shown), with the exception of "vomiting when thinking of MTX" (associative vomiting) and the entire domain of oral pain. These 4 items also reduced the homogeneity of the MISS questionnaire, and removal of these items resulted in a high Cronbach's alpha of 0.919, which confirmed the reliability of the questionnaire. Upon exclusion of the 4 nondiscriminative items (i.e., associative vomiting and the oral pain domain), the total score of the modified 12-item MISS questionnaire remained discriminative between MTX-intolerant patients (median score 13 [interquartile range 7.5–18.5]) and MTX-tolerant patients (median score 1 [interquartile range 0–4]). The modified MISS questionnaire had a minimum possible score of 0 points and a maximum possible score of 36 points. The modified 12-item MISS questionnaire satisfies the criteria of feasibility, face validity, and content validity since it is short and easy to complete by patients or parents, easy to interpret by physicians, and contains all relevant aspects of MTX intolerance as established by a consensus of expert rheumatologists (WA, EPAH, SK, WK, and NMW). This questionnaire was used for further analysis and will be referred to as the MISS questionnaire (see Supplementary Figure 1).

The area under the ROC curve was 0.90 (95% CI 0.83–0.96), indicating that 90% of the patients were classified correctly with the MISS questionnaire. Based on the scores obtained, we expected to find the most discriminative value between 2 and 11 points. Table 1 shows sensitivity, specificity, PPV, and NPV for the scores between 2 and 11 points. Cutoff scores of 5 and 6 showed the highest sum score of sensitivity (88%) and specificity (80%), namely, 168%. At these cutoff points, 88% of the patients diagnosed as having MTX intolerance according to the gold standard would also be identified as intolerant to MTX according to the MISS questionnaire. Furthermore, 80% of the patients who were tolerant to MTX according to the gold standard would indeed be identified as MTX tolerant according to the MISS questionnaire. At these cutoff points, 65% of patients who were intolerant to MTX (PPV) and 94% who were tolerant to MTX (NPV) would be correctly diagnosed. The diagnostic parameters of the cutoff scores 5 and 6 were equal, since there were no patients in the validation cohort with a score of 5. Since MTX-intolerant patients scored high on gastrointestinal symptoms before MTX intake

and when thinking of MTX intake, we defined MTX intolerance as a total score of ≥6 with at least 1 point on anticipatory and/or associative and/or behavioral symptoms.

Table 1. Sensitivity, specificity, PPV and NPV for various cutoff scores on the MISS questionnaire*

Cutoff score	Sensitivity	Specificity	Σ†	PPV	NPV
2	100	59	159	50	100
3	92	67	159	53	95
4	92	72	164	58	96
5	88	80	168‡	65	94
6	88	80	168‡	65	94
7	84	80	164	64	92
8	76	82	158	63	89
9	64	84	148	62	85
10	60	90	150	71	85
11	60	90	150	71	85

*Values are the %. The Methotrexate Intolerance Severity Score (MISS) questionnaire was validated in a cohort of 86 patients. PPV = positive predictive value; NPV=negative predictive value.
† Sum of sensitivity and specificity.
‡ Optimal sum of sensitivity and specificity.

We found that 150 (50.5%) of the patients had MTX intolerance according to the above-mentioned definition (Table 3). Furthermore, the frequency of gastrointestinal adverse events in each domain was significantly higher in patients diagnosed as having MTX intolerance compared to patients without MTX intolerance. The percentages of patients diagnosed as having MTX intolerance who had nausea and behavioral symptoms were 91.3% and 88.7%, respectively. Twenty-eight (18.7%) of the MTX-intolerant patients experienced anticipatory vomiting, whereas this symptom did not occur in patients considered to be tolerant to MTX (Table 3). Twenty-three (56.1%) of the 41 patients who were taking antiemetics were intolerant to MTX.

Patients with MTX intolerance were receiving a slightly higher MTX dosage than patients without MTX intolerance (10.9 mg/m2/week and 9.8 mg/m2/week, respectively [P = 0.002]). Furthermore, MTX-intolerant patients had longer disease durations and received MTX for longer periods than did MTX-tolerant patients (median disease duration 4.3 years in MTX-intolerant patients versus 3.0 years in MTX-tolerant patients [P = 0.026] and median duration of MTX use 2.0 years in MTX-intolerant patients versus 1.2 years in MTX-tolerant patients [P = 0.001]). Patients with MTX intolerance were also somewhat younger than those without MTX intolerance (median age 11 years versus 12 years [P = 0.015]). JIA subtype and physician's global assessment of disease activity did not differ significantly between the 2 groups.

Table 2. Baseline characteristics of 297 patients at the time of completing the MISS questionnaire*

Baseline characteristics	Frequency (%)
Sex, female	204 (68.7)
JIA subtype	
Oligoarticular JIA, persistent	79 (26.6)
Oligoarticular JIA, extended	50 (16.8)
Polyarticular JIA, RF positive	20 (6.7)
Polyarticular JIA, RF negative	95 (32)
Systemic onset JIA	31 (10.4)
Enthesitis-related JIA	5 (1.7)
Psoriatic arthritis	17 (5.7)
Disease characteristics	
ANA positive†	141 (47.5)
RF positive‡	21 (7.1)
HLA B27 positive§	21 (7.1)
Chronic iridocyclitis	52 (17.5)
Age, mean ±SD	10.9 (3.9)
JIA duration in years, mean ±SD	4.9 (4.1)
Duration of MTX use, median (IQR) years	1.6 (0.6-3.6)
Disease activity, toxicity and medication use	
Physician's global assessment of disease activity, median (IQR) (0-10 scale)	0.5 (0-2)
MTX dose (mg/m^2/week), median (IQR)¶	10.2 (8.1-12.8)
Oral route of MTX administration	220 (74.1)
Folic acid	295 (99.3)
NSAIDs	189 (63.6)
Oral steroids	27 (9.1)
Antiemetics	41 (13.8)
Other DMARDS#	49 (16.5)

* Except where indicated otherwise, values are the number (%) of patients. MISS=Methotrexate Intolerance Severity Score; JIA=juvenile idiopathic arthritis; IQR=interquartile range; NSAIDs=nonsteroidal antiinflammatory drugs.

† Antinuclear antibody (ANA) status was determined in 282 patients.

‡ Rheumatoid factor (RF) status was determined in 212 patients.

§ HLA–B27 status was determined in 94 patients.

¶ Data on methotrexate (MTX) dose (mg/m^2) were available for 292 patients.

Of the 49 patients taking other disease-modifying antirheumatic drugs (DMARDs), 32 were taking etanercept, 9 were taking anakinra, 4 were taking adalimumab, and 4 were taking infliximab.

Table 3. Overall and per domain prevalence of MTX-related gastrointestinal complaints in all patients and by route of administration*

Prevalence	Tolerant to MTX	Intolerant to MTX	Oral MTX	Parenteral MTX
Total	147 (49.5)	150 (50.5)	220 (74.1)	77 (25.9)
Cut-off score ≥ 6	0 (0)	150 (100)	98 (44.5)*	52 (67.5)†
Abdominal pain	41 (27.9)	111 (74.0)	72 (73.5)	39 (75.0)
After MTX	34 (23.1)	100 (66.7)	66 (67.3)	34 (65.4)
Anticipatory and associative	12 (8.2)	85 (56.7)	54 (55.1)	31 (59.6)
Nausea	54 (36.7)	137 (91.3)	90 (91.8)	47 (90.4)
After MTX	43 (29.3)	126 (84.0)	83 (84.7)	43 (82.7)
Anticipatory and associative	22 (15.0)	116 (77.3)	75 (76.5)	41 (78.8)
Vomiting	7 (4.8)	74 (49.3)	45 (45.9)	29 (55.8)
After MTX	7 (4.8)	73 (48.7)	45 (45.9)	28 (53.8)
Anticipatory	0 (0)	28 (16.7)	14 (14.3)	14 (26.9)
Behavioural complaints	39 (26.5)	133 (88.7)	82 (83.7) §	51 (98.1)‡
Restlessness	22 (15.0)	116 (77.3)	69 (70.4) #	47 (90.4)§
Crying	2 (1.4)	69 (46.0)	38 (38.8) †	31 (59.6) ¶
Irritability	22 (15.0)	105 (70.0)	67 (68.4)	38 (73.1)
Refusal of MTX	22 (15.0)	116 (77.3)	69 (70.4) &	47 (90.4)§

* Values are the number (%) of patients. Intolerance to methotrexate (MTX) was defined as a score of ≥6 on the MTX Intolerance Severity Score questionnaire. For tolerance and intolerance, the percentages for each symptom are out of the total number of patients; for oral MTX and parenteral MTX, the percentages for each symptom are out of the number of patientswith a cutoff score of ≥6. Anticipatory refers to before MTX intake, and associative refers to when thinking of MTX.
† P=0.001 versus oral MTX, by chi-square test.
‡ P= 0.008 versus oral MTX, by chi-square test.
§ P=0.005 versus oral MTX, by chi-square test.
¶ P=0.015 versus oral MTX, by chi-square test.

Higher prevalence of MTX intolerance in patients receiving parenteral MTX than in patients receiving oral MTX

We determined the prevalence of MTX intolerance in patients receiving oral MTX and in patients receiving parenteral MTX. Table 3 shows that the prevalence of MTX intolerance was significantly higher in patients who were receiving parenteral MTX than in patients who were receiving oral MTX (67.5% and 44.5%, respectively [P = 0.001]). Significantly more patients who were receiving parenteral MTX than those who were receiving oral MTX refused to take the drug (P = 0.005) and exhibited restlessness (P = 0.005) as well as crying (P = 0.015) when taking MTX (Table 3). More patients who were receiving parenteral MTX than patients who were receiving oral MTX experienced vomiting after administration of MTX and anticipatory vomiting as well as anticipatory and associative abdominal pain and nausea, though these differences were not statistically significant. Although the prevalence of MTX intolerance was higher in patients receiving parenteral MTX, the severity of MTX intolerance did not differ between the 2 groups; the median score on the MISS questionnaire was 12 points for both patients receiving oral MTX and patients receiving parenteral MTX.

In The Netherlands, it is common clinical practice to initially treat patients with oral MTX; those patients in need of higher dosages to reach clinical efficacy are treated with parenteral MTX. As expected, patients receiving parenteral MTX received a higher median MTX dosage (13.5 mg/m2/week) than patients receiving oral MTX (9.6 mg/m2/week) ($P < 0.001$). JIA subtype, physician's global assessment of disease activity, duration of JIA, and duration of MTX use did not differ between the 2 groups. In addition, the median age of MTX-intolerant patients who were receiving oral MTX (10.7 years) was similar to that of MTX-intolerant patients who were receiving parenteral MTX (10.0 years).

In a multivariate logistic regression analysis, MTX intolerance was associated with the route of MTX administration only. Patients receiving parenteral MTX had higher odds of having MTX intolerance than patients receiving oral MTX (OR 1.9 [95% CI 1.01–3.58], $P = 0.046$). Moreover, patients receiving a higher MTX dose showed a trend toward increased odds of having MTX intolerance (OR 1.08 [95% CI 1.00–1.16], $P = 0.051$). Significant associations were not found between MTX intolerance and other covariates, such as age, comedication, JIA subtype, physician's global assessment of disease activity, disease duration, and duration of MTX use.

DISCUSSION

We designed and validated the MISS questionnaire. Using this tool we determined that the prevalence of MTX intolerance reached 50.5% in JIA patients.

To date, the only instrument used to determine gastrointestinal symptoms in JIA patients is the Gastrointestinal Symptom Scale for Kids (GISSK).[14] The GISSK assesses the presence and the severity of gastrointestinal symptoms, such as heartburn, bloating, nausea, vomiting, stomach ache, stool consistency, and loss of appetite. In contrast to the GISSK, which screens for gastrointestinal side effects in JIA patients in general and irrespective of treatment, the MISS questionnaire assesses gastrointestinal adverse effects related to MTX use specifically. Furthermore, unlike the GISSK, the MISS questionnaire considers the occurrence of MTX-related anticipatory and associative gastrointestinal adverse effects and behavioral symptoms. The MISS questionnaire is therefore a more appropriate tool to determine the frequency of such symptoms related to MTX intake.

We found a notably high prevalence of MTX intolerance. Several studies have demonstrated that gastrointestinal adverse effects occur frequently in JIA patients receiving MTX.[5, 14, 15] Three previous studies determined the prevalence of MTX-related gastrointestinal adverse effects, which ranged from 10% to 21% depending on the type of gastrointestinal symptoms.[3, 16, 17] The substantially higher prevalence of MTX intolerance in our study compared to the aforementioned findings could have several explanations. We used a specific definition to determine the prevalence of MTX intolerance, which included gastrointestinal symptoms before and after MTX intake as well as behavioral symptoms. Previously, these frequently

occurring symptoms were not taken into account, which could have resulted in the higher prevalence of MTX intolerance in our study. Moreover, we determined the prevalence of MTX intolerance with a structured questionnaire. This could have led the patients and their parents to consider the occurrence of MTX-related symptoms more thoroughly, leading to a higher prevalence.

The prevalence of MTX intolerance was 23% higher in patients receiving parenteral MTX than in patients receiving oral MTX. The higher prevalence of MTX intolerance in patients receiving parenteral MTX originates from a higher prevalence of behavioral symptoms as well as anticipatory and associative abdominal pain, nausea, and vomiting in these patients, although the differences in the prevalence of these gastrointestinal effects were not great. In addition to the aversion to MTX, fear of needles likely contributed to a higher prevalence of these adverse effects and in turn to a higher prevalence of MTX intolerance in patients receiving parenteral MTX.[18] However, the prevalence of these adverse effects in the absence of needles, namely in patients receiving oral MTX, is also very high. This shows that aversion to MTX plays an important role in the induction of anticipatory and associative gastrointestinal adverse effects and behavioral problems for both routes of administration.

The frequent occurrence of the above-mentioned symptoms before MTX intake supports the notion that classic conditioning mechanisms play an important role in the development of MTX intolerance. In classic conditioning terms, an unconditioned stimulus (i.e., MTX) produces an unconditioned response, such as abdominal pain, nausea, or vomiting, once MTX is administered. At that moment, many potential conditioned stimuli are present. The most commonly reported conditioned stimuli at our outpatient clinic are the yellow color of the pill or injection fluid, and the liquid with which MTX is administered, such as orange juice or water. After a number of weeks of taking MTX, the conditioned stimuli mentioned above lead to the conditioned response of anticipatory and associative adverse effects. It is thought that these pretreatment adverse effects occur as a result of stimulation of the higher centers of the central nervous system if a patient experiences the conditioned stimuli.[19] Therefore, cognitive–behavioral therapy may be beneficial in treating JIA patients with MTX intolerance.[6]

Anticipatory and associative adverse effects are clinically not very evident. Consequently, these symptoms cannot easily be detected by physician assessment only, but can be detected with the MISS questionnaire. Therefore, using the MISS questionnaire is advantageous, since it allows early detection of MTX intolerance symptoms, which could create a window of opportunity for the treatment of MTX intolerance before the conditioned response becomes so severe that it negatively affects the patient's quality of life or leads to early cessation of an otherwise effective MTX treatment.

Interestingly, posttreatment gastrointestinal adverse effects were equally prevalent in patients receiving oral MTX and those receiving parenteral MTX. This suggests that MTX, regardless of the route of administration, uses the same mechanism to induce posttreatment

gastrointestinal symptoms. Nausea and vomiting after treatment with cytostatic agents occur as a consequence of stimulation of the chemoreceptor trigger zone, which in turn stimulates the vomiting center in the medulla.[20] It is, however, unknown whether low-dose MTX treatment in JIA can stimulate the chemoreceptor trigger zone, thereby causing nausea and vomiting in these patients.

The findings of the present study do not indicate which clinical variables (i.e., MTX dose, age) are associated with the development of MTX intolerance and how many patients discontinue MTX due to intolerance. Large prospective trials following up JIA patients from the start of MTX treatment are needed to answer such questions. We are presently gathering a large prospective JIA cohort in order to address such issues.

To our knowledge, this is the first study to demonstrate a high prevalence of MTX intolerance in JIA patients, using a newly designed MISS questionnaire. Since the extent of MTX intolerance in JIA patients often remains unrecognized in clinical practice, we suggest that clinicians use the MISS questionnaire in their assessment of the frequency, severity, and type of MTX intolerance symptoms. We showed that anticipatory and associative gastrointestinal adverse effects as well as behavioral symptoms occurred frequently, strongly suggesting that classic conditioning plays a central role in the development of MTX intolerance. Therefore, we are currently performing a randomized controlled trial to compare the effect of cognitive–behavioral therapy on MTX intolerance with the effect of a switch to parenteral MTX or a continuation of oral MTX with a concomitant antiemetic.

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SUPPLEMENTARY INFORMATION

Supplementary Table 1. Methotrexate intolerance severity score – MISS

	NO Complaints	COMPLAINTS (score 1-3 points)		
		Mild	Moderate	Severe
	0	1	2	3
STOMACHACHE				
- My child has a stomachache after taking MTX	□	□	□	□
- My child has a stomachache several hours to one day before taking MTX	□	□	□	□
- My child has a stomachache when thinking of MTX	□	□	□	□
NAUSEA				
- My child is nauseous after taking MTX	□	□	□	□
- My child is nauseous several hours to one day before taking MTX	□	□	□	□
- My child is nauseous when thinking of MTX	□	□	□	□
VOMITING				
- My child vomits after taking MTX	□	□	□	□
- My child vomits hours to one day before taking MTX	□	□	□	□
BEHAVIORAL COMPLAINTS				
- My child is restless when taking MTX	□	□	□	□
- My child cries when taking MTX	□	□	□	□
- My child is irritable when taking MTX	□	□	□	□
- My child refuses to take MTX	□	□	□	□

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8

PREVALENCE OF METHOTREXATE INTOLERANCE IN RHEUMATOID AND PSORIATIC ARTHRITIS

Maja Bulatović Ćalasan[1], Oscar F.C. van den Bosch[1], Marjonne C.W. Creemers[2], Martijn Custers[3], Antonius H.M. Heurkens[4], Jan Maarten van Woerkom[5] and Nico M. Wulffraat[1]

[1]Department of Pediatric Immunology, Room KC 03.063.0, University Medical Center, Wilhelmina Children's Hospital, Utrecht 3508 AB, The Netherlands; [2]Department of Rheumatology, Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands; [3]Department of Rheumatology, Woerden, Maartenskliniek, The Netherlands; [4]Department of Rheumatology, Meander Medical Center, Amersfoort, The Netherlands; [5]Department of Rheumatology, Gelre Hospitals, Apeldoorn and Zutphen, The Netherlands

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ABSTRACT

Introduction

The aim of this study was to determine the prevalence of gastrointestinal and behavioural symptoms occurring before (anticipatory/associative) and after methotrexate (MTX) administration, termed MTX intolerance, in rheumatoid (RA) and psoriatic arthritis (PsA).

Methods

Methotrexate Intolerance Severity Score (MISS), previously validated in juvenile idiopathic arthritis patients, was used to determine MTX intolerance prevalence in 291 RA/PsA patients. The MISS consisted of four domains: abdominal pain, nausea, vomiting and behavioural symptoms, occurring upon, prior to (anticipatory) and when thinking of MTX (associative). MTX intolerance was defined as ≥6 on the MISS with ≥1 point on anticipatory and/or associative and/or behavioural items.

Results

A total of 123 patients (42.3%) experienced at least one gastrointestinal adverse effect. The prevalence of MTX intolerance was 11%. MTX intolerance prevalence was higher in patients on parenteral (20.6%) than on oral MTX (6.2%) ($p < 0.001$).

Conclusion

Besides well-known gastrointestinal symptoms after MTX, RA and PsA patients experienced these symptoms also before MTX intake. RA and PsA patients on MTX should be closely monitored with the MISS for early detection of MTX intolerance, in order to intervene timely and avoid discontinuation of an effective treatment.

INTRODUCTION

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are inflammatory disorders characterized by chronic arthritis.[1,2] In RA and PsA treatment, methotrexate (MTX) is the first-choice disease-modifying anti-rheumatic drug (DMARD) due to low costs, efficacy and an acceptable safety profile.[3,4] Serious adverse effects such as pulmonary toxicity, hepatotoxicity and bone marrow suppression are rare or transient if MTX is stopped.[5] In contrast, gastrointestinal adverse effects are common, affecting as many as 66% of patients.[2,6-11] Due to these adverse effects, up to 12% of RA and PsA patients discontinue MTX after 6 months to 2 years of treatment.[6-8,12]

Previously, we showed in juvenile idiopathic arthritis (JIA) that 50.5% of patients suffered not only from a myriad of gastrointestinal adverse effects after MTX intake, but also from adverse effects before MTX intake (anticipatory) and when thinking of MTX (associative).[13] The latter symptoms arise as a classical conditioning response to gastrointestinal symptoms after MTX administration. Therefore, the nature of MTX-induced gastrointestinal adverse effects, which we termed MTX intolerance, is complex, and could even further impede the use of an otherwise effective drug. Although MTX-induced gastrointestinal adverse effects occur frequently in RA and PsA, severity and the type - in particular the occurrence of anticipatory and associative symptoms - have not been assessed.

The aim of this study was to determine the type and prevalence of MTX-induced gastrointestinal adverse effects, with a standardized questionnaire, in a large cohort of RA and PsA patients.

METHODS

Study design and patients

A cross-sectional descriptive study (ISRCTN13524271) included RA and PsA patients attending the outpatient wards of four general hospitals between May 2011 and June 2012. All patients were treated with MTX for at least 3 months and received weekly folic acid (5 to 15 mg).[5] Patients' data on disease activity, MTX dose and route of administration, co-medication, history of peptic ulcers and smoking was collected. The study was approved by the medical ethics committees of the University Medical Center Utrecht and the four general hospitals in 's-Hertogenbosch, Woerden, Amersfoort and Apeldoorn where the patients were included. As the study burden for patients was low and required no treatment changes, the ethics committees waived the need for informed consent.

MTX intolerance severity score

To determine the prevalence of MTX-induced gastrointestinal adverse effects, patients completed the methotrexate intolerance severity score (MISS), previously developed and validated in JIA.[13] The MISS consists of four domains: abdominal pain, nausea, vomiting and behavioural symptoms, assessing symptoms after MTX administration, anticipatory (before MTX) and associative symptoms (when thinking of MTX). The behavioural symptoms domain includes restlessness, irritability and refusal of MTX, which develop in response to MTX-induced gastrointestinal symptoms and anticipation thereof. A patient could score 0 (no symptoms), 1 (mild symptoms), 2 (moderate symptoms) or 3 (severe symptoms) points on each item. MTX intolerance was defined as ≥6 points, including at least one anticipatory, associative or behavioural symptom.[13]

MTX intolerance prevalence

The prevalence was determined of: a) individual symptoms in all patients; b) MTX intolerance, defined as above; c) individual symptoms in MTX intolerant versus tolerant patients. MTX intolerance prevalence was compared between patients on oral and parenteral MTX (chi-square test). MTX intolerance severity, age, MTX dose, disease activity parameters and medication use were compared in tolerant versus intolerant patients, and in intolerant patients on oral versus parenteral MTX (*t*-test, Mann–Whitney *U*-test). To evaluate associations of MTX intolerance with clinically relevant covariates - disease activity score (DAS)-28, physician global assessment (PGA), age, MTX dose, MTX route and non-steroidal anti-inflammatory drug (NSAID) use - multivariate logistic regression was performed. Statistical analyses were carried out with IBM SPSS Statistics, version 20.

RESULTS

Baseline characteristics

Of 296 patients, 5 were excluded due to diagnosis other than RA or PsA (n = 3 with ankylosing spondylitis; n = 1 with peripheral spondyloarthritis; and n = 1 with scleroderma). Table 1 shows the baseline characteristics of 291 patients; the majority was female (62.2%), 249 (85.6%) had RA and 42 (14.4%) had PsA with low to moderate DAS-28.

Table 1. Baseline characteristics of 291 patients at the time of completing the MISS*

Sex, female	181 (62.2)
Age, mean +/- SD years	59.4 +/- 12.4
Diagnosis	
Rheumatoid arthritis	249 (85.6)
Psoriatic arthritis	42 (14.4)
Disease activity	
Disease activity score 28, median (IQR)[a]	2.5 (1.7-3.2)
Physician's global assessment, median (IQR), (0-10 scale)[b]	2.0 (1.0-3.0)
Erythrocyte sedimentation rate (mm/hour)	11.0 (5.0-22.0)
MTX use	
Route of administration, oral	194 (66.7)
Dose (mg/week), median (IQR)	20.0 (12.5-25.0)
Other medication	
NSAIDs	145 (49.9)
Proton-pump inhibitors	127 (43.6)
Antiemetics	5 (1.7)
Oral steroids	31 (10.7)
Other DMARDs[c]	72 (24.7)

Characteristics are as calculated at the time of completing the MISS except where indicated otherwise. Values are number (%), except where indicated otherwise. [a]Disease activity score 28 was determined in 266 patients (274 rheumatoid arthritis and 19 psoriatic arthritis patients); [b] physician's global assessment was determined in 268 patients; [c]of 72 patients on other DMARDs, 26 were on DMARDs (plaquenil, n = 24; leflunomide, n = 2) and 46 were on biologic agents (infliximab, n = 24; adalimumab, n = 10; etanercept, n = 9; abatecept, n = 2; golimumab, n = 1). MISS, methotrexate intolerance severity score; MTX, methotrexate; NSAIDs, nonsteroidal anti-inflammatory drugs; DMARDs, disease-modifying anti-rheumatic drugs.

MTX intolerance prevalence in RA and PsA

One hundred and twenty-three (42.3%) RA and PsA patients experienced at least one gastrointestinal symptom during MTX treatment. The most prevalent gastrointestinal symptom after MTX administration was nausea, affecting 93 (32.0%) patients, whereas abdominal pain occurred in 11.3% and vomiting in 6.5% (Table 2). Pre-treatment nausea was the most prevalent; 8.6% had anticipatory and 11.0% associative nausea. Anticipatory vomiting was the least prevalent, affecting 1.7% (Table 2). Behavioural symptoms, overall, affected 16.5% of patients, with restlessness being the most prominent symptom in 13.1% of patients (Table 2).

MTX intolerance was found in 32 (11.0%) patients having a median score of 9 (IQR: 6.25 to 12.00). The prevalence and severity of MTX intolerance was similar in RA (n = 26 (10.4%), score 9 (6.8 to 12.3)) and PsA (n = 6 (14.3%), score 7 (6.0 to 13.0)). All intolerant patients (100%) experienced post-treatment nausea, whereas 46.9% had post-treatment abdominal pain and 31.3% had post-treatment vomiting (Table 2). The most prevalent pre-treatment gastrointestinal symptoms were anticipatory and associative nausea, affecting 56.3% and 53.1% of intolerant patients respectively, followed by anticipatory abdominal pain in 37.5% and associative abdominal pain in 34.4%. Anticipatory vomiting occurred in 15.6% of intolerant

patients, whereas this symptom did not occur in tolerant patients. Overall, behavioural symptoms occurred in 81.3% of intolerant patients, of whom 37.5% refused MTX.

MTX-intolerant patients were younger than the MTX-tolerant (mean age 51.6 +/- 12.2 versus 60.4 +/- 12.1 years, $P < 0.001$). MTX dose, DAS-28, PGA, erythrocyte sedimentation rate (ESR), co-medication use, history of peptic ulcers (3.8% of all patients) and smoking (25.8% of all patients) did not differ between the two groups. Gender distribution did not differ between MTX-intolerant and MTX-tolerant patients (female, 75.0% versus 60.6%; male, 25.0% versus 39.4%), but more female (75%) than male patients (25%) were intolerant, although this was not statistically significant.

MTX intolerance prevalence in patients on oral and parenteral MTX

MTX intolerance prevalence was significantly higher in patients on parenteral (20 of 97, 20.6%) than on oral MTX (12 of 194 m 6.2%, $P < 0.001$) (Table 2). Significantly more patients on parenteral than on oral MTX exhibited behavioural symptoms ($P = 0.02$), whereas other symptoms were comparable between the two groups. The median MTX intolerance score was higher in intolerant patients on parenteral than on oral MTX, although not significantly (9.5, IQR 7.0 to 15.5) versus 7.5, IQR 6.0 to 9.0), $P = 0.08$). Patients on parenteral MTX received the same MTX dose (20.0 mg/week, IQR 15.0 to 25.0) as patients on oral MTX (20.0 mg/week, IQR 15.0 to 20.0).

In the multivariate analysis, older patients were less likely to have MTX intolerance (odds ratio (OR) 0.93, 95%m, CI 0.89, 0.97; $P = 0.001$). If age was stratified into two groups, namely ≥65 and <65 years, older patients were again less likely to have MTX intolerance (OR 0.21, 95% CI 0.06, 0.85; $P = 0.03$) whereas patients with higher PGA (OR 1.26, 95% CI 1.05, 1.51; $P = 0.01$) and those receiving parenteral MTX (OR 3.88, 95% CI 1.41, 10.62; $P = 0.01$) were more likely to have MTX intolerance.

Table 2. Prevalence of MTX-related gastrointestinal symptoms in all patients and in intolerant patients by route of MTX administration*

	All patients	Tolerant to MTX	Intolerant to MTX	Oral MTX	Parenteral MTX
Total	291 (100)	259 (89.0)	32 (11.0)	194 (66.7)	97 (33.3)
Cut-off score ≥6	32 (11.0)	0 (0)	32 (100)	12 (6.2)	20 (20.6)[a]
Abdominal pain	44 (15.1)	24 (9.3)	20 (62.5)	7 (58.3)	13 (65.0)
After MTX	33 (11.3)	18 (6.9)	15 (46.9)	5 (41.7)	10 (50.0)
Anticipatory	17 (5.8)	5 (1.9)	12 (37.5)	5 (41.7)	7 (35.0)
Associative	17 (5.8)	6 (2.3)	11 (34.4)	4 (33.3)	7 (35.0)
Nausea	100 (34.4)	68 (26.3)	32 (100)	12 (100)	20 (100)
After MTX	93 (32.0)	61 (23.6)	32 (100)	12 (100)	20 (100)
Anticipatory	25 (8.6)	7 (2.7)	18 (56.3)	6 (50.0)	12 (60.0)
Associative	32 (11.0)	15 (5.8)	17 (53.1)	5 (41.7)	12 (60.0)
Vomiting	22 (7.6)	11 (4.2)	11 (34.4)	5 (41.7)	6 (30.0)
After MTX	19 (6.5)	9 (3.5)	10 (31.3)	5 (41.7)	5 (25.0)
Anticipatory	5 (1.7)	0 (0)	5 (15.6)	2 (16.7)	3 (15.0)
Behavioral symptoms	48 (16.5)	22 (8.5)	26 (81.3)	7 (58.3)	19 (95.0)[b]
Restlessness	38 (13.1)	16 (6.2)	22 (68.8)	6 (50.0)	16 (80)
Irritability	29 (10.0)	7 (2.7)	22 (68.8)	5 (41.7)	17 (85.0)[c]
Refusal of MTX	13 (4.5)	1 (0.4)	12 (37.5)	2 (16.7)	10 (50.0)

Values are number (%) of patients. All domains and individual items differentiate between tolerant and intolerant patients ($P < 0.001$).
[a]$P < 0.001$ versus oral MTX, by chi-square test; [b]$P = 0.02$ versus oral MTX, by chi-square test, [c]$P = 0.02$ versus oral MTX, by chi-square test. MTX, methotrexate.

DISCUSSION

We showed that besides the well-known MTX-induced gastrointestinal symptoms upon MTX administration, RA and PsA patients also had anticipatory and associative gastrointestinal and behavioural symptoms before MTX administration, collectively termed MTX intolerance. MTX intolerance prevalence in RA and PsA patients was 11%.

Studies in RA have found similar occurrence rates compared to our study[7,11]; nausea was the most prevalent symptom, occurring in 14.4 to 28.0% compared to 32.0% in our cohort, followed by abdominal pain in 9.7 to 10.6% compared to 11.3% in our cohort and vomiting in 3.4% compared to 6.5% in our cohort. Of note is that comparisons were made between symptoms occurring only after MTX, as it is likely that previous studies took solely these symptoms into account (not the pre-treatment symptoms).

In contrast to JIA in which the prevalence of MTX intolerance reached 50.5%, the prevalence in RA/PsA was considerably lower at 11%. MTX intolerance severity was lower in adults (score 9) than in children (score 12) ($P = 0.003$). Substantially lower MTX intolerance prevalence in RA/PsA was due to: a) lower percentage of adults with score ≥6, and b) lower percentage of adults (24.4% versus 67% in JIA) with at least one anticipatory, associative and/or behavioural symptoms. As anticipatory and associative symptoms arise as classic conditioning responses to physical symptoms upon MTX use, the lower percentage of RA/PsA patients with pre-treatment symptoms suggests a weaker, classic, conditioning response in adults than in children taking MTX. This is supported by the fact that, whereas 82% of 204 JIA patients with symptoms after MTX also had symptoms before MTX intake, only 51% of 106 RA/PsA patients with symptoms after MTX had symptoms before MTX intake.

MTX intolerance prevalence was higher in patients on parenteral (20.8%) than on oral MTX (6.2%), which we also demonstrated for JIA.[13,14] This difference was caused by more behavioural symptoms in the parenteral group. Aversion towards needles, besides aversion towards MTX, could have contributed to a higher prevalence of these symptoms. It is common to switch patients from oral to parenteral MTX due to gastrointestinal symptoms.[5] Indeed, 13 of 20 intolerant patients on parental MTX had been switched to this route from oral MTX due to gastrointestinal symptoms. Considering their past symptoms on oral MTX, the patients who switched may have been more prone to develop gastrointestinal and behavioural symptoms on parenteral MTX, resulting in higher MTX intolerance prevalence in the parenteral group.

Besides the observed association between parenteral MTX and MTX intolerance, age was also associated with MTX intolerance, namely older patients (>65 years) were less likely to have MTX intolerance than younger patients (≤65 years). In previous studies, neither younger nor older age (>65 years) was associated with occurrence of MTX-related gastrointestinal and other side effects.[15,16] Validation studies are required to determine whether younger age is a risk factor for MTX intolerance.

Anticipatory and associative gastrointestinal symptoms could have a negative impact on patients' quality of life[14] and impede the use of MTX. Nevertheless, these symptoms are clinically not very evident.[13] Consequently, they cannot be easily detected by physician assessment only, but can be detected using the MISS.[13] Therefore, using the MISS is advantageous as it allows early detection of symptoms. This could create a window of opportunity for timely treatment of MTX intolerance, as well as for early treatment of emerging physical symptoms, which could prevent the development of conditioned responses and therefore MTX intolerance. Similar to JIA, treatment of (physical) symptoms could include lowering the MTX dose,[17] switching to parenteral MTX[14,18,19] or starting behavioural therapy[20] or anti-emetics.[19]

Although the MISS was validated and employed to measure MTX intolerance prevalence in JIA, it provided a structured platform to assess the type of MTX-induced gastrointestinal symptoms in RA/PsA. Nevertheless, the MISS should be validated in adults with rheumatic diseases. Furthermore, this study does not reveal variables associated with MTX intolerance development, nor does it demonstrate the frequency of MTX discontinuation or of switching to other medication due to MTX intolerance. Prospective trials are required to address these issues.

This is the first study to demonstrate using a standardized questionnaire, that MTX intolerance occurs in 11%, more frequently in patients on parenteral than on oral MTX, and possibly persists after a switch from oral to parenteral MTX. Since persistent gastrointestinal symptoms are the major reason to discontinue MTX, intolerant patients could be more prone to stop MTX or switch to (less effective) DMARDs or expensive biological agents.[12] Upon validation in adults, the MISS may be used in daily clinical practice to closely monitor patients and to intervene timely using the abovementioned approaches in order to prevent or reduce the negative impact of MTX intolerance on patients' daily lives, compliance and continuation of an effective treatment.

CONCLUSIONS

Using a standardized MISS questionnaire, we showed that besides the well-known MTX-induced gastrointestinal symptoms upon MTX administration, RA and PsA patients also experienced anticipatory and associative gastrointestinal and behavioural symptoms before MTX administration, which develop as a classical conditioning response to physical symptoms after MTX. The prevalence of MTX intolerance was 11%. MTX intolerance occurred more often in patients on parenteral (20.6%) than in those on oral MTX (6.2%) and persisted after a switch from oral to parenteral MTX. As persisting MTX intolerance could have a negative impact on patients' quality of life and hamper the use of MTX, RA and PsA patients on MTX should be monitored with the MISS for early detection of MTX intolerance. This would create a window of opportunity to intervene timely and avoid incompliance and discontinuation of an otherwise efficacious treatment.

ABBREVIATIONS

DAS: Disease activity score; DMARD: Disease-modifying anti-rheumatic drug; ESR: Erythrocyte sedimentation rate; JIA: Juvenile idiopathic arthritis; MISS: Methotrexate intolerance severity score; MTX: Methotrexate; NSAID: Non-steroidal anti-inflammatory drug; OR: Odds ratio; PGA: Physician global assessment; PsA: Psoriatic arthritis; RA: Rheumatoid arthritis.

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9

EFFECT OF ORAL METHOTREXATE (MTX) AND BEHAVIOURAL THERAPY OR PARENTERAL MTX *VERSUS* ORAL MTX AND ANTIEMETICS ON MTX INTOLERANCE IN JUVENILE IDIOPATHIC ARTHRITIS: A RANDOMISED CONTROLLED TRIAL

Maja Bulatović Ćalasan[1], Marleen Verkaaik[1], Wineke Armbrust[2], Michael Frosch[3], Esther P. A.H. Hoppenreijs[4], Sylvia Kamphuis[5], Toine C. G. Egberts[6], Gerben Sinnema[7], Carin M.A. Rademaker[6] and Nico M. Wulffraat[1]

[1]Department of Pediatric Immunology, University Medical Centre Utrecht, Wilhelmina Children's Hospital, Utrecht, The Netherlands; [2]Department of Paediatric Rheumatology, Beatrix Children's Hospital, University Medical Center Groningen, Groningen, the Netherlands; [3]Department of Paediatric Rheumatology and Immunology, University Medical Centre Münster, Germany, [4]Department of Paediatrics/Paediatric Rheumatology, St. Maartenskliniek and University Medical Centre St. Radboud Nijmegen, the Netherlands, [5]Department of Paediatric Rheumatology, Erasmus University Medical Centre Rotterdam, Sophia Children's Hospital, Rotterdam, The Netherlands, [6]Department of Clinical Pharmacy, University Medical Centre Utrecht, The Netherlands, [7]Department of Paediatric Psychology, University Medical Centre Utrecht, The Netherlands

Submitted

ABSTRACT

Objective

To investigate the effect of oral methotrexate (MTX) and behavioural therapy (BT) *or* parenteral MTX compared with oral MTX and antiemetic, on MTX intolerance in juvenile idiopathic arthritis (JIA).

Methods

In a randomised controlled trial, 48 patients with MTX intolerance were allocated to: a) standard of care treatment with oral MTX and an antiemetic or b) switch to parenteral MTX or c) oral MTX combined with BT. Primary outcome measure was MTX intolerance, assessed with MTX Intolerance Severity Score (MISS), during the 3-month intervention period. Secondary outcome measures were MTX intolerance at 6 and 12 months, cross-over to another treatment strategy and MTX discontinuation.

Results

MTX intolerance frequency decreased in all treatment strategies after the 3-month intervention period to: 56.2% in the oral MTX and antiemetic group ($p=0.010$), 58.8% in the parenteral MTX group ($p=0.011$) and 73.3% in the oral MTX and BT group. At 12 months, MTX intolerance frequency declined to 31.2% ($p<0.001$), 29.4% ($p<0.001$) and 60.0% ($p=0.006$) compared to baseline. During follow-up, 20.8% switched to another treatment strategy and 22.9% stopped MTX. MTX intolerance scores decreased in all treatment strategies, which occurred already in the 1st week upon enrolment.

Conclusion

BT and oral MTX or parenteral MTX were not superior to oral MTX and antiemetics in treating MTX intolerance. Fast improvement occurred in all treatment strategies, likely due to a positive impact of trial participation. We recommend clinicians to openly address MTX intolerance and select a treatment strategy that patients and parents are motivated for.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is the most common childhood rheumatic disease.[1;2] In the treatment of JIA, methotrexate (MTX) is the cornerstone disease-modifying anti-rheumatic drug (DMARD), due to its efficacy and safety. Serious adverse effects such as hepatotoxicity and bone marrow suppression occur rarely and are usually transient if MTX is stopped.[3] Conversely, gastrointestinal adverse effects, such as nausea, abdominal pain and vomiting, are common during MTX treatment.[4;5] Folic acid supplementation is an accepted strategy to prevent and treat these adverse effects.[6-8] Despite folic acid use, many JIA patients experience gastrointestinal adverse effects after MTX intake.[4;9-13] JIA patients also develop anticipatory adverse effects, occurring before MTX intake, and associative adverse effects, occurring when thinking of MTX, as well as behavioural symptoms such as crying and restlessness when taking MTX.[5;14] These adverse effects are a result of classical conditioning to the abovementioned physical symptoms experienced after MTX intake.[14] Such combination of symptoms, which we previously termed MTX intolerance[14], represents a significant burden for JIA patients and their parents. Notably, MTX intolerance occurs in up to half of JIA patients on MTX[14], and can negatively affect their quality of life.[9] Moreover, over three-quarter of intolerant patients were reluctant to use MTX or refused it, which, besides leading to incompliance, could lead to premature termination of MTX treatment or even replacement with costly biologicals.[5;15;16] To pre-empt such consequences, effective treatment of MTX intolerance is crucial.

A commonly applied treatment strategy in patients suffering from MTX-related gastrointestinal adverse effects is combination of oral MTX[15] with antiemetic drugs.[17] Furthermore, as parenteral MTX may be associated with fewer gastrointestinal symptoms than oral MTX, patients experiencing persistent gastrointestinal adverse effects are commonly switched to parenteral MTX.[9;13;17] However, in current clinical practice, these treatment strategies are not always successful. This could be due to the fact they target physical symptoms only, rather than anticipatory, associative or behavioural symptoms. Behavioural intervention, on the other hand, do target these conditioned responses[18], and may be particularly beneficial in MTX intolerant patients, as we have previously shown in an uncontrolled study.[5] In the present randomised-controlled study, we investigated the effect of: a) behavioural therapy added to oral MTX and b) switch to parenteral MTX, compared with c) standard of care treatment consisting of oral MTX with an antiemetic, on MTX intolerance in JIA.

PATIENTS AND METHODS

Design

Prospective, open, multicentre, randomised controlled trial (ISRCTN13524271), performed at four University Medical Centres (UMC) in the Netherlands (Utrecht, Rotterdam, Groningen, Nijmegen) and at the UMC in Münster, Germany, between January 2007 and December 2011. It was approved by Ethics Committees of participating centres, and conducted according to good clinical practice guidelines.

Participants

Inclusion criteria were age between 4 and 18 years, confirmed JIA diagnosis,[19] use of oral MTX and MTX intolerance. MTX intolerance was assessed using a 12-item MTX Intolerance Severity Score (MISS), as described previously.[14] Briefly, MTX intolerance included abdominal pain, nausea and vomiting occurring after MTX as well as before (anticipatory) and when thinking of (associative) MTX administration, accompanied by behavioural symptoms.[14] MTX intolerance was defined as a score of ≥5 on the MISS (range 0-36 points), including at least one anticipatory, associative or behavioural symptom.[14] Concomitant treatment with non-steroidal anti-inflammatory drugs, other disease-modifying anti-rheumatic drugs, low-dose systemic corticosteroids (≤0.2 mg/kg/day) and intra-articular corticosteroids was allowed. All patients received weekly folic acid (5 mg/week). Exclusion criteria were current use of parenteral MTX or systemic corticosteroids (>0.2 mg/kg/day), hepatotoxicity, defined as aspartate and alanine aminotransferase levels greater than twice the upper limit of normal,[3] bone marrow suppression defined as lymphocyte count $<0.9x10^9$/l, granulocyte count $<1.5x10^9$/l and/or thrombocyte count $<20\ x10^9$/l.[3]

Randomisation

Participants were randomly assigned to one of the three treatment strategies, using an internet-based method for randomisation (stratified per site) with allocation concealed, developed by Julius Centre for Health Sciences and Primary Care, UMCU.

Treatments and follow-up

The three treatment strategies comprised: a) continuation of oral MTX with addition of domperidone (10 mg (5-15 kg) or 30 mg (>15kg), both twice daily, rectally) (continued, if already used), b) switch to parenteral (subcutaneous or intramuscular) MTX (Metoject, Medac, Germany) or c) oral MTX combined with BT by a paediatric psychologist. Patients <8 years were treated with the "Magic Box" method, based on systemic desensitization by distraction(20), using toys as means of distraction during MTX intake to alleviate anticipatory, associative and behavioural symptoms. Patients >8 received cognitive behavioural therapy[5;21], during which they learnt to overrule negative thoughts about MTX intake with positive thoughts.

Patients were followed for 12 months. At baseline and 3, 6 and 12 months, their clinical data and medication use were documented. To monitor MTX intolerance, patients filled in a diary, weekly, during the first 3 months, and once at 6 and 12 months, which included the MISS and questions on compliance to and continuation of MTX. In addition, patients receiving parenteral MTX documented local side effects at the site of injection, and patients having BT recorded the number of sessions.

The first 3 months represented the intervention period. Upon the 3-month intervention period, cross-over to one of the remaining treatment strategies or discontinuation of MTX treatment due to persisting MTX intolerance, increased disease activity or disease remission was allowed.

Outcomes

The primary outcome measure was MTX intolerance, defined as stated above, following the 3-month intervention period. Secondary outcome measures were MTX intolerance at 6 and 12 months, frequency of cross-over to one of the remaining treatment strategies and frequency of MTX discontinuation.

Statistical analysis

Sample size calculation was performed based on the expectation that the majority of patients receiving standard of care would remain intolerant (i.e. 90%), and on the proportion of patients in our pilot study who stayed intolerant after receiving behavioural therapy, namely 2 of 9 patients (22%, [95%-CI: 3-60%]). Given the imprecision in this effect-estimate, a more conservative effect-estimate was chosen, which equalled the upper limit of the 95% confidence interval. Thus, to detect a reduction to 60% in MTX intolerance rates and assuming MTX intolerance rates of 90% in the standard of care group, 42 patients in each treatment strategy would be adequate to reach significance ($\alpha<0.05$) at the power of 0.80.

Patients were analysed on an intention-to-treat basis. For missing values at 3, 6 or 12 months, the last observation was carried forward. In the intention-to-treat analysis, if patients stopped MTX, the score on the MISS was considered to be 0, as these patients could no longer be intolerant to MTX. Those patients who adhered to the allocated treatment (did not switch or stop MTX) during 3, 6 or 12 months were analysed on per-protocol basis.

To compare MTX intolerance frequencies and cross-over rates to another treatment strategy or MTX discontinuation, during follow-up, in parenteral MTX and oral MTX+BT groups *versus* oral MTX+antiemetic group, chi-square test with continuity correction (as appropriate) was used. To adjust for multiple comparisons, Dunnett's procedure was applied. To determine the likelihood of staying intolerant during follow-up in parenteral MTX and oral MTX+BT groups *versus* oral MTX+antiemetic group, relative risks (RR) and 95% confidence intervals (95%-CI) were computed. To compare MTX intolerance frequency during follow-up with baseline, within each group, chi-square test was used.

In addition, to compare scores on MISS (continuous variable) of parenteral MTX and oral MTX+BT groups with those of the oral MTX+antiemetic group, and to compare the scores within each group during follow-up relative to baseline, linear mixed model analysis was used. This model enables a repeated measurement analysis with unequal periods of time between the visits. Moreover, it considers each patient to have his own pattern of MTX intolerance over time.

Statistical analyses were carried out with SPSS version 20.0.0 (SPSS inc, Chicago, Illinois, USA). All comparisons were two-sided at a 5% alpha level. The linear mixed models were represented as regression coefficients (β) with 95%-CI.

RESULTS

Patients

Of 48 included patients, 3 assigned to MTX+antiemetic, 6 assigned to parenteral MTX and 4 assigned to oral MTX+BT did not receive allocated treatments (Figure 1). Patients receiving BT needed a median of 5 sessions (range: 1-8). One patient on parenteral MTX had mild skin irritation at the injection site. Baseline patient characteristics were similar between the three treatment groups (Table 1). Of note is that in the standard of care group, 3 patients were on domperidone and 2 on ondansetron at baseline, which were continued upon inclusion (Table 1). In the parenteral MTX group, 4 used antiemetics at baseline (3 discontinued upon inclusion), whereas in the BT group 3 were on antiemetics at baseline and during the trial (Table 1).

Primary outcome

Intention-to-treat analysis revealed that MTX intolerance frequency after the 3-month intervention period did not significantly differ in the parenteral MTX (58.8%) and oral MTX+BT (73.3%) groups compared to the oral MTX+antiemetic group (56.2%) (p=0.57) (Table 2). The relative risk (RR) of remaining MTX intolerant after 3 months was 1.05 [95%-CI: 0.58-1.88] for the parenteral MTX group and 1.30 [0.77-2.21] for the oral MTX+BT group compared to the oral MTX+antiemetic group. However, within the 3 groups, MTX intolerance frequency at 3 months decreased compared with baseline, which was significant for oral MTX+antiemetic and parenteral MTX groups (Table 2).

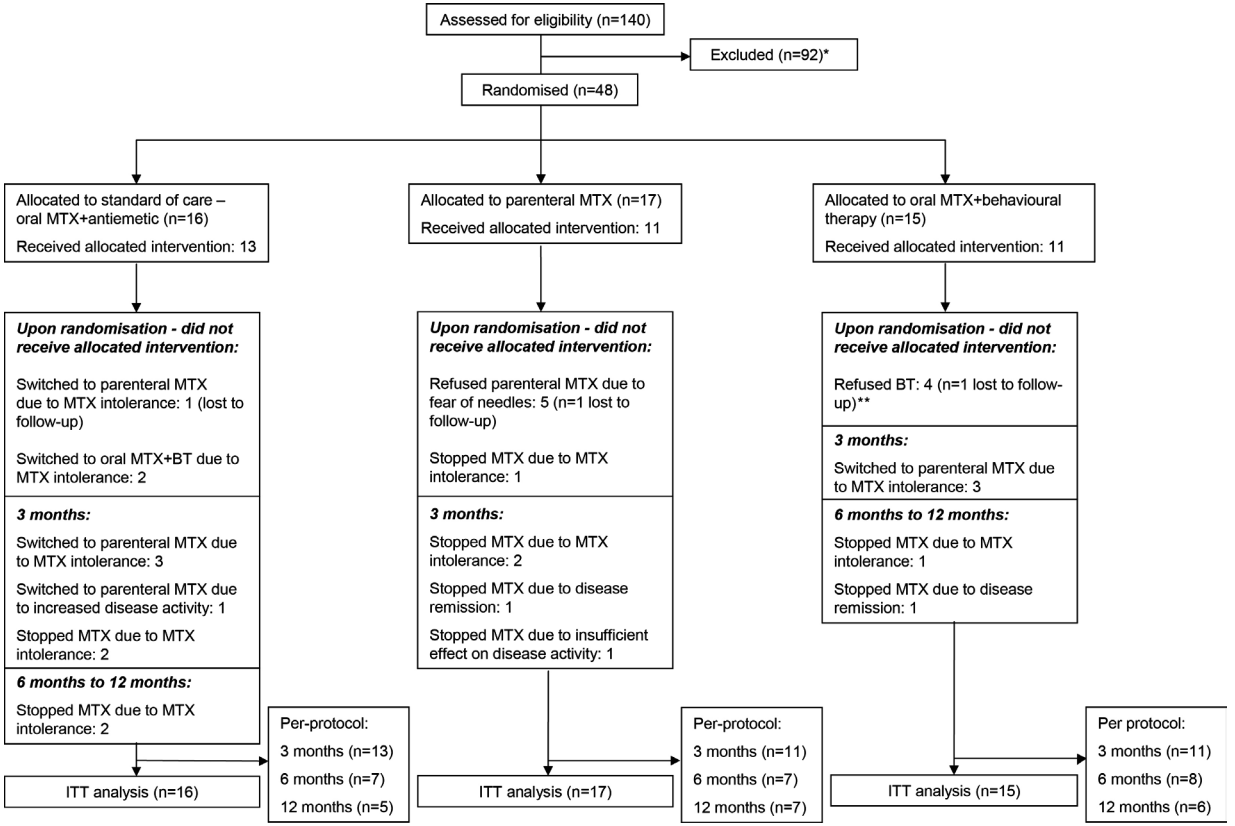

Figure 1. Study flow diagram. *Did not meet inclusion criteria (n=45) and declined participation (n=47) because of: manageable MTX intolerance (n=16), trial considered a burden due to practical constraints (such as traveling time to the clinic) (n=10), fear of needles (n=4), wish to choose treatment (n=4), incompliance to MTX (n=1) and unknown reasons (n=12). ** Reasons for refusal of BT were: long traveling time and disbelief that BT would be helpful. Last observation carried forward was applied in: patients lost to follow-up upon randomisation (n=3), patients with missing MISS at 6 and/or 12 months (oral MTX+antiemetic (n=3), parenteral MTX group (n=4), oral MTX+BT (n=3)). MTX=methotrexate; BT=behavioural therapy; ITT=intention-to-treat.

Table 1. Baseline characteristics

	Oral MTX and antiemetic	Parenteral MTX	Oral MTX and BT
Patients, N	16	17	15
Female, N (%)	11 (68.8)	13 (76.5)	10 (66.7)
Age in years, median (IQR)	13.3 (10.7-15.3)	13.4 (8.1-15.7)	9.8 (8.5-15.0)
Duration of MTX use in years, median (IQR)	1.0 (0.4-1.3)	1.1 (0.4-2.0)	0.9 (0.3-1.6)
JIA subtype, N (%)			
Persistent oligoarticular	5 (31.3)	6 (35.3)	5 (33.3)
Extended oligoarticular	4 (25.0)	1 (5.9)	4 (26.7)
Polyarticular*	5 (31.3)	9 (52.9)	5 (33.3)
Psoriatic	1 (6.3)	0 (0.0)	0 (0.0)
Enthesitis-related	0 (0.0)	1 (5.9)	0 (0.0)
Systemic-onset	1 (6.3)	0 (0.0)	1 (6.7)
Core-Set Criteria, median (IQR)			
Physician global assessment disease activity (0-10)	1.5 (0.6-2.4)	0.5 (0.0-1.8)	1.0 (0.1-1.5)
Joints with active arthritis	1 (0-2.8)	0 (0-2.0)	0 (0-2.0)
Joints with limited range of motion	1.5 (0-2.0)	0 (0-2.5)	0 (0-3.0)
CHAQ disability (0-3) [#]	0.3 (0.1-0.8)	0.3 (0.1-1.1)	0.2 (0.0-1.0)
Parent/patient global assessment of well-being (0-10)[#]	1.3 (0.4-2.9)	3.0 (1.1-6.1)	2.0 (1.0-4.8)
Parent/patient global assessment of pain (0-10) [#]	1.8 (0.5-2.6)	2.5 (1.0-5.2)	1.3 (0.6-3.3)
ESR (mm/hour) [&]	7.5 (4.3-18.0)	8.0 (5.0-18.0)	6.0 (4.0-16.0)
JADAS-27 (0-57) [#]	3.8 (2.4-7.7)	4.0 (1.9-9.2)	5.1 (3.1-6.8)
Medication			
Methotrexate dose, mg/m^2/wk, median (IQR)[~]	9.7 (7.3-10.8)	9.9 (9.0-11.5)	11.2 (9.3-12.9)
Folic acid, N (%)	16 (100)	17 (100)	15 (100)
NSAIDs, N (%)	15 (93.8)	8 (47.1)	9 (60.0)
MTX intolerance score on MISS, median (IQR)	13.0 (7.5-18.5)	13.0 (6.0-18.0)	15.0 (11.0-21.0)

*Rheumatoid Factor (RF) positive: Parenteral MTX: n=1; Oral MTX and BT: n=1. [#]Available in: Oral MTX and antiemetic: 15 patients; Parenteral MTX: 13 patients; Oral MTX and BT: 14 patients. [&]Available in: Parenteral MTX: 15 patients. [~]Concomitant treatments: Oral MTX and antiemetic: oral steroids (n=2), etanercept (n=2), anakinra (n=1), antiemetics (domperidone (n=3), ondanestron (n=2)); Parenteral MTX: oral steroids (n=2), sulfasalazine (n=2), etanercept (n=2), infliximab (n=1), antiemetics (domperidone (n=2), ondanestron (n=2)); Oral MTX and BT: oral steroids (n=1), etanercept (n=2), antiemetics (domperidone (n=3)). JIA, Juvenile Idiopathic Arthritis; BT, behavioural therapy; MTX, methotrexate; JADAS, Juvenile Arthritis Disease Activity Score; CHAQ, Childhood Health Assessment Questionnaire; ESR, erythrocyte sedimentation rate; NSAIDs, non-steroidal anti-inflammatory drugs; MISS, methotrexate intolerance severity score.

Table 2. MTX intolerance frequency (N, %) in intention-to-treat analysis

Time-point	Oral MTX and antiemetic	Parenteral MTX	Oral MTX and BT
Start	16 (100)	17 (100)	15 (100)
3 months	9 (56.2)[a]	10 (58.8)[b]	11 (73.3)
6 months	7 (43.8)[a]	7 (41.2)[b]	10 (66.7)
12 months	5 (31.2)[a]	5 (29.4)[b]	9 (60.0)[c]

[a] Lower frequency of MTX intolerance at 3 (p=0.010), 6 (p=0.002) and 12 (p<0.001) months compared with start. [b] Lower frequency of MTX intolerance at 3 (p=0.011), 6 (p<0.001) and 12 (p<0.001) months compared with start. [c] Lower frequency of MTX intolerance at 12 months (p=0.006) compared with start. MTX, methotrexate; BT, bahavioural therapy

Per-protocol analysis of patients who adhered to the allocated treatment during the 3-month intervention period revealed that MTX intolerance was comparable between the 3 groups (p=0.69) (Table 3). Compared to baseline, MTX intolerance frequency decreased within all three groups, which was statistically not significant, most likely due to low patient numbers (Table 3).

Table 3. MTX intolerance frequency (N, %)[#] in per-protocol analysis

Time-point	Oral MTX and antiemetic	Parenteral MTX	Oral MTX and BT
3 months	7/13 (53.8)	5/11 (45.5)	7/11 (63.6)
6 months	4/7 (57.1)	2/7 (28.6)	4/8 (50.0)
12 months	2/5 (40.0)	2/7 (28.6)	3/6 (50.0)

[#] Presented as number of patients with MTX intolerance out of total number of patients analysed per-protocol during follow-up. MTX, methotrexate; BT, bahavioural therapy

Secondary outcomes

MTX intolerance frequency at 6 and 12 months

Intention-to-treat analysis demonstrated that MTX intolerance frequency, at 6 and 12 months, did not significantly differ between the groups (p=0.29 and p=0.15, respectively) (Table 2). The RR to remain MTX intolerant was 1.08 [0.51-2.28] after 6 months and 0.94 [0.33-2.65] after 12 months in the parenteral MTX group, and 1.52 [0.79-2.95] after 6 months and 1.92 [0.82-4.42] after 12 months in the oral MTX+BT group compared to the oral MTX+antiemetic group. Nevertheless, within all 3 groups, MTX intolerance frequency continued to decrease at 6 and 12 months compared with baseline (Table 2).

Per-protocol analyses of patients who adhered to allocated treatment showed that MTX intolerance frequency remained similar between the groups at 6 (p=0.53) and 12 months (p=0.73) (Table 3). Compared to baseline, MTX intolerance frequency continued to decrease at 6 and 12 months within all three groups; which was statistically not significant, probably due to low patient numbers (Table 3).

Switch of treatment strategy and MTX discontinuation

In total, 10 (20.8%) of 48 enrolled patients switched to another treatment strategy during follow-up (Figure 1). Six patients (37.5%) assigned to standard of care and 3 (20.0%) assigned to BT switched to another treatment strategy because of intolerance (difference not significant, p=0.50). In addition, 9/48 (18.8%) patients refused allocated treatment strategy and continued their initial treatment with oral MTX; 5 (29.4%) assigned to parenteral MTX and 4 (26.7%) assigned to BT (Figure 1). Furthermore, 11/48 (22.9%) stopped MTX treatment during follow-up (Figure 1). Four patients (25.0%) assigned to oral MTX+antiemetic, 2 (11.8%) in the parenteral MTX and 1 (6.7%) in the oral MTX+BT group discontinued MTX due to intolerance (difference not significant, p=0.32).

MTX intolerance severity

Intention-to-treat analysis of scores according to MISS revealed that MTX intolerance severity did not significantly differ between the parenteral MTX and oral MTX+BT groups and oral MTX+antiemetic group throughout follow-up (Figure 2). During the 3-month intervention period, MTX intolerance scores (12 weeks) were on average 1.1 points lower in the parenteral MTX group (β=-1.1 [95%-CI:-5.6 to 3.5]) and 1.8 points higher in the oral MTX+BT group (β=1.8 [-2.8 to 6.5]) than in the oral MTX+antiemetic group, which was not significant (p=0.64 and p=0.42, respectively) (Figure 2). Linear mixed model analysis during 6 months and the entire 12-month follow-up showed comparable results (data not shown).

However within all three groups, MTX intolerance scores declined considerably from start until 3, 6 and 12 months (Figure 2). MTX intolerance scores were significantly lower during the entire 12-month follow-up in oral MTX+antiemetic (β= -0.12 [-0.19 to -0.05], p=0.002), parenteral MTX group (β= -0.10 [-0.19 to -0.01], p=0.037) and oral MTX+BT groups (β= -0.11 [-0.18 to -0.05], p=0.002). MTX intolerance scores decreased already in the 1st week upon enrolment (Figure 2).

In per-protocol analysis MTX intolerance scores did not differ between groups during follow-up (Figure 3). Nevertheless, MTX intolerance scores declined in the 1st week after enrolment in all three groups. The scores decreased in oral MTX+antiemetic (β= -0.07 [-0.22 to 0.08], p=0.288), parenteral MTX (β= -0.06 [-1.54 to 1.42], p=0.571), and statistically significant in the oral MTX+BT group (β= -0.14 [-0.24 to -0.03], p=0.011) (Figure 3).

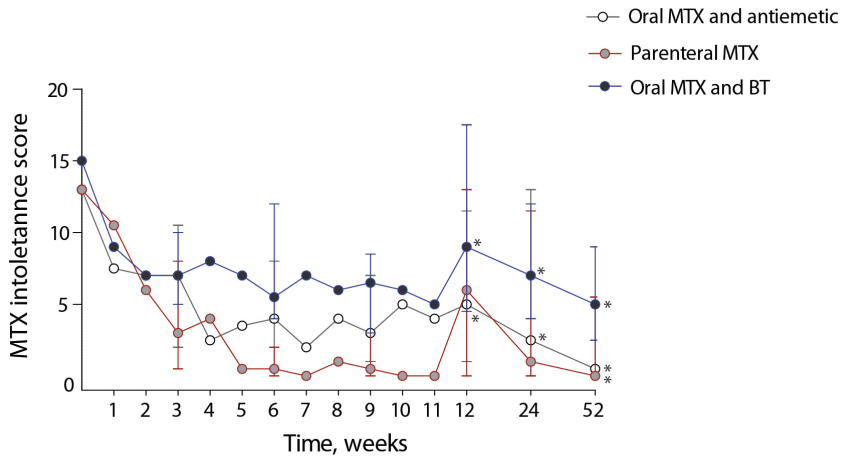

Figure 2. MTX intolerance score according to MISS during 12-month follow-up in intention-to-treat analysis. Between groups, MTX intolerance scores did not differ significantly during follow-up. Within groups, MTX intolerance scores declined over time. Statistical significance is indicated with stars (*). Scores differed significantly compared with baseline for: oral MTX+antiemetic group during 3 (p=0.022), 6 (p=0.005) and 12 months (p=0.002), parenteral MTX group during 12 months (p=0.037), and oral MTX+BT group during 3 (p=0.032), 6 (p=0.003) and 12 (p=0.002) months. Circles show medians with IQRs for oral MTX+antiemetic group (open circles), parenteral MTX group (red light gray circles) and oral MTX+BT (blue dark gray circles). For the first 12 weeks (3 months), IQRs are shown only for 3, 6 and 9 weeks, although MISS' were filled in weekly. MTX, methotrexate; MISS, methotrexate intolerance severity score; BT=behavioural therapy.

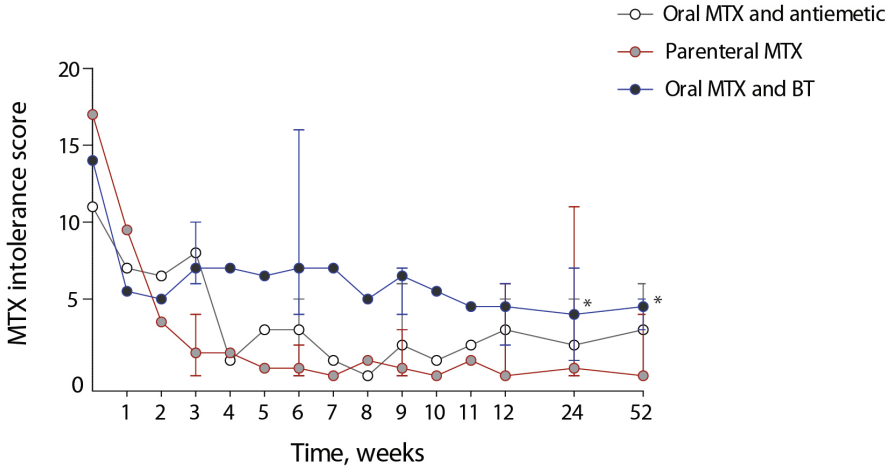

Figure 3. MTX intolerance score according to MISS during 12-month follow-up in per-protocol analysis. Between groups, MTX intolerance scores did not differ significantly during follow-up. Within groups, MTX intolerance scores declined over time. Statistical significance is indicated with stars (*). Scores differed significantly compared with baseline for the oral MTX+BT group during 6 (p=0.034) and 12 (p=0.011) months. Circles show medians with IQRs for oral MTX+antiemetic group (open circles), parenteral MTX group (red light gray circles) and oral MTX+BT (blue dark gray circles). For the first 12 weeks (3 months), IQRs are shown only for 3, 6 and 9 weeks although MISS' were filled in weekly. MTX, methotrexate; MISS, methotrexate intolerance severity score; BT=behavioural therapy.

DISCUSSION

This randomised controlled trial in JIA patients with MTX intolerance demonstrated that BT or switch to parenteral MTX did not have a beneficial effect on MTX intolerance over standard of care treatment with oral MTX+antiemetic. Instead, beneficial effects on MTX intolerance frequency and severity were observed in each treatment strategy.

Various behavioural interventions have been applied to treat chemotherapy-related anticipatory nausea and vomiting in adult and paediatric cancer patients, and were (moderately) effective in decreasing the rate and severity of such adverse effects.[18;21] In our open study in JIA, BT was fully or partially effective in 7 (77.8%) of 9 treated patients.[5] Similar efficacy was hypothesized in the present controlled clinical trial. Here, however, BT was somewhat less efficacious, notably in 26.7% in intention-to-treat or 36.4% in per-protocol analysis after the 3-month intervention period, which increased, at 12 months, to 40% and 50%, respectively. The lower efficacy could be explained by differences in evaluation of MTX intolerance between the two studies: the uncontrolled study evaluated (anticipatory) nausea and behavioural distress only, based on medical file records and interviews, whereas the clinical trial assessed a large panel of symptoms using a validated questionnaire.

BT did not target MTX intolerance more effectively than oral MTX+antiemetic, as continuation of the standard of care treatment was effective in more than 40% of patients after 3 months and in around 60% after 12 months, as opposed to the hypothesized 10%. Furthermore, switch to parenteral MTX yielded a similar efficacy rate as the standard of care. In another trial in JIA, switch to parenteral from oral MTX led to complete resolution of nausea in 9 (81.8%) of 11 patients and decreased the severity in the remaining 2.[13] However, the effect of continuing the standard of care treatment or of parenteral MTX on anticipatory/associative nausea and on other (conditioned) symptoms was not evaluated.

In our clinical trial, all three treatment strategies had beneficial effects on MTX intolerance. While this was expected of BT whose primary target are conditioned adverse effects, it is less obvious why parenteral MTX and oral MTX+antiemetics would also have favourable effects. An explanation could be that circumventing the gastrointestinal mucosa in case of parenteral MTX or using an antiemetic diminished the physical symptoms, which in turn resulted in reduction of conditioned response and behavioural distress. Indeed, conditioned responses cease if physical symptoms are absent.[18]

Although plausible that improvement was the direct result of treatment strategies, a striking decline in scores, in the first week after enrolment, strongly suggests that participation in the trial, rather than given treatments, exerted beneficial effects observed in all three groups. Willingness to participate in the trial could demonstrate patients' motivation and positive expectations, which in turn could have led to a swift change in reported symptoms. Interestingly, patients' expectations may play an important role in the development of conditioned responses.[18;22;23] An illustrative study showed that cancer patients expecting

to develop anticipatory nausea during chemotherapy, did develop it, whereas patients not expecting anticipatory nausea, did not develop it.[24] In the same vein, positive expectations of resolving MTX intolerance upon inclusion in the trial, could have contributed to a fast decrease in MTX intolerance severity, independent of the treatment strategy. Nevertheless, treatment strategies could have been important in reducing MTX intolerance severity, since beneficial effects observed in the first week after enrolment continued during follow-up. This lasting effect could have, in turn, been strengthened by additional aspects of trial participation, such as increased attention of clinicians for patients and their MTX intolerance, and frequent monitoring of adverse effects through weekly questionnaires.

A limitation of the present study is that the calculated sample size was not achieved; 48 (38.1%) of 126 intended patients were included. Insufficient samples size could impede the validity of our conclusion that BT and parenteral MTX were not superior to the standard of care treatment in treating MTX intolerance. However, the observation that MTX intolerance symptoms improved in all treatment strategies remains valid, in spite of the limited samples size. Patient inclusion was indeed particularly difficult due to: sufficiently handled MTX intolerance using other (preferred) strategies (partitioning the doses given, concealing pills or injection liquid in food), or preference to choose one of the treatment strategies rather than to be randomised. Therefore, selection bias towards patients suffering from severe MTX intolerance could be present, although baseline MTX intolerance score in this clinical trial (13.6 in all patients) was similar to the score (12 points) in our cross-sectional study of 297 patients.[14] In addition, around 20% of included patients refused the allocated treatment immediately upon randomization or during follow-up or switched to another treatment strategy. Taken together, difficulties in inclusion and treatment adherence depict how challenging it is to recruit (paediatric) patients with a complex problem in a clinical trial, which has been reported before.[25]

This randomized controlled trial does not support the use of BT or parenteral MTX over standard of care for the treatment of MTX intolerance in JIA. All three strategies improved MTX intolerance symptoms, which is likely due to trial participation. We, therefore, recommend clinicians to address the issue of MTX intolerance openly and monitor it regularly, together with patients, through MISS questionnaires. Furthermore, clinicians should engage in dialogue with patients and their parents, in search of a treatment strategy that both parties are motivated for, since motivation, positive expectations and frequent self-monitoring appear important for the control of MTX intolerance.

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10

PREDICTION OF METHOTREXATE INTOLERANCE IN JUVENILE IDIOPATHIC ARTHRITIS

E.H. Pieter van Dijkhuizen,*[1] Maja Bulatović Ćalasan,*[1] Saskia M.F. Pluijm,[2] Maurits C.F.J. de Rotte,[3] Sebastiaan .J. Vastert,[1] Sylvia M.M. Kamphuis,[4] Robert de Jonge,[3] and Nico M. Wulffraat[1]

*contribited equally

[1]Department of Pediatric Immunology, University Medical Centre Utrecht, Wilhelmina Children's Hospital, Utrecht, The Netherlands; [2]Department of Pediatric Hemato-Oncology, Erasmus University Medical Centre Rotterdam, Sophia Children's Hospital, Rotterdam, The Netherlands; [3]Department of Clinical Chemistry, Erasmus University Medical Centre Rotterdam, Rotterdam, The Netherlands; [4]Department of Pediatric Rheumatology, Erasmus University Medical Centre Rotterdam, Sophia Children's Hospital, Rotterdam, The Netherlands

Submitted

ABSTRACT

Objectives

Methotrexate (MTX) is a safe drug in the treatment of juvenile idiopathic arthritis (JIA). Despite its safety, MTX-related gastrointestinal adverse effects before and after MTX administration, termed MTX intolerance, occur frequently, leading to incompliance and potentially premature MTX termination. The aim of this study was to construct a risk model to predict MTX intolerance.

Methods

In a prospective JIA cohort, clinical variables and single nucleotide polymorphisms were determined at MTX start. The Methotrexate Intolerance Severity Score was employed to measure MTX intolerance in the first year of treatment. MTX intolerance was most prevalent at 6 or 12 months after MTX start, which was defined as the outcome for the prediction model. The model was developed in 152 patients using multivariate logistic regression analysis and subsequently internally validated using bootstrapping.

Results

The prediction model included the following predictors: JIA subtype, antinuclear antibody, parent/patient assessment of pain, Juvenile Arthritis Disease Activity Score-27, thrombocytes, alanine aminotransferase and creatinine. The model classified 77.5% of patients correctly, and 66.7% of patients after internal validation by bootstrapping. The prediction model was transformed into a risk score (range 0-17). At a cut-off of ≥6, sensitivity was 82.0%, specificity 56.1%, positive predictive value was 58.7% and negative predictive value 80.4%.

Conclusions

This clinical prediction model showed good predictive power to detect MTX intolerance. This easy-to-use tool could assist clinicians in identifying patients at risk to develop MTX intolerance, and in turn to monitor them closely and intervene timely in order to prevent the development of MTX intolerance.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is the most common childhood rheumatic disease.[1;2] In JIA, methotrexate (MTX) is the cornerstone treatment, due to its efficacy and safety. Serious adverse effects such as hepatotoxicity and bone marrow suppression occur rarely.[3] In contrast, MTX-related gastrointestinal adverse effects, such as nausea, abdominal pain and vomiting, occur frequently.[4-10] Folic acid supplementation is an accepted strategy to prevent and treat these adverse effects.[11-13] Despite folic acid use, many JIA patients experience gastrointestinal adverse effects after MTX intake.[4-10] JIA patients also experience anticipatory adverse effects, occurring before MTX administration (at the sight of MTX), and associative adverse effects, occurring when thinking of MTX administration (its colour or smell).[4;5;14] These adverse effects are a result of classical conditioning to the abovementioned physical symptoms experienced after MTX intake.[14] Importantly, if physical symptoms are absent, conditioned responses cannot develop.[15] Such a combination of symptoms, which we previously termed MTX intolerance[14], is a significant burden for JIA patients and their parents. Notably, MTX intolerance occurs in up to half of JIA patients on MTX[14], and can negatively affect their quality of life.[6] Moreover, over three-quarters of intolerant patients reluctantly used or even refused MTX[14], which, besides leading to incompliance, could lead to premature discontinuation of MTX, and even replacement by costly biologicals.[5;16;17] Such consequences could be avoided, if the development of MTX intolerance is prevented.

To prevent MTX intolerance, it is crucial to predict which patients starting MTX will be at risk to develop it. Therefore, in patients at risk, clinicians would be able to prevent MTX intolerance by immediate treatment of emerging physical symptoms, which normally give rise to conditioned responses, thus preventing the development of MTX intolerance. Treatment of physical symptoms could include lowering the MTX dose[4], switching to parenteral MTX[6;10;18] or starting behavioural therapy[5] or antiemetics.[18] Predicting MTX intolerance would enable clinicians to apply such treatment strategies only in those patients who are likely to develop MTX intolerance.

Although single nucleotide polymorphisms (SNPs) involved in the MTX metabolic pathways, have been associated with MTX-related gastrointestinal adverse effects in rheumatoid arthritis (RA) and JIA,[19-29] to date no model has been constructed to predict MTX intolerance in JIA. The aim of this large cohort study was to develop and internally validate such a prediction model, using clinical and genetic predictors.

Table 1. Prevalence, univariate ORs (95%-CI) and p-values for potential predictors of MTX intolerance at MTX start

Variables		Cohort, n=152 Frequency n (%)[a]	OR (95%-CI)	p-value
Demographics				
Female		92 (60.5)	1.34 (0.64-2.82)	0.432
Age at disease onset	>8 years	80 (52.6)	0.68 (0.34-1.36)	0.271
Age at MTX start*	>12 years	72 (47.4)	0.54 (0.27-1.07)	0.073
Disease duration at MTX start	>0.5 years	103 (67.8)	0.79 (0.37-1.70)	0.535
JIA subtype*[b]				
Oligoarticular (persistent/extended)		62 (40.8)	Reference	
Polyarticular (RF negative/positive)		64 (42.1)	1.91 (0.86-4.24)	0.094
Other (systemic/psoriatic/enthesitis)		26 (17.1)	0.78 (0.27-2.31)	
Disease characteristics				
ANA*[b,c]	Positive	84 (55.3)	1.98 (0.97-4.07)	0.057
RF[c]	Positive	16 (10.5)	1.52 (0.62-3.72)	0.352
HLA-B27[c]	Positive	11 (7.2)	0.78 (0.29-2.12)	0.510
Uveitis	Present	21 (13.8)	1.44 (0.55-3.78)	0.455
Disease activity				
CHAQ disability score[c]	≤0.250	36 (23.7)	Reference	
	0.250-1.875	88 (57.9)	0.61 (0.24-1.55)	0.395
	>1.875	15 (9.9)	0.72 (0.18-2.80)	
	≤3 cm	58 (38.2)	Reference	
Parent/patient assessment of pain*[b,c]	3-6 cm	36 (23.7)	2.19 (0.84-5.67)	0.086
	>6 cm	42 (27.6)	0.78 (0.30-2.02)	
Parent/patient global assessment[c]	>2.5 cm	90 (59.2)	0.79 (0.36-1.72)	0.494
Active joints*	>2	92 (60.5)	2.00 (0.91-4.41)	0.070
Limited joints*	>1	108 (71.1)	2.02 (0.92-4.46)	0.072
PGA[d]	≤2 cm	50 (32.9)	Reference	
	2-5 cm	86 (56.6)	1.35 (0.53-3.47)	0.496
	>5 cm	16 (10.5)	0.87 (0.21-3.60)	
ESR[c]	>15 mm/hr	74 (48.7)	1.46 (0.66-3.25)	0.341
CRP[c]	>10 mg/L	49 (32.2)	0.83 (0.40-1.74)	0.544
JADAS-27*[b,c]	≤5	16 (10.5)	Reference	
	5-15	59 (38.8)	0.40 (0.11-1.40)	0.048
	>15	52 (34.2)	0.93 (0.25-3.44)	
Biochemical variables[c]				
Haemoglobin	>7.5 mmol/L	78 (51.3)	1.18 (0.60-2.32)	0.620
Leucocytes	>7 $\times 10^9$/L	96 (63.2)	1.21 (0.59-2.47)	0.606
Thrombocytes*[b]	> 350 $\times 10^9$/L	74 (48.7)	1.61 (0.82-3.16)	0.161
AST	>17 IU/L	96 (63.2)	1.08 (0.50-2.36)	0.635
ALT*[b]	>12 IU/L	101 (66.4)	0.41 (0.19-0.88)	0.019
Creatinine*[b]	>50 µmol/L	56 (36.8)	0.51 (0.24-1.08)	0.069
Medication				
MTX dose, median (IQR)	mg/m^2/week	9.9 (9.0-11.2)	NA	
MTX route	oral	148 (97.4)	NA	
MTX restarted		31 (20.4)	1.22 (0.48-3.11)	0.554
Folic acid		150 (98.7)	NA	
Anti-emetics		5 (3.3)	NA	
NSAID		120 (78.9)	0.93 (0.38-2.28)	0.655

Single nucleotide polymorphisms[c]				
MTHFR rs1801133 C>T	TT	15 (9.9)	0.60 (0.21-1.69)	0.322
MTHFR rs1801131 A>C	CC/AC	79 (52.0)	1.65 (0.76-3.62)	0.201
MTRR rs1801394 A>G*	GG/AG	117 (77.0)	0.53 (0.24-1.20)	0.123
RFC/SLC19A1 rs1051266 C>T*	TT	17 (11.2)	1.77 (0.74-4.25)	0.194
ITPA rs1127354 C>A	AA/CA	15 (9.9)	0.62 (0.22-1.74)	0.350
AMPD1 rs17602729 G>A	AA/GA	41 (27.0)	1.46 (0.70-3.05)	0.304
ATIC rs2372536 C>G	GG/CG	93 (61.2)	0.84 (0.39-1.83)	0.614
ADA22 rs73598374 C>T	TT/CT	13 (8.6)	NA	
ADORA2A rs5751876 C>T	TT	28 (18.4)	1.54 (0.65-3.64)	0.319
MDR-1/ABCB1 rs1128503 G>A*	AA	32 (21.1)	1.73 (0.75-3.98)	0.190
MDR-1/ABCB1 rs1045642 G>A	AA	44 (28.9)	1.40 (0.65-3.01)	0.376
MDR-1/ABCB1 rs2032582 C>A/T	AA/TT	24 (15.8)	1.51 (0.63-3.64)	0.344
MRP-1/ABCC1 rs35592 T>C	CC/TC	52 (34.2)	0.79 (0.39-1.57)	0.494
MRP-1/ABCC1 rs3784862 A>G	GG/AG	73 (48.0)	0.97 (0.50-1.91)	0.824
MRP-2/ABCC2 rs4148396 C>T	TT	18 (11.8)	1.57 (0.60-4.08)	0.349
MRP-2/ABCC2 rs717620 C>T	TT/CT	44 (28.9)	0.82 (0.37-1.82)	0.626
MRP-3/ABCC3 rs4793665 T>C	CC/TC	92 (60.5)	0.73 (0.36-1.49)	0.381
MRP-3/ABCC3 rs3785911 A>C*	CC/AC	78 (51.3)	1.67 (0.84-3.32)	0.136
MRP-4/ABCC4 rs868853 T>C	CC/TC	22 (14.5)	0.88 (0.35-2.18)	0.734
MRP-4/ABCC4 rs2274407 C>A	AA/CA	20 (13.2)	1.33 (0.48-3.73)	0.514
MRP-5/ABCC5 rs2139560 G>A	AA/GA	92 (60.5)	1.31 (0.64-2.68)	0.450
BCRP/ABCG2 rs13120400 T>C	CC/TC	63 (41.4)	0.77 (0.38-1.59)	0.470
BCRP/ABCG2 rs2231142 G>T	TT/GT	30 (19.7)	0.96 (0.42-2.20)	0.744
FPGS rs4451422 A>C	CC/AC	102 (67.1)	1.37 (0.63-2.94)	0.417
GGH rs10106587 A>C	CC/AC	73 (48.0)	1.20 (0.59-2.46)	0.508
GGH rs3758149 G>A	AA/GA	77 (50.7)	1.20 (0.57-2.55)	0.602
PCFT/SLC46A1 rs2239907 C>T	TT/CT	104 (68.4)	1.49 (0.69-3.23)	0.306

*Variables associated with the outcome at p<0.20 in the univariate logistic regression analysis. Variables with observed frequencies of <5 in the cross-tabulation with the outcome were excluded from the univariate logistic analysis: MTX route, use of folic acid, use of anti-emetics and *ADA22* rs73598374.

[a] Frequencies are based on observed data, not imputed data.

[b] JIA subtype, ANA, parent/patient assessment of pain, JADAS-27, thrombocytes, ALT and creatinine were included in the multivariate logistic regression analysis.

[c] MICE was used to impute missing values in the following variables (percentage of missing values): HLA-B27 (60.5), RF (19.1), JADAS-27 (16.4), CRP (15.8), parent/patient global assessment (11.8), *RFC/SLC19A1* rs1051266 (11.8), creatinine (11.2), parent/patient assessment of pain (10.5), CHAQ disability score (8.6), *MDR-1/ABCB1* rs2032582 (8.6), ALT (7.9), AST (7.2), ESR (5.3), *GGH* rs3758149 (4.6), *MRP-2/ABCC2* rs717620 (3.9), *MRP-4/ABCC4* rs868853 (3.9), *MRP-5/ABCC5* rs2139560 (3.9), *GGH* rs10106587 (3.9), *MTHFR* rs1801131 (3.3), *ATIC* rs2372536 (3.3), *ADORA2A* rs5751876 (3.3), *MRP-1/ABCC1* rs3784862 (3.3), *MRP-2/ABCC2* rs4148396 (3.3), *MRP-3/ABCC3* rs4793665 (3.3), *BCRP/ABCG2* rs13120400 (3.3), *PCFT/SLC46A1* rs2239907 (3.3), *MTHFR* rs1801133 (2.6), *MTRR* rs1801394 (2.6), *ITPA* rs1127354 (2.6), *AMPD1* rs17602729 (2.6), *ADA22* rs73598374 (2.6), *MDR-1/ABCB1* rs1128503 and rs1045642 (2.6), *MRP-1/ABCC1* rs35592 (2.6), *MRP-3/ABCC3* rs3785911 (2.6), *MRP-4/ABCC4* rs2274407 (2.6), *BCRP/ABCG2* rs2231142 (2.6), *FPGS* rs4451422 (2.6), thrombocytes (2.0), ANA (2.0), hemoglobin (1.3), leucocytes (1.3).

[d] PGA was determined retrospectively by an experienced physician (SJV) in 20 visits (13.2%).

ALT, alanine aminotransferase; ANA, antinuclear antibody; AST, asparagine aminotransferase; CHAQ, childhood health assessment questionnaire; CI, confidence interval; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HLA, human leucocyte antigen; IQR, interquartile range; IU, international units; JADAS, juvenile arthritis disease activity score; JIA, juvenile idiopathic arthritis; MICE, multivariate imputation by chained equations; MTX, methotrexate; NSAID, non-steroidal anti-inflammatory drug; OR, odds ratio; PGA, physician global assessment; RF, rheumatoid factor.

PATIENTS AND METHODS

Patients and study design

An investigator-initiated observational prospective study on efficacy and adverse effects of MTX in patients starting MTX (ISRCTN13524271) was performed at the University Medical Centre Utrecht and Erasmus University Medical Centre Rotterdam, The Netherlands, between January 2008 and October 2012. It was approved by the Ethics Committees of the participating centres and the Central Committee on Research involving Human Subjects, and was conducted according to good clinical practice guidelines.

Patients aged 1-18 years, with a confirmed diagnosis of JIA according to International League of Associations for Rheumatology (ILAR) criteria,[30] who started MTX, were included. Those who had stopped MTX, but re-started MTX due to a relapse, were also included. At the time of MTX start, their clinical data (Table 1) were documented in case report forms and blood for the analysis of SNPs was drawn.

All patients completed the previously developed and validated MTX Intolerance Severity Score (MISS) at 3, 6 and 12 months after MTX start.[14] Those with a score of ≥6, including at least one anticipatory, associative or behavioural symptom, were defined as MTX intolerant.[14]

Development of MTX intolerance over time

To define the outcome for the prediction model, the development of MTX intolerance at 3, 6 and 12 months after MTX start was assessed. For this analysis, of 175 patients starting MTX treatment, 8 patients were excluded due to a diagnosis other than JIA (n=4: Lyme disease, colitis, sarcoidosis, 22q11 deletion syndrome) and use of biologicals at MTX start (n=3: anakinra; n=1: etanercept), resulting in 167 eligible patients (Figure 1). Additionally, 25 patients who completed only one MISS during follow-up were excluded, as their development of MTX intolerance could not be determined. Therefore, the development of MTX intolerance was assessed in 142 patients (Figure 1). The majority of patients developed MTX intolerance at 6 or 12 months after MTX start (Results; Table 2). Consequently, the outcome for the prediction model was defined as MTX intolerance at 6 or 12 months. For the construction of the prediction model, patients with a completed MISS at 6 or 12 months were re-selected from the eligible cohort of 167 patients, resulting in 152 included patients (Figure 1).

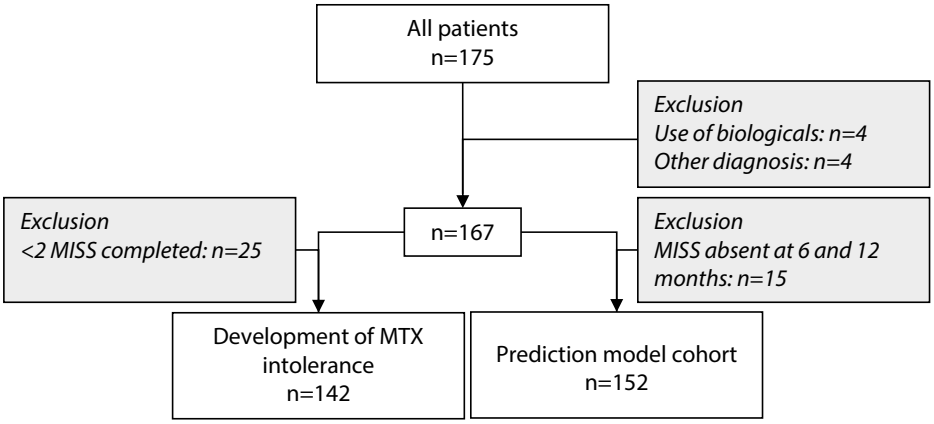

Figure 1. Flowchart
MISS, Methotrexate Intolerance Severity Score; MTX, methotrexate.

Potential clinical and genetic predictors

Potential clinical predictors (demographics, JIA subtype, disease characteristics, disease activity and biochemical measurements) were identified at baseline (Table 1). Potential genetic predictors were SNPs, selected for their involvement in the MTX metabolic pathways, a high polymorphic allele frequency and documented functional effects [31]. SNPs were determined in the following genes: methylenetetrahydrofolate reductase (MTHFR), reduced folate carrier (RFC), methionine synthase reductase (MTRR), inosine triphosphatase (ITPA), adenosine monophosphate deaminase (AMPD), aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC), adenosine-deaminase (ADA), adenosine A2A receptor (ADORA2A), multidrug resistance (MDR) 1, multidrug resistance protein (MRP) 1-5, breast cancer resistance protein (BCRP), folylpolyglutamate synthase (FPGS), gamma glutamyl hydrolase (GGH) and proton-coupled folate transporter (PCFT) (Table 1).

Statistical analysis

Prediction model construction

The prediction model was constructed in several steps. First, missing values were imputed using multivariate imputation by chained equations (MICE).[32] Second, to facilitate implementation of the model in daily clinical practice, continuous variables were dichotomised or categorised, according to patterns in the data or the risk gradients across percentiles, and the cut-off points with the lowest p-value on the log-likelihood test were chosen.[33] Third, all variables were entered in a univariate logistic regression analysis. The results are presented as regression coefficients (β) and odds ratios (OR) with 95% confidence intervals (95%-CI). Variables with a p-value <0.20 on the log-likelihood test were eligible for inclusion in the multivariate logistic

regression analysis. The maximum number of included variables equalled the square root of cases (MTX intolerant patients) in the imputed sample. If more variables were eligible than the allowed maximum, or if variables correlated (Spearman's |rho| >0.40), those with the lowest p-value were included in the multivariate analysis. In addition, an interaction term resulting in a significant change in -2 log-likelihood was added to the final prediction model.

Predictive power of the model was assessed with the C-statistic (Coriginal), which reflects the percentage of patients classified correctly. To determine whether the model fit the data well, the Hosmer-Lemeshow test was employed. Multicollinearity was tested with variance inflation factors (VIF).

Prediction model validation and risk score computation

The model was internally validated using bootstrapping,[34-36] which was performed by randomly drawing 200 samples (of equal size as the original dataset), with replacement, from the original dataset. Multivariate models were fitted for the 200 bootstrap samples and the corresponding C-statistics (Cboot) were determined, as described above. Furthermore, each bootstrap model was fitted in the original dataset, resulting in the corresponding C-statistics (Cboot-original). Next, the Cboot-original values were subtracted from the Cboot values, yielding the so-called optimism values. These were then averaged and subtracted from Coriginal, which resulted in the adjusted C-statistic, indicating the performance of the model in the population.[36] Furthermore, to correct for overfitting, the βs were reduced with a shrinkage factor, calculated from the bootstrap re-sampling.

The abovementioned procedures were performed twice. Firstly, only the routinely available clinical variables were considered as potential predictors. Secondly, SNPs were also considered as potential predictors in order to determine whether they contributed to the prediction of MTX intolerance.

To compute a risk score of becoming MTX intolerant, the shrunken βs were multiplied and rounded off to obtain simple scores that sum up to a total risk score. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of various cut-off points were calculated. Statistical analyses were carried out with R statistics version 2.15.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Development of MTX intolerance over time

Development of MTX intolerance was assessed in 142 patients. In the first year after MTX start, 59 (41.5%) patients were intolerant (score ≥6 with at least 1 anticipatory, associative or behavioural complaint) (Table 2). At 3 months, 22 (15.7%) patients were intolerant. However, intolerance resolved in the majority (13 [59.1%]) at 6 months. At 6 months, the number of

intolerant patients increased to 33 (24.1%), of whom 24 (72.7%) were newly intolerant. At 12 months, 14 (42.4%) of those intolerant at 6 months stayed intolerant, whereas the other 19 either had less than 6 points on the MISS (n=8; range: 0-5 points) or did not complete it.

The total number of intolerant patients at 12 months was 30 (23.3%), of whom 13 (43.3%) were newly intolerant (Table 2).

Taken together, the majority of patients developed MTX intolerance at 6 or 12 months after MTX start. Consequently, the outcome for the prediction model was defined as MTX intolerance at 6 or 12 months after MTX start.

Table 2. MTX intolerance development

Time point	*N*	Intolerance, n(%)[a]
3 months	140[b]	22 (15.7)
6 months	137[b]	33 (24.1)
12 months	129[b]	30 (23.3)
First treatment year	142	59 (41.5)
6 or 12 months[d]	152[c]	51 (33.6)

[a] Frequencies are based on observed data; [b] Patients still on MTX; [c] Cohort for prediction model construction; [d] Outcome was imputed in 21.7% of cases MTX, methotrexate; n, number of patients

Baseline characteristics of the prediction model cohort

The prediction model was constructed in 152 patients. According to the outcome as defined above, 51 (33.6%) patients were MTX intolerant (Table 2). Intolerant and tolerant patients did not differ on frequency of MTX re-start, MTX dose, route of administration, concomitant medication use or disease activity (Juvenile Arthritis Disease Activity score [JADAS-27]) at 6 and 12 months after MTX start (data not shown).

Nineteen (12.5%) patients discontinued MTX treatment during the follow-up, because of MTX intolerance (n=8), disease remission (n=3), insufficient effect (n=2), MTX toxicity (increased liver enzymes: n=1) or other reasons (n=5). Patients also switched the route of administration due to gastrointestinal complaints (either from oral to subcutaneous or vice versa): 8 patients after 3 months, 6 patients after 6 months and 1 patient after 12 months.

Baseline characteristics are depicted in Table 1. Thirty-one patients (20.4%) had re-started the MTX treatment after a relapse. The majority of patients had either oligoarticular or polyarticular JIA (82.9%), with high disease activity (median JADAS-27 of 12.7 [interquartile range 7.6-18.2]). Median MTX dose was 9.9 mg/m2/week, administered mostly as oral MTX (97.4%) with concomitant use of folic acid (98.7%).

Clinical prediction model

Ten clinical variables were univariately associated with MTX intolerance (p<0.20; Table 1). The maximum number of variables allowed in the multivariate analysis was 7. Those with the lowest p-value were selected for the clinical prediction model, namely JIA subtype, JADAS-27, parent/patient assessment of pain, antinuclear antibody (ANA), alanine aminotransferase (ALT), thrombocytes, creatinine and an interaction term between creatinine and JIA subtype. The C-statistic of the clinical prediction model was 77.5% (Table 3). The model fit the data well, as shown by a non-significant Hosmer-Lemeshow test (p=0.705). There was no multicollinearity (data not shown).

Table 3. Prediction model and scores for MTX intolerance

Predictors		OR (95%-CI)	p-value	β[a]	Score[b]
JIA subtype					
Oligoarticular (persistent/extended)		Reference			0
Polyarticular (RF negative/positive)		4.99 (1.36-18.34)	0.016	0.914	5
Other (systemic/psoriatic/enthesitis)		0.93 (0.16-5.49)	0.935	-0.042	0
ANA	Positive	1.98 (0.83-4.68)	0.122	0.387	2
	≤3 cm	Reference			0
Parent/patient assessment of pain	3-6 cm	2.06 (0.72-5.89)	0.175	0.412	2
	>6 cm	0.60 (0.17-2.07)	0.421	-0.288	-1
	≤5	Reference			0
	5-15	0.35 (0.08-1.56)	0.168	-0.599	-3
	>15	0.77 (0.14-4.32)	0.766	-0.150	-1
Thrombocytes	>350 $\times 10^9$/L	1.27 (0.49-3.27)	0.621	0.136	1
ALT	>12 IU/L	0.39 (0.16-0.96)	0.040	-0.534	-3
Creatinine	>50 µmol/L	1.37 (0.33-5.67)	0.665	0.179	1
Interaction term creatinine*JIA subtype					
>50 µmol/L & polyarticular arthritis		0.17 (0.02-1.35)	0.093	-1.022	-5
>50 µmol/L & other JIA subtype		0.82 (0.07-9.74)	0.878	-0.110	-1
Constant				-0.039	7
C-statistic		77.5%			
C-statistic (optimism-corrected by bootstrap)		66.7%			
Hosmer-Lemeshow test (p-value)		0.705			

[a] These are shrunk coefficients (by factor 0.5688) to correct for overfitting.

[b] Shrunk coefficients were multiplied by 5 and rounded off to the nearest integer. The constant was adjusted to obtain the minimum score of 0.

ALT, alanine aminotransferase; ANA, anti-nuclear antibody; CI, confidence interval; JADAS, juvenile arthritis disease activity score; JIA, juvenile idiopathic arthritis; MTX, methotrexate; OR, odds ratio; RF, rheumatoid factor.

Clinical-genetic prediction model

SNPs were considered as potential predictors in order to determine their contribution to MTX intolerance prediction. Four SNPs in the MTRR, RFC, MDR-1 and MRP-3 genes had univariate p-values of <0.20, however these p-values (range: 0.123-0.194) were generally higher than those of the clinical model variables (range: 0.048-0.161) (Table 1). Hence, since 7 variables with the smallest p-values were selected for multivariate analysis, only the MTRR rs1801394 SNP, next to 6 clinical model variables (excluding thrombocytes), was included in the model. The model's C-statistic was 77.7%.

Prediction model validation

The clinical and the clinical-genetic prediction models were internally validated using bootstrapping. Upon internal validation, the corrected C-statistic of the clinical model was 66.7%, whereas the corrected C-statistic of the clinical-genetic model was 64.6%.

Since the clinical-genetic model did not perform better than the model with clinical variables, the latter was given preference as clinical variables are readily available at MTX start, making it easier to apply the model in clinical practice.

Risk score

To enable health care professionals to use the model easily, the shrunken βs of the clinical model's predictors, transformed into simple scores, were used to compute an individual risk score for being MTX intolerant. This score ranged from 0 to 17 points, with a higher score reflecting a higher probability of MTX intolerance (Table 3). The lowest predicted risk of being MTX intolerant was 18.8%, if the following predictors were present: oligoarticular JIA, negative ANA, parent/patient assessment of pain >6 cm, JADAS-27 of 5-15 points, thrombocytes ≤350×109/L, ALT >12 IU/L and creatinine ≤50 μmol/L. The combination of these predictors resulted in a score of 0 [7 (the constant) + 0 + 0 + (-1) + (-3) + 0 + (-3) + 0] (Table 3). On the other hand, the highest predicted risk of being MTX intolerant was 85.9%, if the following predictors were present: polyarticular JIA, positive ANA, parent/patient assessment of pain of 3-6 cm, JADAS-27 ≤5 points, thrombocytes >350×109/L, ALT <12 IU/L and creatinine ≤50 μmol/L. The combination of these predictors resulted in a score of 17 [7 + 5 + 2 + 2 + 0 + 1 + 0 + 0]. The score of the interaction term was added to the rest of the score, if both predictors within the interaction were present (Table 3). For example, the same patient as above with >50 μmol/L creatinine had a risk score of 13, computed as the sum of individual predictors' scores [7 + 5 + 2 + 2 + 0 + 1 + 0 + 1] plus the score (-5) of the interaction term.

Within the 0-17 range, the diagnostic accuracy of different cut-off scores for predicting the risk of being MTX intolerant was evaluated by computing the corresponding sensitivity, specificity, PPV, NPV, and accuracy (Table 4). Our goal was to correctly identify as many future MTX intolerant patients as possible (high sensitivity), while attempting to avoid misidentification

of tolerant patients as intolerant patients (moderate specificity). This was reached at the cut-off score ≥6, where 82% of intolerant patients and 56.1% of tolerant patients were identified correctly.

Table 4. Diagnostic parameters of the risk score for various cut-off scores

Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
≥4	93.4	29.9	50.3	85.7	57.3
≥5	87.8	46.1	55.3	83.3	64.1
≥6	82.0	56.1	58.7	80.4	67.3
≥7	69.2	69.9	63.6	74.9	69.6
≥8	58.7	80.3	69.4	71.9	71.0
≥9	46.0	86.8	72.6	67.9	69.2

NPV, negative predictive value; PPV, positive predictive value.

DISCUSSION

We developed and internally validated a prediction model for MTX intolerance at 6 or 12 months after MTX start in a large JIA cohort, consisting of routine clinical variables: JIA subtype, JADAS-27, parent/patient assessment of pain, ANA, ALT, thrombocytes, creatinine and an interaction term between creatinine and JIA subtype. The model classified 77.5% of patients correctly, and 66.7% after internal validation.

To our knowledge, no previous studies have developed a similar model and a corresponding risk score to predict the occurrence of MTX-induced gastrointestinal adverse effects in JIA. In our model, patients who had more pain (>6 cm), higher baseline disease activity assessed with JADAS-27 and higher ALT, had a lower risk to become MTX intolerant. On the other hand, patients with positive ANA, who had less pain (3-6 cm), higher thrombocyte levels and higher creatinine, had an increased risk of MTX intolerance. The relationship between JIA subtype, creatinine and MTX intolerance was complex; polyarticular JIA was a strong predictor in younger patients (median age: 7.5 years) with creatinine ≤50 µmol/L (score 5, Table 3), whereas oligoarticular JIA was a moderate predictor in older patients (median age: 13.7 years) with creatinine >50 µmol/L (score 1, Table 3).

In RA, two studies identified combinations of risk genotypes to predict adverse effects in general and gastrointestinal adverse effects in particular.[20;27] In our study, SNPs did not significantly contribute to the prediction of MTX intolerance, since only 4 of 27 SNPs were moderately associated with MTX intolerance and only one SNP could be included in the clinical-genetic model, which had comparable predictive power as the clinical model. Previously, in RA and JIA, significant associations ($p<0.05$) were reported between SNPs in the MTHFR, ATIC, ADORA, MRP2/ABCC2 and GGH genes and gastrointestinal adverse effects.[19-29] SNPs in these genes were not associated with MTX intolerance in our study, which could be due to

disparities in patient groups (RA versus JIA), cohorts (cross-sectional versus longitudinal), and the definition of MTX-induced gastrointestinal complaints (after MTX versus before and after MTX use).

The outcome of the prediction model was defined as MTX intolerance at 6 or 12 months after MTX start, since the majority of patients developed MTX intolerance at these time-points. The later onset of MTX intolerance is consistent with the notion that the development of MTX intolerance is governed by a classical conditioning response, which strengthens over time.[5;14] Moreover, our previous cross-sectional study demonstrated higher prevalence of MTX intolerance (50.5-67.5%) in patients with longer MTX use (IQR: 0.6-3.6 years) compared to the prevalence of 34.1% in the present longitudinal study during the first year of MTX treatment.[14] This also supports the notion that MTX intolerance takes time to develop and that longer MTX use may increase the risk of MTX intolerance. To determine whether the risk of MTX intolerance indeed increases with longer MTX use, development of MTX intolerance should be monitored beyond one year of MTX use. Nevertheless, MTX intolerance ensued in 15.8% of patients already after 3 months of MTX use. Interestingly, patients who had restarted MTX had a higher risk of becoming intolerant after 3 months than those newly starting MTX (36% versus 12.7%, p=0.015).

To predict which patients are prone to develop MTX intolerance, the affordable and accessible risk score could be readily used by clinicians, based on the knowledge of clinical variables, which are routinely determined and available for all JIA patients before MTX start. At the cut-off score of ≥6, as many as 82% of intolerant patients were classified correctly (high sensitivity), while maintaining correct classification of 56.1% of tolerant patients (moderate specificity).

Identification of patients at risk increases patients' and clinicians' awareness of MTX intolerance. In patients at risk, clinicians should frequently (i.e. every 4 weeks) monitor MTX-related gastrointestinal adverse effects, using the MISS, from the very start of MTX treatment. This would enable clinicians to treat the emerging physical symptoms immediately, for example by lowering MTX dose,[4] switching to parenteral MTX,[6;10;18] adding antiemetics[18] or applying behavioural therapy,[5] thus preventing the development of a classical conditioning response[15] and hence MTX intolerance. The effect of these timely interventions on the development of MTX intolerance should be determined in a clinical trial.

The study's strengths were that MTX intolerance was assessed using a validated questionnaire. In addition, the model was constructed and internally validated in a large prospective JIA cohort. Internal validation using bootstrapping is an established method, comparable to external validation in an independent cohort, to estimate the performance of a prediction model in the population.[34-36]

In conclusion, we developed and internally validated a clinical prediction model for MTX intolerance in a large JIA cohort. It is an easy-to-use tool to identify patients at risk of developing

MTX intolerance, and in turn to monitor them closely and intervene timely, in order to prevent MTX intolerance and its negative impact on patients' daily lives, compliance and continuation of an effective treatment.

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PART III

METHOTREXATE EFFECTS ON T CELLS

11

METHOTREXATE TREATMENT AFFECTS EFFECTOR, BUT NOT REGULATORY T CELLS IN JUVENILE IDIOPATHIC ARTHRITIS

Maja Bulatović Ćalasan*, Sebastiaan J. Vastert*, Rianne C. Scholman, Frederik Verweij, Mark Klein, Nico M. Wulffraat, Berent J. Prakken** and Femke van Wijk**

*contributed equally
**contributed equally

Center for Molecular and Cellular Intervention, Department of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Centre Utrecht, The Netherlands

Submitted

ABSTRACT

Objective

The balance between regulatory (Treg) and effector T cells (Teff) is crucial for immune regulation in juvenile idiopathic arthritis (JIA). How methotrexate (MTX), the cornerstone treatment in JIA, influences this balance *in vivo* is poorly elucidated. The aim of this study was to investigate quantitative and qualitative effects of MTX on Treg and Teff in JIA patients during MTX treatment.

Methods

Peripheral blood samples were obtained from JIA patients at MTX start and 3 and 6 months thereafter. Treg numbers and phenotype were determined by flow cytometry and suppressive function in allogeneic suppression assays. Teff proliferation upon stimulation with anti-CD3, activation status and intracellular cytokine production were determined by flow cytometry. Effector cell responsiveness to suppression was investigated in autologous suppression assays. Effector cell cytokines in supernatants of proliferation and suppression assays and in plasma were measured by cytokine multiplex assay.

Results

MTX treatment in JIA did not affect Treg phenotype and function. Instead, MTX treatment enhanced, rather than diminished, $CD4^+$ and $CD8^+$ T cell proliferation of JIA patients after 6 months of therapy, independent of clinical response. Effector cells during MTX treatment were equally responsive to Treg-mediated suppression. MTX treatment did not attenuate Teff activation status and their capacity to produce IL-13, IL-17, TNFα and IFNγ. Similarly to Teff proliferation, plasma IFNγ concentrations after 6 months were increased.

Conclusion

This study provides a novel insight that MTX treatment in JIA does not attenuate Teff function but conversely, enhances T cell proliferation and IFNγ plasma concentrations in JIA patients.

INTRODUCTION

In the past decade, intensive research has focused on FOXP3$^+$ regulatory T cells (Treg) in chronic autoimmune inflammation in rheumatic diseases.[1;2] The question whether Treg number and function are altered in autoimmune inflammation is still a matter of debate.[3] In juvenile idiopathic arthritis (JIA), the most common childhood autoimmune diseases, Treg are present in high numbers and are capable of suppressing CD4$^+$ and CD4$^+$ T cells *in vitro*.[4-6] Notwithstanding the abovementioned, the suppressive function of Treg can be hampered *in vivo* by the inflammatory environment in the joint and the resistance of CD4$^+$ and CD8$^+$ effector T (Teff) cells to suppression.[2;5;6] Therefore, the balance between Treg and Teff is crucial for immune regulation in JIA.

The question arises whether and how effective current treatments, such as methotrexate (MTX), influence this balance. MTX, the cornerstone disease modifying anti-rheumatic drug in JIA, can induce disease remission in up to 70% of JIA patients.[7-11] Furthermore, 50% remains in drug-free remission for more than 2 years upon MTX discontinuation.[7] In spite of its convincing efficacy, delineation of MTX's effects on the balance between Treg and Teff in JIA patients during MTX therapy is missing.

In animal models, MTX's effects have been attributed to MTX-induced anti-inflammatory adenosine, whose production is mediated by CD39 and CD73 ectoenzymes.[12-16] In humans, the *in vitro* binding of adenosine to receptors on Treg and adenosine production by CD39/CD73 expressing-Treg leads to increased Treg numbers and suppressive function.[17-20] Furthermore, *in vitro* exposure to MTX has been shown to induce (sensitivity to) apoptosis of activated T cells.[21-23] This phenomenon is attributable to the inhibition of folate metabolism and *de novo* purine and pyrimidine synthesis,[24] resulting in anti-proliferative effects, which is the most prominent feature of MTX. Although animal models and *in vitro* experiments offer clues on the effects of MTX of Treg and Teff, such systems are not representative of the clinical reality of JIA patients on MTX. As opposed to animal models, in which the effects of MTX are observed in a matter of days or weeks, and to cell culture systems, in which MTX effects are observed within hours or days, the full blown effects of MTX in patients can be reliably evaluated only after 3 or even 6 months of treatment.[25] Such delayed clinical effect is in part due to the time-dependent accumulation of long-chain MTX polyglutamates, MTX clinical efficacy mediators,[26-28] whose accumulation does not occur during short exposure to MTX *in vitro*. Therefore, *ex vivo* data from patients using MTX is required to clarify the effects of MTX on Treg and Teff.

Here, we studied quantitative and qualitative effects of MTX treatment on Treg and Teff of JIA patients at MTX start and while on MTX for 3 and 6 months. Our data indicate that MTX treatment does not alter Treg phenotype or suppressive function. Instead, MTX leads to enhanced T cell proliferation and higher IFNγ levels in plasma, independently of clinical response. Taken together, low-dose MTX treatment does not target regulatory T cells; instead it enhances, rather than attenuates, the function of effector cells.

PATIENTS AND METHODS

Patients and study design

A prospective investigator-initiated clinical trial on MTX in JIA (ISRCTN13524271), approved by the ethics committee, was performed at the University Medical Center Utrecht between August 2007 and February 2013.

Patients aged 2-18 years, with a confirmed JIA diagnosis,[29] starting MTX without concomitant biological treatment, were included (Table 1). Patients, who stopped MTX for >6 months, but re-started MTX due to a relapse, were also included. At MTX start, 3 and 6 months after MTX start, clinical data was collected and blood was sampled. In some patients with active disease, synovial fluid (SF) was acquired during therapeutic joint aspirations. Due to limited cell numbers, not all patients could be included in all experiments.

MTX clinical response was determined at 6 months after MTX start, as this is a commonly used time point to establish MTX efficacy, using the ACR pediatric criteria.[30] MTX responders were defined as patients who satisfied at least ACR50 criteria (50% improvement in at least 3 of the 6 core-set criteria, with no more than 30% worsening in more than 1 of the remaining criteria.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC) at time-points 0, 3 and 6 or synovial fluid mononuclear cells (SFMC) were isolated using Ficoll Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences, AB) and frozen in fetal calf serum (FCS) (Invitrogen) containing 10% DMSO (Sigma-Aldrich) until further experimentation. Cells were cultured (37°C and 5% $CO_{2)}$ in 10% human AB serum (Invitrogen) with RPMI 1640 (2 mM L-glutamine and 100 U/ml penicilline-streptomycine) and stimulated either with 1.5 µg/ml plate-bound anti-CD3 (clone OKT3, eBioscience) or with anti-CD2/anti-CD3/anti-CD28 beads (Treg Suppression inspector, Miltenyi Biotect, Germany).

Suppression assays

Allogeneic: To study suppressive capacity of Treg from time-points 0, 3 and 6, allogeneic assay was performed, in which patient $CD4^+CD25^+$ $CD127^{low}$ Treg were co-cultured with healthy donor (HD) $CD4^+CD25^-$ T cells, sorted by flow cytometry on FACS Aria (BD Biosciences) (Supplementary Figure 1). Treg were co-cultured with 25.000 T cells at 1:8 and 1:4 ratios in 100 µl of culture volume, and stimulated with anti-CD2/anti-CD3/anti-CD28 beads (Treg Suppression inspector, Miltenyi Biotect, Germany). To control for cell density, $CD4^+CD25^-$ T cells instead of Treg were added at a 1:4 ratio.

Autologous: To study responsiveness of effector cells at time-points 0 and 6 to Treg-mediated suppression, autologous assays were performed. Total PBMC were used as effector

cells and co-cultured with sorted $CD4^+CD25^+$ $CD127^{low}$ Treg from time-points 0 and 6 and vice versa (cross-over assay). Effector cells (10.000 cells) were co-cultured with Treg at 1:8, 1:4 and 1:2 ratios, and stimulated with plate bound anti-CD3.

In both assays, at day 5, supernatants were collected to measure cytokine production. Subsequently, ^3H was added during the last 16-19 hours, and its uptake measured by liquid scintillation beta counter, to quantify effector cell proliferation.

Table 1. Patient characteristics

Female, N/total (%)	53/76 (69.7)
Age at MTX start, years, mean (+/- SD)	11.2 (3.9)
Age at onset, years, mean (+/- SD)	8.1 (4.6)
JIA subtype, N (%)	
Persistent oligoarticular	21 (27.6)
Extended oligoarticular	14 (18.4)
Polyarticular*	35 (46.1)
Enthesitis-related	6 (7.9)
Core-Set Criteria, median (IQR)	
Physician global assessment disease activity (0-10)[#]	3.0 (2.0-3.5)
Joints with limited range of motion	2 (1-4)
Joints with active arthritis	3 (1-7)
CHAQ disability (0-3)[£]	0.9 (0.3-1.5)
Parent/patient global assessment of well-being (0-10)[&]	4.0 (1.0-6.6)
Parent/patient global assessment of pain (0-10)[&]	3.3 (1.0-6.5)
ESR (mm/hour)[$]	15.0 (7.0-40.0)
Medication	
Methotrexate dose, mg/m^2/wk, median (IQR)[~]	10.0 (9.2-11.0)
Folic acid, N (%)	76 (100)
NSAIDs, N (%)	60 (78.9)
Local steroids[¶], N (%)	12 (15.8)
Responder status (≥ACR50), N (%)	
3 months	47 (61.8)
6 months	51 (67.1)

*Rheumatoid Factor (RF) positive n=8 (22.9% of all polyarticular JIA patients)
[#]Available in 74 (97.4%) patients; [£]Available in 72 (94.7%) patients; [&]Available in 71 (93.4%) patients; [$]Available in 72 (94.7%) patients
[~]Two patients (3.6%) on parenteral MTX
[¶] One patient (polyarticular JIA) was on low dose oral steroids (0.4 mg/kg/day) at MTX start
JIA; Juvenile Idiopathic Arthritis; CHAQ ; Childhood Health Assessment Questionnaire, ESR; erythrocyte sedimentation rate; NSAIDs; Nonsteroidal anti-inflammatory drugs.

T cell proliferation and effector cell cytokine production

To measure T cell proliferation, PBMC were labeled with 2 μM CFSE (Invitrogen) for 10 minutes at 37°C and washed. CFSE-labeled PBMC (60.000 cells) were plated into anti-CD3-coated wells. In some experiments, increasing concentrations of MTX (Emthexate, 2.5mg/ml) were added *in*

vitro to either 200.000 PBMC at time-point 0 or to 200.000 SFMC, from the start of culture. At day 5, proliferation of effector cells was analyzed with flow cytometry by gating $CFSE^+$ cells. Proliferation of $CD4^+$ and $CD8^+$ T cells was measured by gating $CD3^+$ cells, followed by gating $CD4^+$ and $CD8^+$. Simultaneously, PBMC (60.000 cells), not labeled with CFSE, were plated into anti-CD3-coated wells in order to collect supernatants at day 4 to measure cytokine production. Furthermore, plasma was obtained by centrifugation of peripheral blood (PB) at 150g for 10 minutes, and then stored at -80°C. Cytokine concentrations were measured with the Bio-Plex system combined with the Bio-Plex Manager Version 4.0 software (Bio-Rad laboratories), emploting the Luminex technology, as previously described.[31]

Flow cytometry

To determine the phenotype of Treg and T cells and to detect intracellular cytokine production, cells were stained *ex vivo* and measured with flow cytometry. To detect intracellular cytokine production, cells were stimulated with PMA (20 ng/ml; MP Biomedicals) and ionomycin (1 µg/ml, Calbiochem) for 5 hours (+ 4.5 hours of Golgistop (1/1500; BD Biosciences)). The staining protocol is described elsewhere.[6] Cells were acquired on FACSCanto II and analyzed using FACS Diva Version 6.13 software (BD Biosciences). Flow cytometry antibodies are described in supplementary information.

Statistical analysis

To analyze patient samples, t-test or Mann-Whitney U test was used, as appropriate. To analyze paired patient samples, paired T test or Wilcoxon matched pairs test was used, as appropriate. Statistical analysis was performed using GraphPad Prism Version 5.03 (Graphpad software) and SPSS version 20.0.0 (SPSS inc, Chicago, Illinois, USA).

RESULTS

Treg numbers are not increased during MTX treatment

Previous studies showed that Treg frequency in rheumatoid arthritis (RA) increased during treatment with anti-TNFα drugs.[32;33] We investigated whether treatment with MTX also leads to increased Treg numbers. The frequency of $CD4^+FOXP3^+$ Treg in PB of JIA patients, at 3 (mean±SEM: 4.1±0.5%) and at 6 months (3.5±0.3%), did not increase compared to their frequency at MTX start (4.1±0.3%) (Figure 1A). Instead, Treg frequency at 6 months was lower than at MTX start ($p<0.05$), which was the case in MTX responders and non-responders (not statistically significant) (Figure 1B). Taken together, Treg numbers do not increase during MTX treatment in JIA, and do not correlate with clinical efficacy of MTX.

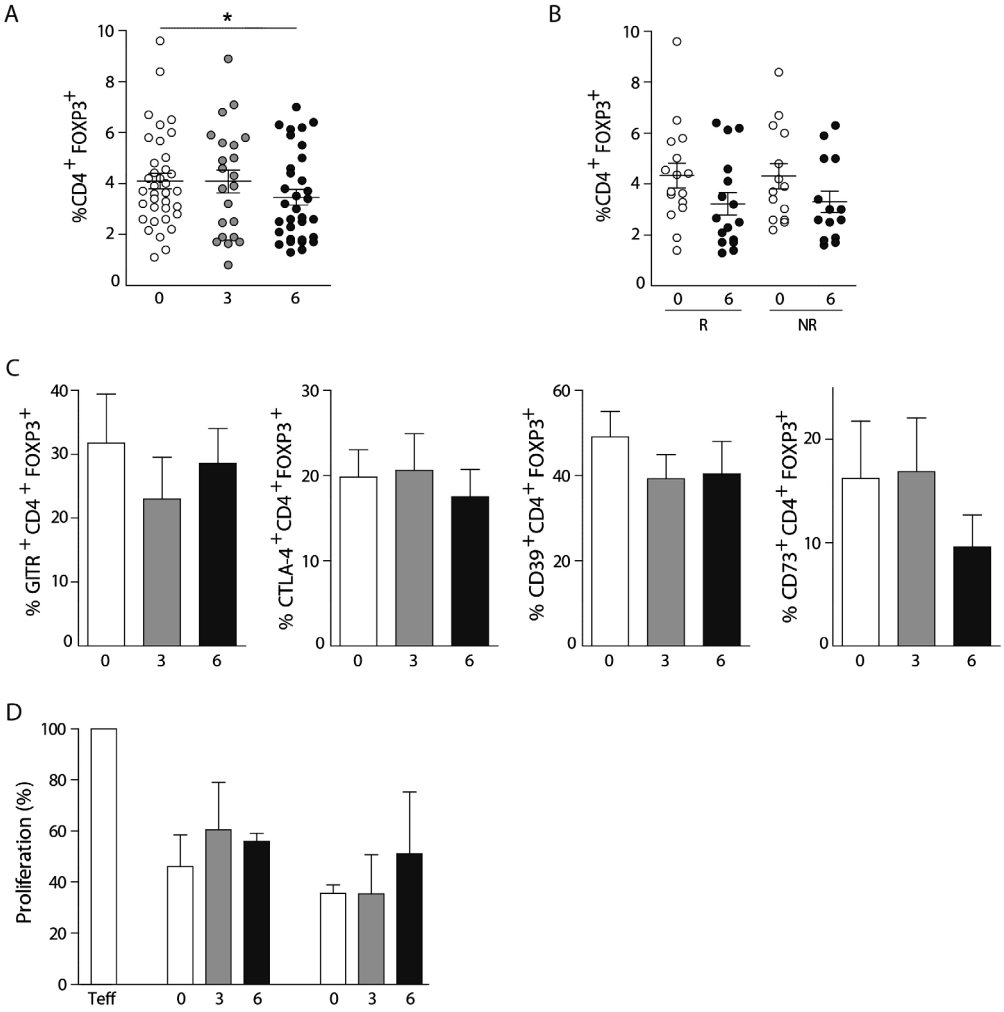

Figure 1. MTX treatment in JIA patients does not alter Treg phenotype and function. A-C. PBMC were isolated from JIA patients at start and 3 and 6 months after start of MTX therapy. PBMC were stained for CD4, FOXP3 and other marker expression, measured ex vivo by flow cytometry. A. Percentage of CD4+ FOXP3+ cells (mean ± SEM) in PBMC of JIA patients at MTX start (0 – white bars), 3 months (3 – gray bars) and 6 months (6 – black bars) after MTX start (n=42). B. Percentage of CD4+ FOXP3+ cells (mean ± SEM) in PBMC of JIA patients at MTX start who will be MTX responders (R) at 6 months after MTX start (n=16), and of JIA patients who will be MTX non-responders (NR) at 6 months after MTX start (n=14). C. Percentage of GITR, CTLA-4, CD39 and CD73 expressing cells in CD4+ FOXP3+ cells at time-points 0, 3 and 6. D. Allogeneic suppression assay: Patient CD4+CD25+ CD127low Treg and healthy donor (HD) CD4+CD25- effector T cells (Teff) from healthy donors (HD) were sorted from PBMC by flow cytometry and co-cultured in the presence of anti-CD2/anti-CD3/anti-CD28 beads. At day 5, 3H was added and its uptake measured by liquid scintillation beta counter to determine suppression of proliferation. Proliferation of HD Teff in the presence of Treg from JIA patients at time-points 0, 3 and 6 at 1:8 (Treg:Teff) or 1:4 ratios. The results show percentage of proliferation in the presence of Treg relative to proliferation of Teff (set at 100%) cultured alone. Bars represent mean ± SEM of n=3. *p<0.05 compared with time-point 0.

Methotrexate treatment does not alter Treg phenotype and function

As MTX treatment did not increase Treg numbers, we investigated Treg phenotype and suppressive capacity. We examined the expression of CTLA-4 and GITR as well as of ectonucleases CD39 and CD73, which have important roles in Treg suppressive function.[17-20;34-37] These markers were not altered during MTX treatment (Figure 1C), in both responders and non-responders (data not shown).

To investigate Treg suppressive capacity during MTX treatment, allogeneic suppression assays were performed, in which sorted $CD4^+CD25^+CD127^{low}$ Treg from JIA patients were cultured with sorted $CD4^+CD25^-$ effector T cells (Teff) from a HD. As depicted in Figure 1D, suppressive capacity of Treg was not altered upon MTX treatment. Moreover, Treg from all three time-points were equally capable of suppressing the Teff production of IL-13, IFNγ and TNFα (Supplementary Figure 2). Taken together, MTX treatment does not affect Treg phenotype and function.

MTX treatment leads to increased T cell proliferation in JIA patients after 6 months of therapy

As MTX has anti-proliferative properties,[24] we hypothesized that MTX has inhibitory effects on T cell proliferation in JIA. First, we asked whether *in vitro* exposure to MTX inhibited T cell proliferation at MTX start. We demonstrated that 1 and 10 nM concentrations, corresponding to low-dose MTX treatment in patients,[38;39] did not inhibit the proliferation of either PB $CD4^+$ and $CD8^+$ T cells (Figure 2A, *left panels*) or of the highly activated SF T cells[6] (Figure 2A, *right panels*). However, higher MTX concentrations (50 and 100 nM), corresponding to high-dose MTX used for malignancies, did inhibit proliferation of both PB and SF T cells (Figures 2A).

To determine whether MTX treatment exerted anti-proliferative effects on T cells *ex vivo*, proliferation of $CD4^+$ and $CD8^+$ T cells was determined at time-points 0, 3 and 6. $CD4^+$ and $CD8^+$ T cell proliferation at 3 months was comparable to that of MTX start, whereas $CD4^+$ and $CD8^+$ T cell proliferation at 6 months (mean±SEM: 75.3±4.1% and 75.6±4.1%, respectively) was significantly higher than at MTX start (58.5±5.0% % and 58.1±5.1%, respectively) ($p<0.05$) (Figure 2B and 2C, *right and left panel*). The observed increase in proliferation was independent of response to MTX (Figure 2D). At MTX start, however, future responders showed higher $CD4^+$ (mean 67.7%) and $CD8^+$ (67.7%) T cell proliferation compared with future non-responders ($CD4^+$and $CD8^+$: 47.7%, $p<0.05$).

All together, this data demonstrates that low-dose MTX treatment does not lead to inhibition, but rather to enhancement of T cell proliferation in JIA patients.

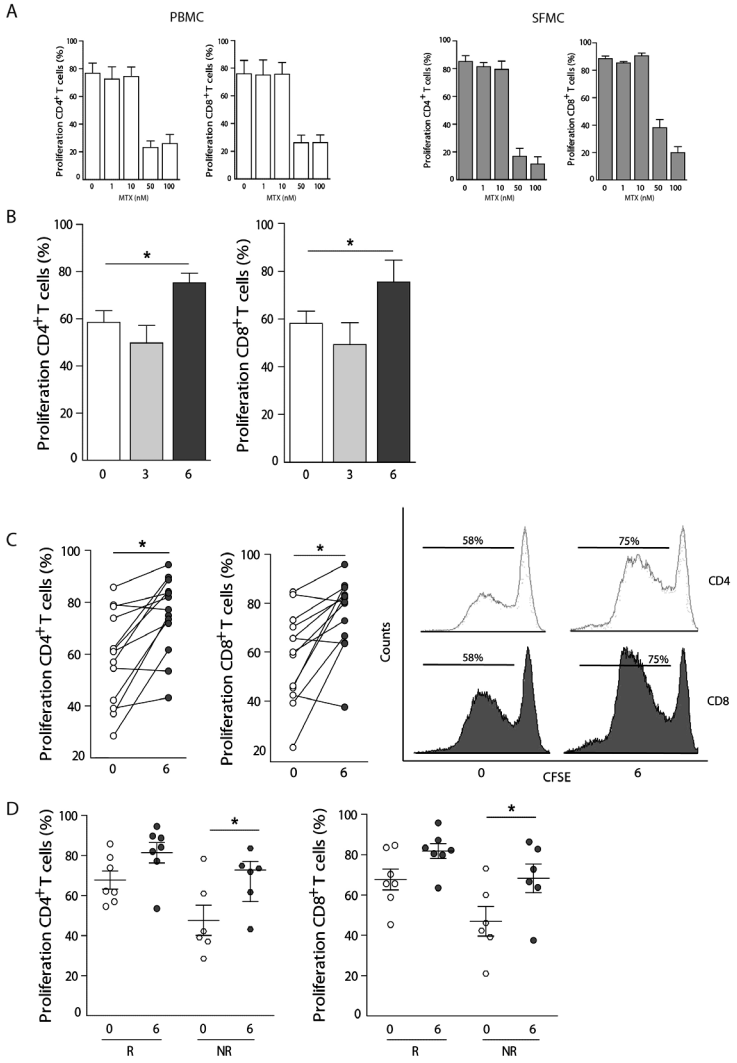

Figure 2. MTX treatment leads to increased T cell proliferation in JIA patients after 6 months of therapy. A-D. CFSE-labeled PBMC or SFMC of JIA patients at MTX start, 3 and 6 months after MTX start were cultured in the presence of anti-CD3. At day 5, PBMC were stained for CD4 and CD8 and proliferation of CD4+ and CD8+ T cells was measured by flow cytometry. Bars and ranges represent mean ± SEM. A. Proliferation of CD4+ (left upper panel) and CD8+ T cells (right upper panel) from peripheral blood of JIA patients at MTX start during in vitro exposure to increasing concentrations of MTX (n=9). Proliferation of CD4+ (left lower panel) and CD8+ T cells (right lower panel) from synovial fluid of JIA patients with active disease during in vitro exposure to increasing concentrations of MTX (n=3). B. Proliferation of CD4+ (left panel) and CD8+ T cells (right panel) of JIA patients at MTX start (0 – white bars), and 3 months (3 – gray bars) and 6 months (6 – black bars) after MTX start (n=13). C. Proliferation of CD4+ (left panel) and CD8+ T cells (right panel) of individual JIA patients at MTX start (0 – white circles) and at 6 months (6 – black / circles). Rightmost panel are representative histograms with percentages indicating the percentage of proliferating cells, 1 representative of n=13. D. Proliferation of CD4+ (left panel) and CD8+ T cells (right panel) of JIA patients at MTX start who will be responders (R) at 6 months after MTX start (n=7), and of JIA patients who will be MTX non-responders (NR) at 6 months after MTX start (n=5). The results show the percentage of proliferating CD4+ or CD8+ T cells. *p<0.05 compared with time-point 0.

MTX treatment does not diminish effector (T) cell activation status or cytokine production in JIA patients

We hypothesized that T cells during MTX treatment showed reduced activation status and lower cytokine production. However, *ex vivo* measured T cell proliferation marker Ki-67 and activation markers CD25, CD69 and HLA-DR did not decrease after 6 months (Supplementary Figure 3). Furthermore, *ex vivo* measured $CD4^+$ production of IL-10, IL-17, IFNγ and TNFα, and $CD8^+$ production of IFNγ and TNFα were not lower at 3 or 6 months (Supplementary Figure 4). In addition, upon anti-CD3 stimulation, effector cells did not produce less IL-10, IL-13, IL-17, IFNγ and TNFα in culture supernatants at time-points 3 and 6 (Figure 3). There were no differences between MTX responders and non-responders (data not shown). Moreover, exposure to 1 and 10 nM of MTX did not inhibit production of IL-13, IL-17, IFNγ and TNFα by effector cells from PB and SF (Supplementary Figure 5). Taken together, MTX treatment does not attenuate activation status and cytokine production of effector (T) cells.

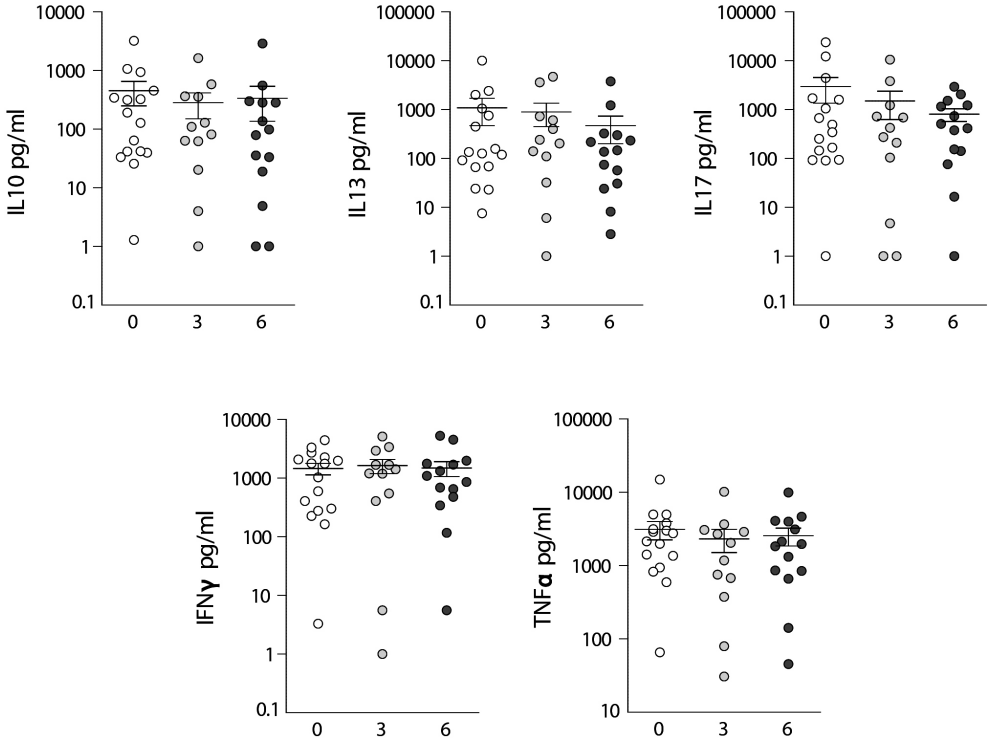

Figure 3. MTX treatment does not lead to decreased effector cell cytokine production in JIA patients after 6 months of therapy. PBMC of JIA patients at MTX start and at 3/6 months after MTX start were cultured in the presence of anti-CD3. At day 4, culture supernatants were harvested to measure cytokine production. IL-10, IL-13, IL-17, IFNγ and TNFα were measured with the Bio-Plex system, which employs the Luminex technology. Ranges show mean ± SEM of n=16.

Effector cells at 6 months are equally responsive to Treg-mediated suppression as effector cells at MTX start

Since T cell activation and cytokine production were not affected by MTX treatment, we investigated whether responsiveness of effector cells to Treg-mediated suppression differed between time-points 0 and 6. As T cells at time-point 6 showed heightened responsiveness to anti-CD3 upon MTX treatment, we hypothesized that these effector cells would also be more responsive to Treg-mediated suppression than effector cells at MTX start. Therefore, we performed cross-over autologous suppression assays, in which PBMC (effector cells) from time-point 6 were co-cultured with sorted $CD4^+CD25^+$ $CD127^{low}$ Treg from time-point 0, and vice versa (Figure 4A, *white and black striped bars*). In addition, effector cells were also co-cultured with Treg from the corresponding time-points (Figure 4A, *white and black bars*). Contrary to our hypothesis, time-point 6 effector cells were equally responsive to suppression of proliferation by Treg from both time-points, compared to time-point 0 effector cells.

In RA, it has been shown that despite proficient suppression of proliferation, Treg-mediated suppression of T cell-cytokine production was compromised.[32] We therefore asked whether time-point 6 effector cells were more responsive to Treg-mediated suppression of cytokine production compared to effector cells at MTX start. Time-point 6 effector cells were, however, equally responsive to suppression of cytokine production (IL-13, TNFα and IFNγ) as effector cells from time-point 0, as shown both in own and cross-over experiments (Figure 4B). IL-17 was resistant to Treg-mediated suppression at both time-points (Figure 4B), which was observed before.[33;40] Taken together, in spite of their enhanced proliferation, effector cells 6 months after MTX start were equally responsive to Treg-mediated suppression of proliferation and cytokine production compared to effector cells at MTX start.

Enhanced IFNγ concentrations in plasma of JIA patients after 6 months of therapy

Cytokine levels in plasma during MTX treatment were quantified. While TNFα concentrations were not affected by MTX treatment, concentrations of another pro-inflammatory cytokine IL-6 decreased at 3 and 6 months (Figure 5C), independent of clinical response. Conversely, IFNγ, a T cell-derived cytokine associated with T cell proliferation, increased at 6, but not at 3 months after MTX start (Figure 5A). Similarly to T cell proliferation, IFNγ was increased in both responders and non-responders, although the increase in non-responders was not statistically significant (Figure 5B). Therefore, MTX treatment also enhances effector cell function with respect to IFNγ levels in plasma.

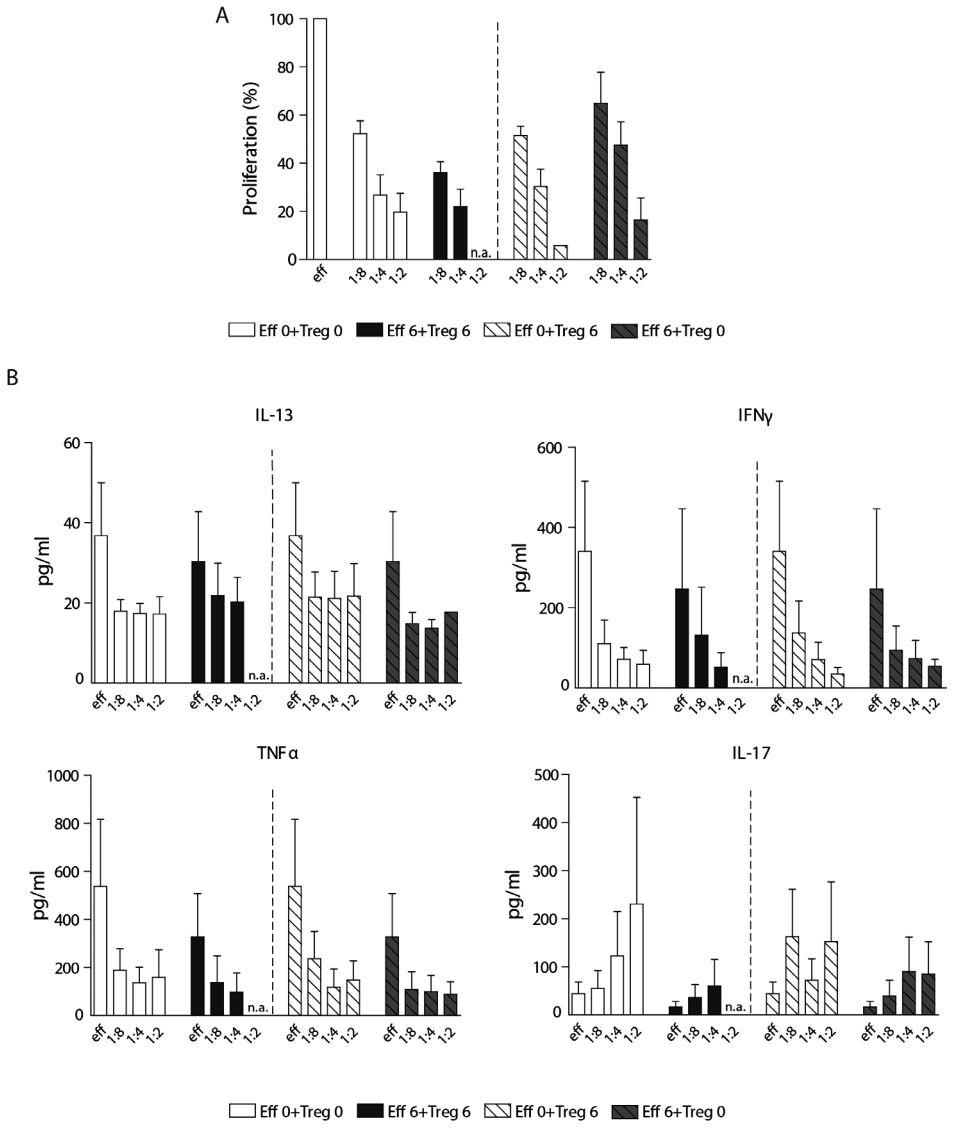

Figure 4. Effector cells at 6 months after MTX start are equally responsive to suppression as effector cells at MTX start. A-B Autologous suppression assays: CD4+CD25+ CD127low Treg from time-points 0 and 6 were sorted from PBMC by flow cytometry and co-cultured with PBMC (effector cells, Eff) from the corresponding time-points (white and black bars) at 1:8, 1:4 and 1:2 ratios in the presence of anti-CD3. In cross-over experiments, effector cells from time-point 0 were co-cultured with Treg from time-point 6 (white striped bars) and vice versa, effector cells from time-point 6 were co-cultured with Treg from time-point 0 (black striped bars). At day 5, culture supernatants were harvested to measure cytokine production and suppression of cytokine production. Subsequently, 3H was added and its uptake measured by liquid scintillation beta counter to determine suppression of proliferation. A. The results show percentage proliferation in the presence of Treg relative to proliferation of effector cells alone (set at 100%). Bars represent mean ± SEM of n=4. B. IL-13, IFNγ, TNFα and IL-17 levels in the absence (eff) or presence of Treg at 1:8, 1:4 and 1:2 ratio. Data represent mean cytokine levels in pg/ml ± SEM of n=4.

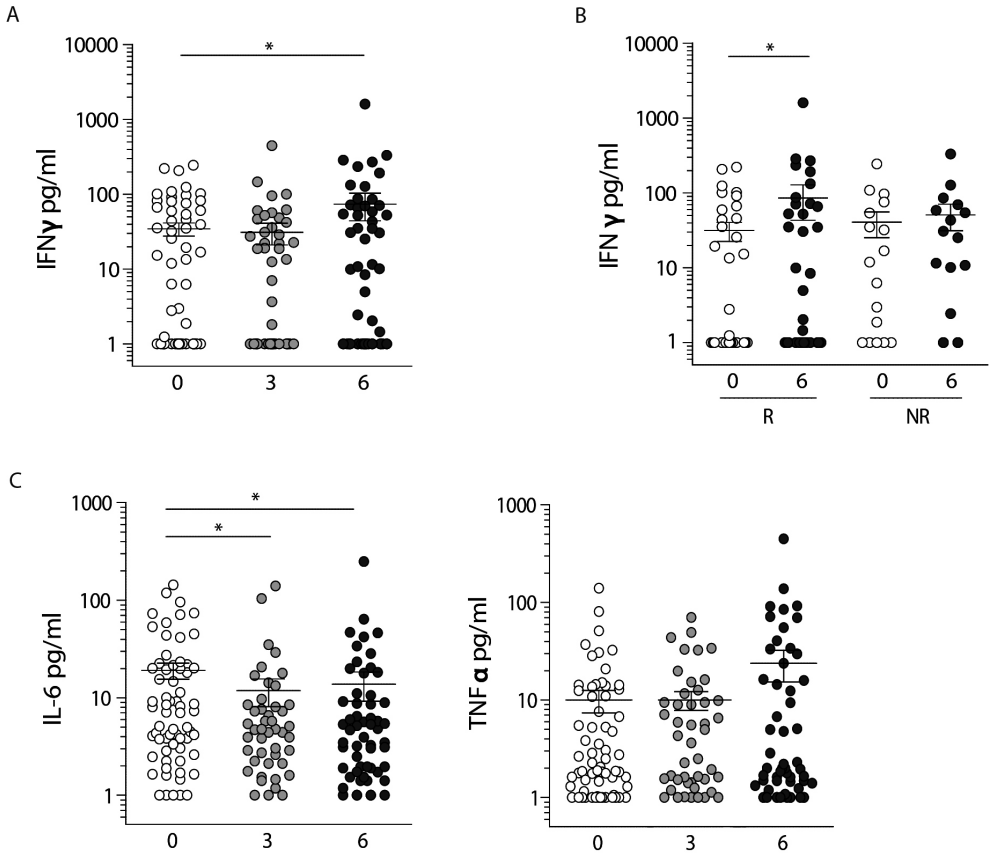

Figure 5. MTX treatment leads to increased IFNγ after 6 months, but decreased IL-6 concentrations after 3 and 6 of therapy in plasma of JIA patients. A-C. Plasma of JIA patients was collected. IFNγ, IL-6 and TNFα were measured with the Bio-Plex system, which employs the Luminex technology. A-B. IFNγ levels of JIA patients during MTX treatment (A) and of JIA patients at MTX start who will be responders (R) at 6 months after MTX start and of those who will be MTX non-responders (NR) at 6 months after MTX start. C. IL-6 and TNFα levels during MTX treatment. Ranges show mean ± SEM of n=64, *p<0.05 compared with time-point 0.

DISCUSSION

About three decades ago, MTX revolutionized the treatment of rheumatic diseases.[25] In JIA treatment, MTX became an anchor drug due to its safety and efficacy.[11] Although the clinical effect of MTX on inflammation has been firmly established in numerous clinical trials in JIA,[7-10] investigation of MTX's effects on Treg and Teff which control and drive the inflammation, has been lacking. Here we studied these compartments in JIA patients before and after the start of MTX therapy. We showed that Treg phenotype and function were not affected by MTX treatment. Conversely, $CD4^+$ and $CD8^+$ T cell proliferation was enhanced, independent

of clinical response to MTX, and plasma IFNγ levels were increased after 6 months of MTX treatment. Taken together, MTX treatment does not attenuate but rather enhances effector cell function.

Previously, no difference in Treg numbers was shown in MTX-treated RA patients.[32] Treg numbers in JIA[33] and RA[41] were also not altered upon treatment with an anti-TNFα agent etanercept, although expansion of Treg was demonstrated in RA after treatment with other anti-TNFα agents (infliximab and adalimumab).[32;33] These biologicals also restored the compromised Treg-mediated suppression of TNFα, IFNγ [32] and IL-17 in RA[33], whereas we found that Treg-mediated suppression of proliferation and cytokine production in JIA was not increased by MTX treatment. Nevertheless, Treg failed to suppress IL-17. In fact co-cultures of Treg and effector cells produced more IL-17 than effector cells alone, suggesting that Treg may produce IL-17, which has been observed recently.[42;43]

Because of its known folate, purine and pyrimidine antagonism, we expected MTX to exhibit anti-proliferative effects on T cells. However, neither *in vitro* exposure to low nanomolar concentrations of MTX, corresponding to low-dose MTX treatment[14;38;39], nor the low-dose MTX treatment itself inhibited proliferation of T cells *ex vivo* and upon T cell receptor stimulation. Instead, we observed enhanced proliferation of $CD4^+$ and $CD8^+$ T cells at 6 months compared with T cell proliferation at MTX start, in both responders and non-responders. This suggests that T cell proliferation could be directly affected by MTX, rather than by clinical improvement. The mechanism by which MTX enhances T cell proliferation remains elusive; nonetheless this concept is interesting in the light of earlier findings that (SF) T cells in RA patients with active disease were hyporesponsive to antigen or mitogen stimulation compared with healthy controls.[44;45] In the present study, T cell proliferation during active disease was similar to that of healthy controls (data not shown). This suggests that T cells in JIA, in contrast to RA, were not hyporesponsive, although their responsiveness could still be enhanced by MTX treatment.

We also demonstrated increased concentrations of IFNγ in plasma of JIA patients after 6 months, which paralleled enhanced proliferation of T cells. The increased plasma concentration of this T-cell cytokine could reflect increased T cell proliferation *in vivo* during MTX treatment. Increased plasma levels of IFNγ, in concert with increased T cell proliferation, suggest that MTX enhances the effector T cell function in JIA patients during MTX treatment. The question remains whether MTX mediates these effects by directly targeting effector T cells or perhaps other immune cell compartments. Since, total mononuclear cells were used in our assays to mimic the *in vivo* situation as closely as possible; MTX may have mediated the observed effects on effector T cells through antigen-presenting cells (APCs).

In conclusion, the present study provides evidence that MTX treatment in JIA does not target Treg, but does target effector cells. Our results provide a novel insight that low-dose MTX treatment does not attenuate Teff function but conversely, enhances T cell proliferation and plasma concentrations of IFNγ in JIA patients. This immunological data is contrary to the

common belief that low-dose MTX treatment in rheumatic diseases has immunosuppressive properties.

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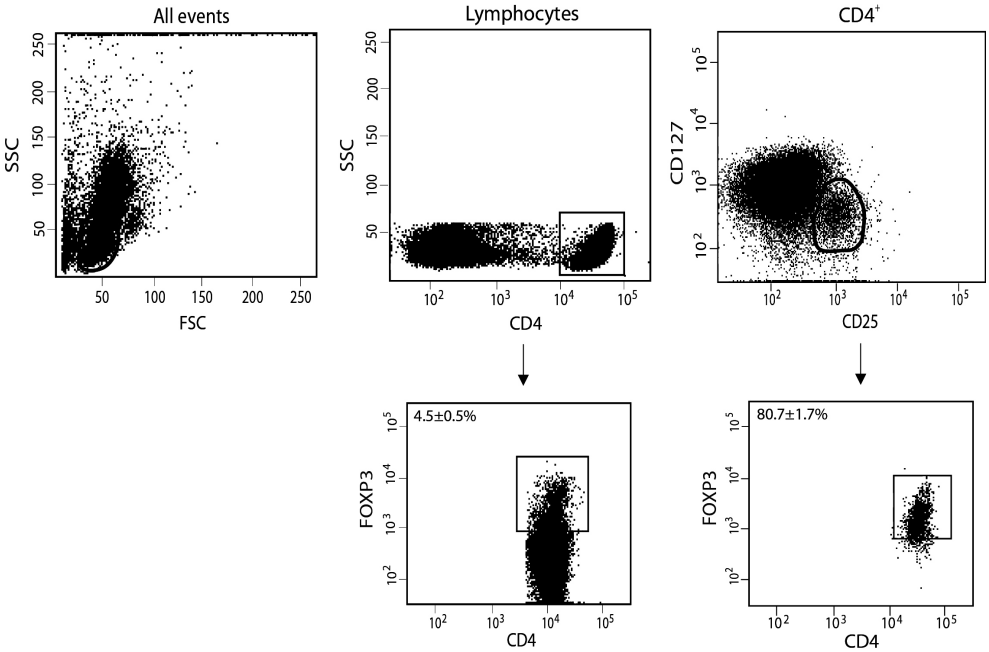
We wish to acknowledge: A. Blaauw and M.J.W. van Opdorp for valuable assistance during patient inclusion, case report form completion and investigator site file maintenance; J. Meerding for experimental assistance, and all members of the Center for Molecular and Cellular Intervention for blood sample processing.

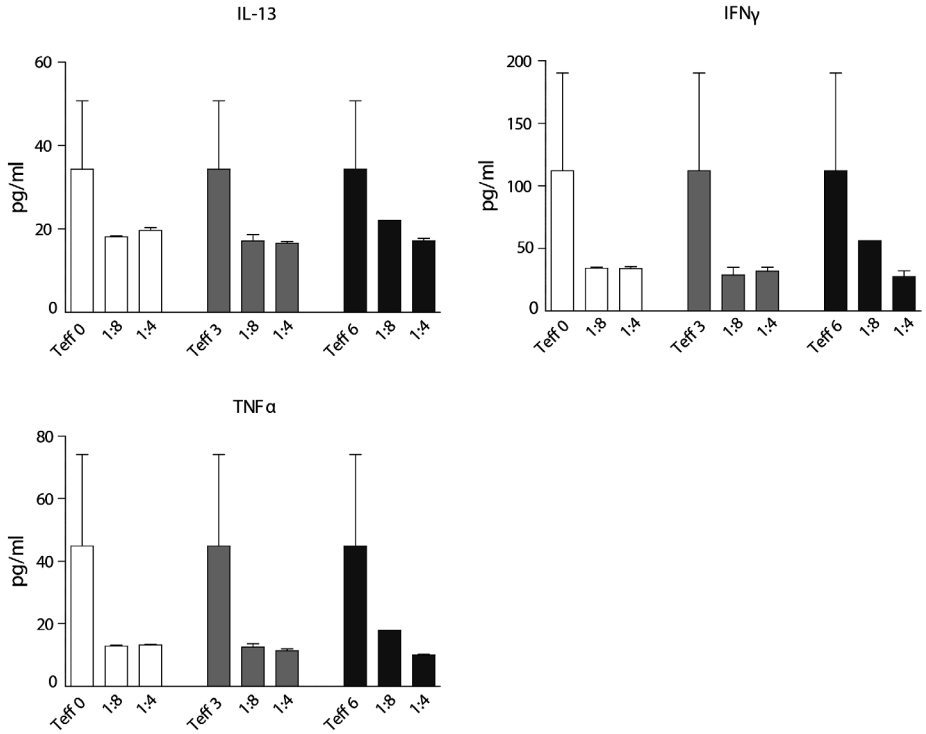
SUPPLEMENTARY INFORMATION

Antibodies used for flow cytometry

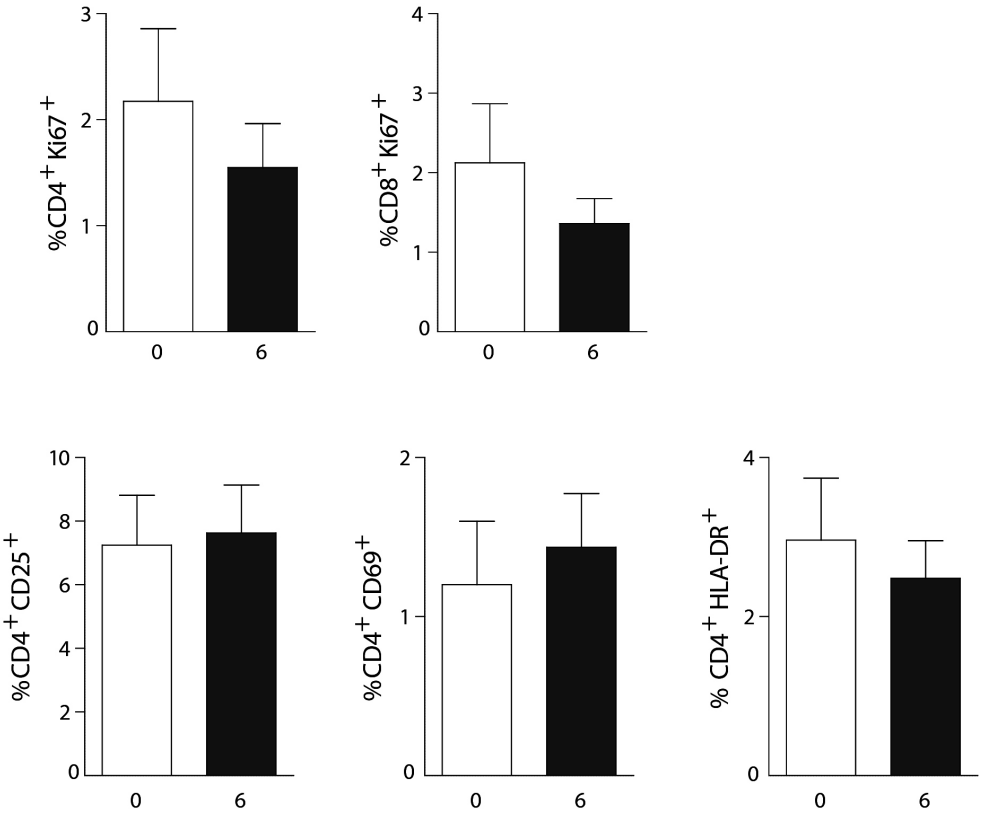
To stain PBMC for phenotyping and upon PMA and Ionomycin stimulation, PBMC were stained with the following monoclonal antibodies: anti-CD3-PerCP-Cy5.5, anti-CD3-PE-Cy7 or CD3-APC-Cy7 or anti-CD3-PcBlue (all UCHT1, BioLegend); anti-CD4-PerCP-Cy5.5 and anti-CD4-PcBlue (RPA-T4, BD Biosciences) or anti-CD4-APC (RPA-T4, eBioscience); anti-CD8-APC (SK1, BD Biosciences) or anti-CD8-PerCP (SK1, Becton Dickinson); anti-CD25-PE-Cy7 (M-A251, BD Biosciences); anti-CD69-FITC (FN50, BD Biosciences), anti-HLA-DR-PE (L243, BD Biosciences) or anti-HLA-DR-PE-Cy7 (L243, BioLegend); anti-CD39-FITC (A1, Bio-Connect); anti-CD73-PE (AD2, BD Biosciences); anti-GITR-FITC (110416, R&D Systems); anti-CD152 (CTLA-4)-APC (BNI3, BD Biosciences); anti-Ki67-FITC (B56, BD Biosciences); anti-FOXP3-APC or anti-FOXP3-PcBlue (both PCH101, eBioscience); anti-IL-13-APC (JES10-5A2, BioLegend); anti-IL-17-FITC (eBio64DEC17, eBioscience); anti-IFNγ-PE (4S.B3, BD Biosciences) or anti-IFNγ-PE-Cy7 (4S.B4, BD Pharmigen); anti-TNFα-PE (Mab11, eBioscience) or anti-TNFα-APC (Mab11, BD Biosciences). To distinguish between CD4+ and CD8+ T cells within CFSE-labeled effector cells, the following antibodies were used: anti-CD3-APC (all UCHT1, BioLegend) and anti-CD4-PcBlue (RPA-T4, BD Biosciences) or anti-CD8-PerCP (SK1, Becton Dickinson). To stain for cell sorting of Treg and Teff, the following monoclonal antibodies were used: anti-CD3-PcBlue (all UCHT1, BioLegend); anti-CD4-APC (RPA-T4, BD Biosciences); anti-CD25-FITC (M-A251, BD Biosciences) and anti-CD127-PE (hiL-7R-M21, BD Bioscience). To check for FOXP3 expression in sorted CD4+CD25+ CD127low Treg, the following antibodies were used: anti-CD4-APC (RPA-T4, BD Biosciences); anti-CD25-FITC (M-A251, BD Biosciences); anti-CD127-PE (hiL-7R-M21, BD Bioscience) and anti-FOXP3-eFluor450 (PCH101, eBioscience).



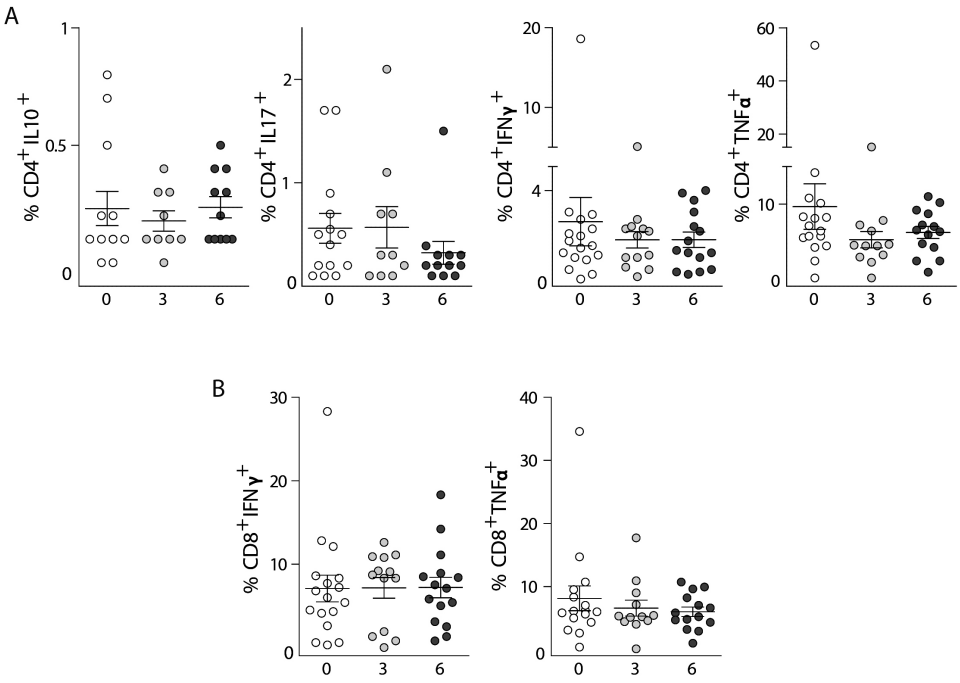
Supplementary Figure 1. Purity of sorted Treg from MTX start, 3 and 6 months after MTX start. Strategy applied to sort CD4+CD25+ CD127low Treg from time-points 0,3 and 6. The areas depicted represent the gates used to sort the cells or analyze the percentage of FOXP3+ cells (mean ± SEM). One representative of n=7.



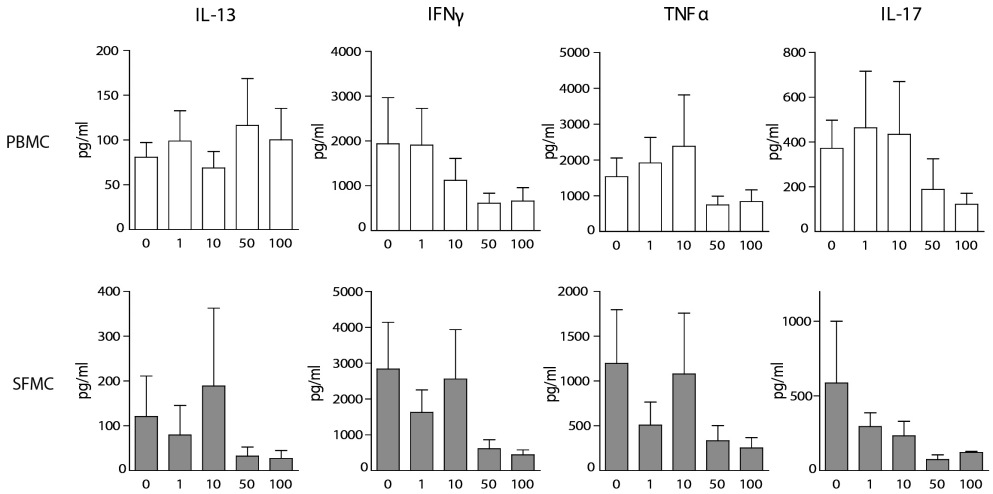
Supplementary Figure 2. Suppression of cytokine production of healthy donor effector T cells by Treg during MTX treatment. Allogeneic suppression assay: IL-13, IFNγ and TNFα levels in the absence (Teff) or presence of Treg at time-point 0 (white bars), time-point 3 (gray bars) and time-point 6 (black bars) at 1:8 and 1:4 ratios. Data represent mean cytokine levels in pg/ml ± SEM of n=2.



Supplementary Figure 3. CD4+ and CD8+ T cells during MTX treatment do not have a diminished activation status ex vivo compared to T cells at MTX start. A. Percentage of Ki67 expressing cells in CD4+ and CD8+, measured ex vivo by flow cytometry (n=8) B. Percentage of CD25, CD69, and HLA-DR expressing cells in CD4+ measured ex vivo by flow cytometry (n=9). Bars represent mean ± SEM.



Supplementary Figure 4. MTX treatment does not lead to decreased CD4+ and CD8+ T cell cytokine production in JIA patients after 6 months of therapy. A-B. PBMC were stained for cytokine expression, measured ex vivo by flow cytometry after 5 hours of PMA and Ionomycin stimulation. A. Percentage of IL-10, IL-17, IFNγ and TNFα-positive CD4+ cells. B. Percentage of IFNγ and TNFα-positive CD8+ cells. Ranges show mean ± SEM.



Supplementary Figure 5. Cytokine production by PBMC and SFMC during in vitro exposure to MTX. IL-13, IFNγ, TNFα and IL-17 levels in the absence or presence of increasing concentrations of MTX Data represent mean cytokine levels in pg/ml ± SEM of n=7 (PBMC) and n=3 (SFMC).

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GENERAL DISCUSSION

Methotrexate treatment response in juvenile idiopathic arthritis

in the past three decades, methotrexate (MTX) has been the cornerstone disease-modifying anti-rheumatic drug in the treatment of rheumatic diseases[1-3], including juvenile idiopathic arthritis.[4,5] Moreover, even with the emergence of biological drugs, MTX retained its central role in the treatment of JIA due to its efficacy and safety.[6] Nevertheless, MTX is not sufficiently efficacious in all JIA patients[6-10] and leads to adverse effects such as gastrointestinal intolerance[4,11-14], which compromises the establishment of tight disease control early on during disease course. In such cases, concomitant therapies with biologicals are given, 3 to 6 months upon MTX start[4,5], the time-points at which MTX's clinical effects are evaluated. High efficacy of combination therapies has also resulted in applying biologicals early in the treatment of JIA, at MTX start, before knowing the patients' response to MTX monotherapy.[9,15] Ideally, however, clinicians should practice precision medicine, thus directing the most appropriate treatment to individual patients based on their knowledge of how these patients will respond to a given therapy. In other words, clinicians should be able to determine, before the start or early (at 3 months) during MTX therapy, which patients will be responsive to MTX and will therefore benefit from MTX monotherapy, and which patients will be partially responsive or unresponsive to MTX, thus requiring fast MTX dose escalation or addition of biologicals. In order to make such tailor-made treatment decisions, clinicians necessitate tools (biomarkers) to optimize JIA treatment with MTX and in turn JIA treatment in general. Different tools for steering tailor-made therapeutic decisions are researched in **Chapter 3, Chapter 4, Chapter 5** and **Chapter 6.**

Genetic determinants and prediction of MTX response

As elaborated on in **Chapter 2,** alterations in genes (single nucleotide polymorphisms (SNPs)) encoding for enzymes in the folate/purine/pyrimidine pathways[16-25] and/or MTX transporters[26,27], could influence their expression level and activity or MTX uptake and retention, respectively, and in turn MTX efficacy. We were especially interested in the association of SNPs in MTX transporters with MTX response, as effective uptake and cellular retention is of particular importance for MTX efficacy and such studies were scarce in JIA.[17-22,25] In a large longitudinal cohort of 287 JIA patients, we showed that two polymorphisms in genes of MTX efflux transporters *ABCB1* and *ABCC3* were associated with MTX response, whereas a polymorphism in a gene of an MTX influx transporter *RFC* was associated with MTX non-response according to ACR paediatric (ACRpedi) criteria during one year of treatment (**Chapter 3**). These SNPs could be used as fast (within 1 week) and relatively-affordable-to-determine, early (before MTX start), objective biomarkers of MTX efficacy. Although they could be used to differentiate between likely to-be MTX responders *versus* non-responders and therefore contribute to optimization of MTX treatment in JIA, the abovementioned associations cannot be used in daily clinical practice to gear individualized treatment decisions in individual patients.

On the other hand, a prediction model for MTX response could be applied in clinical practice to steer tailor-made therapeutic decisions. Therefore, we took a step forward in this direction by transforming associations of SNPs with MTX efficacy into a prediction model for MTX non-response. In **Chapter 4**, we developed and validated a model to predict which patients will be unresponsive to MTX monotherapy before MTX start.[28] Using the prediction model, patients who are likely to be unresponsive to MTX will receive early additional treatment with biologicals, whereas those responsive to MTX are spared costly drug with potentially serious adverse effects. Indeed, the annual costs of a biological i.e. etanercept can exceed the annual cost of MTX by 15-fold (~1000$ for MTX versus ~15.000$ for etanercept). Moreover, the long-term adverse effects of biologicals, particularly TNFα blockers, are largely unknown and could include development of autoimmune phenomena such as inflammatory bowel disease (IBD) and malignancies such as leukaemia and lymphoma.[29-34] In spite of the abovementioned, it remains crucial to treat MTX non-responders adequately with biologicals early during disease course in order to prevent joint destruction and long-term disabilities.[29,35-37]

Our prediction model, developed in a cohort of 183 JIA patients and subsequently validated in 104 patients, included erythrocyte sedimentation rate (ESR) and four SNPs in genes coding for enzymes and transporters of the MTX metabolic pathway – *MTRR, PCFT, ABCB1 and ABCC1*.[28] The prediction model classified 72% of patients correctly in the derivation cohort and 65% in the validation cohort as either responders or non-responders to MTX treatment. SNPs were essential for adequate prediction of MTX non-response because clinical parameters or the laboratory parameter ESR alone were not able to predict MTX non-response. The same was shown for a clinical-genetic prediction model in RA patients.[38] The prediction model was subsequently converted into a risk score-system, ranging from 0 to 11 points, whereby each risk score carried a certain probability of being an MTX non-responder. For different cut-offs within the risk-score range, sensitivity, specificity, positive and negative predictive value for predicting the risk of being an MTX non-responder were computed. We chose a cut-off ≥3 as the optimal score, with a 78% sensitivity and a 49% specificity, as we considered it crucial to adequately treat as many future non-responders as possible with biologicals (high sensitivity), and at the same time attempting to restrict their use as much as possible to those patients who really need them (reasonable specificity). In an ideal situation, the physicians would use the risk score to tailor-make their therapeutic strategies to individual patients depending on their clinical goal and according to the probability of MTX (non-) response.

To use this prediction model in daily clinical practice, additional efforts have to be made. First, the impact of the model on clinicians' therapeutic behaviour as well as on clinical outcomes should be assessed.[39,40] An impact analysis needs to be performed to determine whether the prediction model can be used broadly and whether it performs better than clinical judgment.[39,40] The impact study should be conducted as a trial randomizing clinicians

to an intervention group, exposed to the prediction model, or the control group having no knowledge of it (Figure 1). Such a study will determine whether clinicians knowing the risk score will apply more aggressive therapy in predicted non-responders, for example faster MTX dose escalation or rapid addition of biologicals and whether those blinded for the risk score will adhere to the step-up approach, namely adding biologicals in patients with insufficient response to MTX after 3 to 6 months. The impact analysis would also show whether the use of the risk score, and in turn more aggressive therapy, will lead to better and/or earlier disease activity control in these JIA patients.

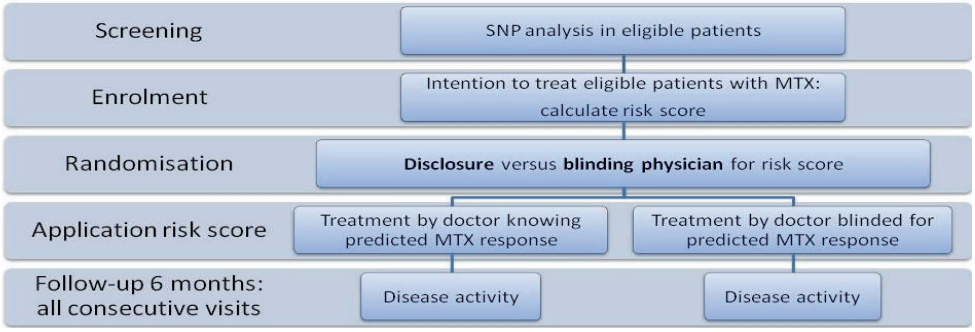

Figure 1. Set-up of the impact study to assess the performance of the prediction model for MTX non-response in daily clinical practice

Furthermore, the model's direct clinical use is impeded by its moderate predictive power of 65% in the relatively small validation cohort. This warrants the model's validation in a larger international JIA cohort and/or refinement with new biomarkers. Such biomarkers could include myeloid related-proteins, MRP-8 and MRP-14[41,42], whose increased concentrations predicted disease relapse after stopping MTX treatment[6] as well as better response to MTX treatment[43], or novel SNPs associated with MTX response, which will be provided by an upcoming large international genome wide association study in JIA patients on MTX.

Therapeutic drug monitoring to measure MTX treatment response

To date, as described above, we (**Chapter 3**) and others identified clinical and genetic determinants[17,18,20-22,44,45], associated with MTX response, and constructed a model for MTX non-response[28] (**Chapter 4**), which could assist clinicians in making individualized treatment decisions. In **Chapter 5**, we turned towards a different tool that could steer tailor-made therapeutic decisions *directly* – measurement of MTX concentrations in blood, the so-called therapeutic drug monitoring (TDM) of MTX. MTX polyglutamates (MTX-PGs) could be a suitable TDM tool in JIA patients[46,47], since polyglutamated MTX mediates MTX's anti-inflammatory effects[24], as elaborated on in **Chapter 2**, and could thus be a biomarker of MTX efficacy in JIA,

if measured early after MTX start. Previous studies in JIA and RA showed conflicting results on the association between MTX-PGs and disease activity[48-55], which could be due to their cross-sectional design, thus impacting the reliability of evaluated disease activity, and variable duration of MTX use, hence compromising the comparison of MTX-PG concentrations between patients, given that MTX-PG accumulation is dependent on the time of exposure to MTX.[56]

In **Chapter 5**, we therefore set out to determine whether erythrocyte MTX-PGs, measured at 3 months after MTX start, were associated with disease activity in a large prospective JIA cohort, followed for one year after MTX start. We determined that long-chain MTX-PG3, MTX-PG4, MTX-PG5 and their sum (MTX-PG3-5), measured after 3 months of MTX use, were associated with lower disease activity at 3 months and during one year of MTX treatment, which was in line with a concomitantly performed longitudinal study in RA.[57,58] The applicability of MTX-PGs as a TDM tool can be illustrated in patients who stopped MTX (n=4) and those who received additional medication (n=7), due to insufficient effect. Patients that discontinued MTX had significantly lower MTX doses and lower long-chain MTX-PG concentrations than those who continued MTX. Instead of stopping MTX, these patients may have benefited from MTX dose escalation. On the other hand, patients on additional medication at 6 months had similar MTX doses and MTX-PG concentrations at 3 months, as patients on MTX monotherapy. They remained non-responders, in spite of optimal MTX treatment (reflected by adequate polyglutamation). If timely monitored with TDM, they could have received additional medication earlier than 6 months after MTX start. TDM of MTX-PGs in JIA could guide clinicians to escalate MTX dose in patients with a low polyglutamation rate, and to offer biologicals to patients with insufficient response to MTX with adequate polyglutamation. Future research should focus on determining MTX-PG pharmacokinetics in response to MTX dose escalation and (changes in) MTX route of administration, with sequential MTX-PG measurements during the first year of MTX treatment. Knowing how to influence accumulation and concentrations of MTX-PGs, with the aim of maximising response to MTX, could enable optimisation of MTX treatment for individual patients.

Disease activity score to measure and compare treatment response

It is noteworthy that, in contrast to **Chapters 3** and **4**, in which the ACRpedi criteria for disease activity are used as the primary outcome measure; **Chapter 5**[57] employed a recently developed and validated composite disease activity measure[59,60], the juvenile arthritis disease activity score in 27 joints (JADAS-27) as the primary outcome.[61] In contrast to relative measures like the ACRpedi criteria, the JADAS-27 is an absolute disease activity measure, which can be used to determine, evaluate and compare disease activity status and course in and between individual patients. In order facilitate the use of JADAS-27 in therapeutic decision-making in clinical practice and trials, we showed in **Chapter 6** that JADAS-27 had moderate to good responsiveness to changes in disease activity status, changed in face of clinical improvement

(median of -5.5 points) and worsening (median of +1.7 points), and had cut-off scores for low (≤2.7) and high disease activity (≥6).[61] If these results are validated in an independent JIA cohort, these JADAS-27 interpretations could be potentially applicable in clinical practice and trials for monitoring and comparison of disease activity (changes) in and between individual patients in response to MTX or other treatments. Given the abovementioned, JADAS-27 also emerges as an important tool in tailoring treatment to individual patients.

The first part of this thesis showed that the central role, which MTX has in the treatment of JIA, should be seen as an opportunity to bring MTX from patients' bedside back to the bench with the goal of optimizing MTX treatment. The research described in the first part of this thesis does not only have a potential to optimize treatment with MTX, but also the treatment with biologicals, as it aims to determine which patients will be responsive to MTX only, and which patients will need more aggressive treatment with biologicals. The latter is particularly important, since, regardless of their high efficacy, biological use at disease onset in all patients is not plausible or desirable due to their high costs, poorly elucidated, but potentially serious, long-term adverse effects and inevitable over-treatment of patients who would have benefited from MTX only. Moreover, such use of biologicals does not satisfy the goal of tailor-made treatment for JIA patients. In the future, the challenge will lie in gathering a large well-defined JIA cohort followed-up from the start of MTX treatment in order to validate the prediction model and MTX-PGs, described in these thesis, as tools for steering tailor-made therapeutic decisions, and to generate new genetic (SNPs or gene expression patterns) and immunological (i.e. MRPs) biomarkers and integrate them into improved tools for steering tailor-made therapeutic decisions. Down the road, applying these tools in daily clinical practice will be the greatest challenge. If this challenge is taken on, the therapeutic goal of achieving clinical remission in tailor-made fashion could be reachable.

Methotrexate intolerance in juvenile and adult arthritis

While optimization of MTX efficacy using the tools, described in the first part of this thesis, is crucial for tailoring treatment to individual patients, identification, prediction and treatment of MTX-related adverse effects are equally important for tailor-made therapeutic decision-making, as shown in the second part of the thesis. Adverse effects, such as hepatotoxicity and bone marrow suppression are infrequent and usually transient if MTX is stopped.[62] We therefore turned towards identification, prediction and treatment of the common MTX-related adverse effect, namely gastrointestinal MTX intolerance in **Chapter 7, Chapter 8, Chapter 9 and Chapter 10.**

MTX intolerance severity score and prevalence of MTX intolerance

In spite of folic acid supplementation[63-65], used for prevention and treatment of gastrointestinal adverse effects, JIA patients still experience gastrointestinal symptoms, such as abdominal discomfort, nausea, vomiting and diarrhoea after MTX intake.[8,12,37,66-68] In **Chapter 7**[11], we focused not only on these symptoms, but also on anticipatory and associative symptoms occurring before MTX intake[13] and when thinking of MTX as well as on behavioural symptoms such as restlessness and crying when taking MTX. These adverse effects arise as a conditioned response to the physical symptoms following MTX intake. In classical conditioning terms, otherwise known as Pavlovian conditioning, an unconditioned stimulus (i.e. MTX) produces an unconditioned physical response, at which moment many potential conditioned stimuli are present (Table 1).

Table 1. Pavlovian conditioning

	Pavlovian conditioning in original dog experiment	**Pavlovian conditioning during MTX use**
Unconditioned stimulus	Food	Methotrexate pill or injection
Unconditioned response	Salivation	Physical complaints after MTX use (pathophysiology unknown)
Conditioned stimuli	Ringing of the bell	Yellow colour of pill/injection fluid Liquid MTX is administered with
Conditioned response	Salivation on bell ringing	Physical complaints before and when thinking of MTX

The most commonly reported conditioned stimuli at our out-patient ward are the yellow colour of the pill or injection fluid, or the liquid (water or juice) MTX is administered with. These conditioned stimuli lead to conditioned response of anticipatory and associative adverse effects. A very fitting description of the abovementioned, written by a JIA patient, was given in the novel "SchEef" by Marlies Allewijn: "*Ik word er steeds misselijker van, lijkt het wel. Ik maak me al dagen van tevoren druk over het innemen van die rotpillen. Het klinkt heel gek, maar ik heb nu al het gevoel dat ik misselijk ben. Dat kan niet, want ik heb ze nog niet ingenomen, maar toch voel ik me niet lekker. Het is zelfs nog gekker: als ik er iemand over vertel of ik schrijf erover, word ik al misselijk."[free translation: I get more and more nauseous, it seems. I am getting upset about taking these terrible pills days before having to take them. It sounds odd, but I already have a feeling that I am nauseous. That can't be since I did not take them yet, but I really do not feel well. It's even crazier: I even get nauseous if I talk or write about it].*

In contrast to cancer patients in whom anticipatory nausea and vomiting are well-known conditioned reactions in response to strongly hematogenic chemotherapy regimens[69,70], these symptoms were unrecognized in JIA and RA patients on low-dose MTX treatment, even though the presence of these symptoms in particular could compromise the use and thus efficacy

of MTX treatment and patients' quality of life.[14] Keeping such consequences in mind, we first designed and validated a questionnaire (Methotrexate Intolerance Severity Score (MISS)), determining that adverse effects after MTX intake as well as the conditioned and behavioural adverse effects are frequent, thus establishing a definition of MTX intolerance, which included both types of adverse effects (a total score of ≥6 with at least 1 point on anticipatory and/or associative and/or behavioural complaints). Using this definition, we subsequently determined that the prevalence of MTX intolerance in a large cohort of 297 JIA patients on MTX reached a high 50.5%. Strikingly, the prevalence of MTX intolerance was 23% higher in patients on parenteral (67.5%) than on oral MTX (44.5%). This difference originated from a higher occurrence rate of behavioural symptoms and conditioned pre-treatment abdominal pain, nausea and vomiting. In keeping with our findings, a recent study showed that taking MTX subcutaneously was associated with a greater risk of feeling sick before, vomiting after MTX administration and anxiousness about injections compared to oral MTX.[14] In our study, aversion towards needles, besides evident aversion towards MTX (the prevalence of adverse effects in the absence of needles, namely in patients on oral MTX was also high) could have also contributed to a higher prevalence of these symptoms in the parenteral group. This finding is especially striking in the light of a common clinical practice of switching patients from oral to parenteral MTX because of gastrointestinal complaints, as parenteral MTX is thought to give rise to fewer gastrointestinal symptoms. In our cross-sectional JIA cohort, we had no knowledge of whether patients used parenteral MTX from MTX start or whether they were switched to parenteral MTX due to insufficient response to MTX or gastrointestinal adverse effects.

In **Chapter 8**, we showed that the occurrence of anticipatory and associative gastrointestinal complaints was not restricted to JIA patients only, but also occurred in adult patients with RA and psoriatic arthritis (PsA). However, while prevalence of MTX intolerance reached 55.5% in JIA, the prevalence in RA and PsA (n=291), measured with the same tool (MISS), was considerably lower amounting to 11%. MTX intolerance was also less severe in adults (median score of 9) than in children (median score of 12). Substantially lower MTX intolerance prevalence in adult patients was due to: lower percentage of adults with a score ≥6 (definition of MTX intolerance) and lower percentage of adults (24.4% versus 67% in JIA) with at least one anticipatory, associative and/or behavioural symptom. The latter suggests a weaker classic conditioning response in adults than in children taking MTX, which is supported by the fact that only 51% of 106 RA/PsA patients with symptoms after MTX had symptoms also before MTX intake, while this was the case in 82% of 204 JIA patients. Interestingly, adults are though to be less prone to classical conditioning than children due to ageing-related changes in hippocampus and cerebellum (fewer synaptic connection, volume reduction and Purkinje cell loss).[71-74] However, similarly to JIA, MTX intolerance prevalence was higher in patients on parenteral (20.8%) than on oral MTX (6.2%). As stated above, it is common to switch patients from oral

to parenteral MTX due to gastrointestinal symptoms. In contrast to the JIA cohort, we could establish that the majority of intolerant RA patients (13 of 20) on parenteral MTX had been switched to this route of administration from oral MTX due to gastrointestinal symptoms. In order to establish whether parenteral MTX, intrinsically, carries a higher risk of gastrointestinal complaints, patients using parenteral MTX from the treatment start should be compared to those using oral MTX from the start. Indeed, our preliminary results in a German JIA cohort showed that patients on parenteral MTX from the treatment start had a higher prevalence of MTX intolerance than those on oral MTX (personal communication).

Chapter 7 and **Chapter 8** showed that anticipatory and associative gastrointestinal complaints are clinically not very evident as they cannot easily be detected by the clinician's assessment only, and that such symptoms possibly persist after a switch from oral to parenteral MTX. Since persistent gastrointestinal symptoms are the major reason to discontinue MTX, intolerant patients could be more prone to stop MTX or switch to (less effective) DMARDs or expensive biological.[4,75-79] Therefore, arthritis patients on MTX should be monitored with the MISS, as it allows early detection of MTX intolerance. This could create window of opportunity not only for timely treatment of MTX intolerance, but also for early treatment of emerging physical symptoms, which could prevent the development of conditioned responses and therefore MTX intolerance.

Treatment of MTX intolerance

In **Chapter 9** we embarked on investigating the effect of three therapeutic strategies on MTX intolerance in a randomized clinical trial. A commonly applied strategy in patients experiencing MTX-related gastrointestinal adverse effects is addition of antiemetic drugs to oral MTX.[4,80] As stated above, patients having gastrointestinal symptoms on oral MTX are commonly switched to parenteral MTX, as parenteral MTX may be associated with fewer gastrointestinal symptoms.[12,67,81] Nevertheless, these strategies are not always successful, which has also been suggested in **Chapter 7** showing higher prevalence of MTX intolerance in JIA on parenteral MTX and in **Chapter 8** demonstrating persistence of MTX intolerance in the majority of RA patients being switched from oral to parenteral MTX due to intolerance. Efficacy of these strategies could be compromised due to their targeting of physical symptoms only, but not of anticipatory, associative and behavioural symptoms as well. On the other hand, behavioural interventions do target these conditioned responses[69], as we showed previously in an uncontrolled pilot study in which 7 (77.8%) of 9 patients were treated successfully with behavioural therapy.[13] Therefore **Chapter 8** investigated the effect of oral MTX and behavioural therapy *or* parenteral MTX compared with the standard of care treatment consisting of oral MTX with an antiemetic, on MTX intolerance in JIA.

Contrary to our hypothesis, behavioural therapy did not target MTX intolerance more successfully than the other two strategies. Unexpectedly, all three treatment strategies had

beneficial effects on MTX intolerance. Such effect was expected of behavioural therapy whose primary target are conditioned adverse effects, but not of parenteral MTX or oral MTX with antiemetics. A plausible explanation could be that circumvention of gastrointestinal mucosa with parenteral MTX and antiemetic use diminished the physical symptoms, resulting in reduction of conditioned responses and behavioural distress, as it is known that conditioned responses cease if physical symptoms are absent. Even more striking was the finding that a sharp decline in MTX intolerance scores occurred in the first week of enrolment, strongly suggesting that participation in the trial, rather than given treatments, mediated the observed beneficial effects in all three groups. Participating in the trial reflects patient's motivation and positive expectations leading to a rapid change in reported symptoms.[82-84] Patients' expectations play an important role in the development of conditioned responses, as was shown for cancer patients who did not develop anticipatory nausea if they were not expecting to develop it.[83] Similarly, positive expectations of resolving MTX intolerance upon entering the trial could have been the main driving force behind a swift decrease in MTX intolerance severity, independent of treatment strategy. Nevertheless, the beneficial effect continued during the entire follow-up, which could be contributed to the direct effect of treatment strategies as well.

Besides illustrating the phenomenon of trial participation, **Chapter 9** also illustrates how challenging it is to recruit paediatric patients with a complex problem in a clinical trial. We included 48 patients – only 38.1% of the initially calculated samples size. A similar number (47) declined to participate despite eligibility, due to sufficiently handled MTX intolerance using other preferred strategies (partitioning the doses given, concealing pills or injection liquid in food), or wish to choose one of the treatment strategies rather than to be randomised. In addition, around 20% of included patients refused the allocated treatment immediately upon randomization or during follow-up or switched to another treatment strategy.

It could be hypothesised that the same mechanism of expectations and motivations led to: on the one hand, fast resolution of MTX intolerance in the trial participants who may have had high expectations and were motivated, and, on the other hand, low recruitment rate and refusal of the allocated treatment upon randomization in those patients (and parents) having low expectations and lack of motivation and/or a different preferred treatment strategy from the allocated one. Indeed, a recent study showed that mothers could still appreciate the medication despite the experienced difficulties in taking the drug.[14] Therefore, the treatment strategies for MTX intolerance should be tailored in such a manner that a treatment strategy is chosen which both parties are motivated for, as motivation and positive expectations appear to be important for the control MTX intolerance.

Prediction and prevention of MTX intolerance

Controlling MTX intolerance could be done by predicting its occurrence and thus preventing its development, as elaborated on in **Chapter 10.** Similarly to **Chapter 4**, we developed and internally validated a prediction model for MTX intolerance (occurring at 6 or 12 months after MTX start) in a cohort of 152 JIA patients. Our prediction model consisted of routine clinical variables: JIA subtype, JADAS-27, parent/patient assessment of pain, antinuclear antibody (ANA), alanine transaminase (ALAT), thrombocyte count, creatinine and an interaction term between creatinine and JIA subtype. The model classified 77.5% of patients correctly, and 66.7% after internal validation by bootstrapping. In contrast to **Chapter 4** where SNPs were essential for predicting MTX non-response[28], here SNPs did not significantly contribute to the prediction of MTX intolerance.

The prediction model was subsequently transformed into an accessible risk score, ranging from 0 to 17 points, which could readily be used by clinicians based on the knowledge of clinical variables, which are routinely determined and available for all JIA patients at MTX start. We chose a cut-off ≥6 as the optimal score, at which 82% of intolerant patients were classified correctly (high sensitivity), while maintaining correct classification of 56.1% of tolerant patients (moderate specificity). Patients at risk of developing MTX intolerance would be identified at MTX start using the prediction model. These patients could then be frequently monitored (using the MISS) and their physical gastrointestinal symptoms identified early and treated timely, thus preventing the development of a classical conditioning response and hence the development of MTX intolerance. Similarly to patients already suffering from MTX intolerance, the treatment strategies, which could include lowering MTX dose, switching to parenteral MTX, starting antiemetics or behavioural therapy, should be tailored to patients' and parents' motivations and expectations. Prevention of these gastrointestinal symptoms with fitting strategies is very likely to preserve and improve the patients' quality of life.[14]

Taken together, the second part of this thesis demonstrated that MTX intolerance is a complex adverse effect, which affects not only JIA but also RA/PsA patients. In daily clinical practice, patients should be frequently monitored with the MISS, particularly those at high risk of developing MTX intolerance (as identified by the prediction model), as this would create a window of opportunity for timely detection and treatment of emerging physical symptoms, which could prevent the development of conditioned responses and therefore of MTX intolerance. Importantly, treatment of MTX intolerance, being with anti-emetic drugs, a switch to parenteral MTX or cognitive-behavioural therapy, should be tailored to satisfy patients' and parents' expectations and motivation, as they seemed to be important for the control of MTX intolerance.

Methotraxate effects on T cells – and now for something completely different

In spite of its firmly established role in the treatment of JIA, the effect of MTX on the balance between regulatory (Treg) and effector (Teff) cells, which control and drive inflammation[85-89], is poorly understood in humans. In animal models, MTX's effects have been attributed to anti-inflammatory adenosine, whose production is mediated by CD39 and CD73 ectoenzymes.[90-94] In turn, adenosine production by $CD39^+/CD73^+$ Treg leads to increased Treg numbers and suppressive function.[95-98] In humans, *in vitro* exposure to MTX has been shown to induce (sensitivity to) apoptosis of activated T cells.[99-101] This is attributable to the inhibition of folate metabolism and *de novo* purine and pyrimidine synthesis, resulting in anti-proliferative effects, which is the most prominent feature of MTX.[24] Despite the abovementioned evidence, animal models and *in vitro* experiments are not representative of the clinical reality of JIA patients on MTX because: as opposed to MTX's effects in hours or days in cell culture systems and animal models, the therapeutic effects of MTX can be evaluated only after 3 or 6 months of treatment, which is in part due to time-dependent accumulation of long-chain MTX-PGs, which mediate MTX's efficacy (**Chapter 5**). Therefore, *ex vivo* data from patients using MTX is required to clarify the effects of MTX on Treg and Teff. In **Chapter 11,** we investigated quantitative and qualitative effects of MTX treatment on Treg and Teff of JIA patients at MTX start and while on MTX for 3 and 6 months.

Similarly to JIA and RA upon treatment with an anti-TNFα agent etanercept[102,103], but in contrast to other anti-TNFα agents, such as infliximab and adalimumab[102,104], which restored the compromised Treg-mediated suppression of cytokines, we showed that Treg phenotype (amongst others, also CD39 and CD73) and suppressive function of Teff proliferation and cytokine production were not affected by MTX treatment after 3 and 6 months. Contrary to our hypothesis that MTX would have an anti-proliferative effect on $CD4^+$ and $CD8^+$ T cell proliferation, we determined that neither *in vitro* exposure to low nanomolar concentrations of MTX, corresponding to low-dose MTX treatment, nor the low-dose MTX treatment itself inhibited proliferation of T cells *ex vivo* and upon T cell receptor stimulation. Instead, $CD4^+$ and $CD8^+$ T cell proliferation at 6 months was enhanced in both responders and non-responders. This suggested that T cell proliferation could be directly affected by MTX, rather than by clinical improvement. Finally, we showed increased concentrations of IFNγ, but not of pro-inflammatory IL-6 and TNFα, in plasma of JIA patients after 6 months, which paralleled enhanced proliferation of T cells. Increased plasma levels of IFNγ, in concert with increased T cell proliferation, suggest that MTX does not attenuate but rather enhances the effector T cell function in JIA patients during MTX treatment. The immunological data in **Chapter 10** is contrary to the common belief that low-dose MTX treatment in rheumatic diseases has immunosuppressive properties. Future efforts should focus on answering whether MTX mediates these effects by directly targeting effector T cells or perhaps other immune cell compartments, such as the antigen-presenting cells (APCs).

CONCLUSION

The ultimate goal in the treatment of JIA is to provide effective treatment as early as possible during disease course for all JIA patients, which is crucial in preventing joint destruction and long-term disabilities. MTX, which revolutionized the treatment of rheumatic diseases about three decades ago, satisfies this goal in the majority of JIA patients. In some, however, early effective treatment cannot be achieved with MTX only due to its insufficient efficacy or (gastrointestinal) side effects. In such patients, biological agents are given (concomitantly with MTX). Over the past years, prompted by the need to establish tight disease control or even disease remission early after disease onset, paediatric rheumatologists began applying concomitant treatment with biologicals, not only in patients in whom MTX was not sufficiently efficacious, but also in patients at MTX start in whom MTX efficacy was not yet established. However, early use of biologicals is not plausible or desirable due to their high costs, poorly elucidated, but potentially serious, long-term adverse effects and inevitable over-treatment of patients who would have benefited from MTX only. Ideally, clinicians should practice precision medicine by giving MTX monotherapy to patients who will be responsive to MTX, and combination therapy with biologicals to those patients who will be unresponsive to MTX.

In order to make such tailor-made therapeutic decisions, MTX treatment needs to be optimized first. We show that JIA treatment can be tailored to fit individual patients by predicting the (non)-response to MTX at MTX start using a prediction model containing SNPs in genes encoding enzymes targeted by MTX and MTX transporters. We also establish that long-chain MTX-PGs could be utilized as a TDM tool in order to guide therapeutic decision-making towards MTX continuation, dose escalation or addition of concomitant biological treatment. Optimization of MTX treatment also includes treatment and prediction of MTX-induced gastrointestinal adverse effects – MTX intolerance, which occurs in over one half of JIA patients on MTX. We demonstrate that all three treatment strategies successfully tackle MTX intolerance, likely owing to patients' and parents' positive expectations and motivation, which treatment of MTX intolerance should be tailored to. We further show that MTX intolerance can be predicted at MTX start using routine clinical variables, offering a window of opportunity to preventing the development of MTX intolerance in patients at risk. Finally, enhancement of effector T cell function during MTX treatment points at immunomodulatory and not immunosuppressive mode of action of MTX in JIA. Future research should focus on gathering a large well-defined JIA cohort in order to validate the tools for steering tailor-made therapeutic decisions, described in this thesis, and to refine these tools with novel genetic and immunological biomarkers, followed by their application in daily clinical practice, in order to provide tailor-made treatment for each JIA patient.

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ADDENDUM 1

DO SNAPSHOT STATISTICS FOOL US IN MTX PHARMACOGENETIC STUDIES IN ARTHRITIS RESEARCH?

Maurits C. F. J. de Rotte[1], Jolanda J. Luime[2], Maja Bulatović[3], Johanna M. W. Hazes[2], Nico M. Wulffraat[3] and Robert de Jonge[1]

[1]Department of Clinical Chemistry; [2]Department of Rheumatology, Erasmus University Medical Center, Rotterdam; [3]Department of Pediatric Immunology, University Medical Center Utrecht, Wilhelmina Children's Hospital, Utrecht, The Netherlands

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SIR, Recently, an interesting discussion was published in *Rheumatology* on discrepant literature results concerningMTX pharmacogenetics in 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 (SNPs) in the *ATIC* gene (rs4673993 and rs2372536, bothin linkage disequilibrium): the 347 C-allele[4,5] and the G-allele[2,6] were both associated with increased efficacy of MTX. Similar discrepancies for SNPs in the methylenetetrahydrofolate reductase (*MTHFR*) gene were reported in a meta-analysis earlier this year.[7] Intrying to explain the discrepancy, Dervieux[1] pointed out the challenges and difficulties that 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 thesnapshot 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 monthsin 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; Fig. 1). From Figure 1 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 1-year 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 lessinformative because, at the next hospital visit, a substantial number of patients may become non-responders and vice-versa. Second, thesnapshot approach only evaluates patients that are still available at the analysed time point and hence, ignoring dropouts or missing data. Often, missing data are 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 studiesmay 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 (Fig. 1) and (ii) to perform sophisticated repeated measurement statistics that are not affected by the disadvantages ofsnapshot 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.

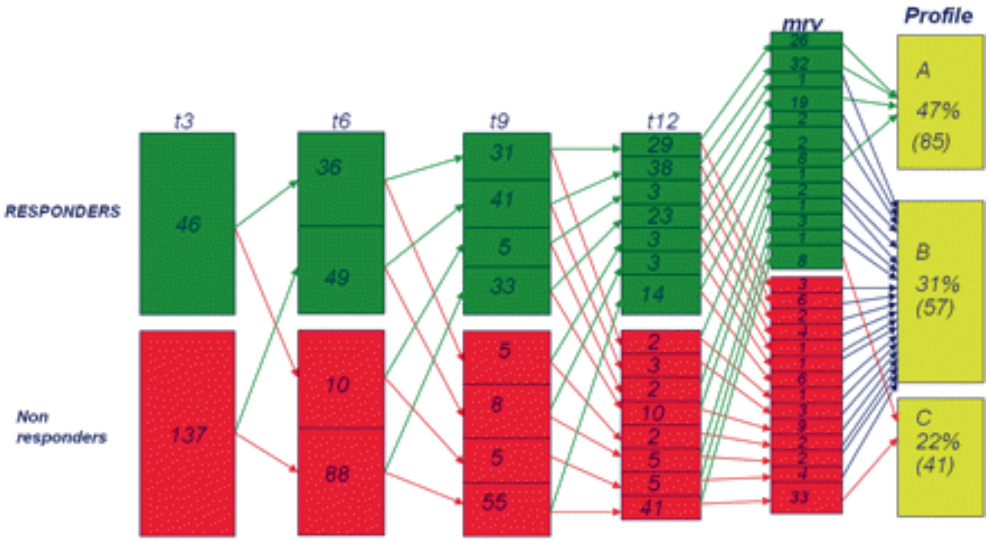

Figure 1. Responders and non-responders in 183 JIA patients following Paediatric American College of Rheumatology 30% (ACRped30) criteria in 3-month intervals up to the most recent visit after start of treatment with MTX. Response is divided into three profiles: (A) patients who will respond towards treatment at any time point between start of treatment and 1-year 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 first year of treatment (22%). t3, t6, t9, t12 = time points 3, 6, 9 and 12 months, respectively, after start of MTX treatment; mrv = most recent visit.

KEY MESSAGE

- Longitudinal designs and repeated measures statistics *vs* cross-sectional analysis prevent false-positive findings in MTX pharmacogenetics

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ADDENDUM 2

SUMMARY

SAMENVATTING

ACKNOWLEDGMENTS

CURRICULUM VITAE

LIST OF PUBLICATIONS

SUMMARY

Methotrexate (MTX) is the cornerstone treatment in juvenile idiopathic arthritis (JIA). Nevertheless, MTX is insufficiently efficacious and leads to adverse effects in some JIA patients, which compromises attainment of complete disease control. In such cases, combination therapies with biologicals are given. High efficacy of combination therapies has resulted in applying biologicals early during JIA treatment, even at MTX start, before knowing the patients' MTX response. However, clinicians should know, before or early after MTX start, which patients will benefit from MTX only and which patients will not, thus requiring addition of biologicals. To make such tailor-made treatment decisions, clinicians necessitate tools to optimise JIA treatment with MTX and in turn JIA treatment in general. This and other unmet needs of MTX have been addressed in chapter 2 and further elaborated on in the rest of this this.

In part I of this thesis, we investigate tools for steering tailor-made therapeutic decisions in JIA. In chapter 3, we showed in 287 JIA patients that single nucleotide polymorphisms (SNPs) in genes of MTX efflux transporters *ABCB1* and *ABCC3* were associated with MTX response, whereas a SNP in an MTX influx transporter *RFC*-gene was associated with MTX non-response during one year of treatment. These SNPs could be used as early and affordable objective biomarkers of MTX efficacy. However, in order to utilise SNPs to gear individualised treatment decisions, we transformed associations of SNPs with MTX efficacy into a prediction model in chapter 4. To predict which patients will be unresponsive to MTX monotherapy before MTX start, we developed a model in 183 JIA patients and validated it 104 patients. The model included erythrocyte sedimentation rate and four SNPs in genes encoding for enzymes and transporters of the MTX metabolic pathway – *MTRR, PCFT, ABCB1 and ABCC1*. The prediction model classified 72% of patients correctly in the derivation cohort and 65% in the validation cohort. The model was converted into a risk score-system, ranging from 0 to 11 points, whereby each risk score carried a certain probability of being an MTX non-responder. We chose a cut-off ≥3 as the optimal score, with a 78% sensitivity and a 49% specificity, as we considered it crucial to adequately treat as many future non-responders as possible with biologicals (high sensitivity), and at the same time attempting to restrict their use as much as possible to those patients who really need them (reasonable specificity). Using the model, patients who are likely not to respond to MTX will receive biologicals early, whereas those responsive to MTX will be spared costly biologicals with potentially serious adverse effects. In chapter 5, we investigated the association of MTX polyglutamates (MTX-PGs), measured at 3 months after MTX start, with disease activity (measured using the juvenile arthritis disease activity score (JADAS-27) researched in chapter 6) of 113 JIA patients followed for one year after MTX start. We determined that long-chain MTX-PG3, MTX-PG4, MTX-PG5 and their sum were associated with lower 3-month and one-year disease activity. We showed that patients that discontinued MTX (n=4) had lower long-chain MTX-PG concentrations than those that continued MTX.

Patients on additional medication at 6 months (n=7) had similar MTX-PG concentrations at 3 months to patients on MTX monotherapy, but nonetheless remained MTX non-responders nonetheless. Therapeutic drug monitoring of MTX-PGs could guide clinicians to escalate MTX dose in patients with low polyglutamation rate or to give biologicals early to patients with adequate polyglutamation, but insufficient MTX response.

In part II, we focus on the most common MTX-related adverse effect, MTX intolerance, in JIA as well as in rheumatoid (RA) and psoriatic arthritis (PsA). In chapter 7, we determined the prevalence of MTX intolerance, which includes gastrointestinal symptoms (abdominal pain, nausea and vomiting) occurring after, before (anticipatory) and when thinking of (associative) of MTX intake or injection (the latter two being the conditioned responses to physical symptoms), as well behavioural symptoms such as restlessness and crying when taking MTX. In a cohort of 297 JIA patients, we showed that the prevalence of MTX intolerance, determined using a newly validated Methotrexate Intolerance Severity Score (MISS), reached a high 50.5%. The prevalence of MTX intolerance was 23% higher in patients on parenteral (67.5%) than on oral MTX (44.5%). In chapter 8, we demonstrated that MTX intolerance also occurred in RA and PsA patients, although to a much lower extent, namely in 11% of 291 patients, suggesting a weaker classic conditioning response in adults than in children taking MTX. Similarly to JIA, however, MTX intolerance prevalence was higher in patients on parenteral (20.8%) than on oral MTX (6.2%). We conclude that arthritis patients on MTX should be monitored with the MISS, as it allows early detection of MTX intolerance and offers opportunity for timely MTX intolerance treatment.

In chapter 9, we compared the therapeutic effect of oral MTX and behavioural therapy (n=15) *or* parenteral MTX (n=17) with oral MTX combined with an antiemetic (n=16) on MTX intolerance. Contrary to our hypothesis, behavioural therapy was not superior in targeting MTX intolerance; instead, all strategies were beneficial in the first enrolment week, suggesting the crucial role of trial participation. We conclude that treatment strategies for MTX intolerance should be tailored individually, based on motivations and expectations of patients and their parents. In chapter 10, we developed and internally validated a prediction model for MTX intolerance (occurring at 6 or 12 months after MTX start) in a cohort of 152 JIA patients. The model consisted of clinical variables: JIA subtype, JADAS-27, parent/patient assessment of pain, antinuclear antibody, alanine transaminase, thrombocyte count, creatinine and an interaction term between creatinine and JIA subtype. The model classified 77.5% of patients correctly, and 66.7% after internal validation, and was transformed into a risk score, ranging from 0 to 17 points. We chose a cut-off ≥6 as the optimal score, at which 82% of intolerant patients were classified correctly (high sensitivity), while maintaining correct classification of 56.1% of tolerant patients (moderate specificity). With this prediction model, patients at risk of developing MTX intolerance could be identified at MTX start and thus treated timely, preventing the development of MTX intolerance.

In part III, chapter 11, we focus on quantitative and qualitative effects of MTX treatment on the key players of autoimmune inflammation in JIA – the regulatory (Treg) and effector (Teff) T cells – at MTX start and at 3 and 6 months upon MTX start. We showed that Treg phenotype and suppressive function on Teff proliferation and cytokine production were not affected by MTX treatment. Contrary to our hypothesis, we showed that neither *in vitro* exposure to low nanomolar concentrations of MTX, nor the low-dose MTX treatment itself inhibited proliferation of T cells *ex vivo* and upon T cell receptor stimulation. Instead, Teff proliferation and IFNγ plasma-concentrations at 6 months were enhanced. These findings indicate that MTX does not attenuate but rather enhances the effector T cell function in JIA patients during MTX treatment. This immunological data is contrary to the common belief that low-dose MTX treatment in rheumatic diseases has immunosuppressive properties.

The data described in this thesis demonstrates that tailor-made MTX treatment is possible and should be applied in order to offer optimal treatment to all JIA patients. Future studies should focus on validation and refinement of described tools with novel genetic and immunological biomarkers in large cohorts, followed by their application in daily clinical practice.

SAMENVATTING

Jeugdreuma, ook wel juveniele idiopathische arthritis (JIA) genoemd, komt voor bij ongeveer 1 op de 1000 kinderen. Bij JIA valt het eigen afweersysteem gewrichten aan, waardoor ontsteking van bijvoorbeeld knieën, enkels en handen ontstaat. Methotrexaat (MTX) is de standaardbehandeling bij JIA. Ondanks zijn centrale rol is MTX onvoldoende effectief en veroorzaakt bijwerkingen bij sommige JIA patiënten, wat het behalen van remissie (=tot rust komen van ontsteking) kan belemmeren. In dat geval wordt combinatietherapie met andere middelen, de zogenaamde biologicals, gegeven. Tegenwoordig gebeurt dit zelfs bij de start van MTX, voordat de respons op MTX bekend is. Echter, behandelaars zouden vóór of vlak na de aanvang van MTX moeten kunnen weten welke patiënten baat zullen hebben bij MTX en welke patiënten niet, waardoor ze met biologicals behandeld zouden moeten worden. Om behandeling op maat te bieden, hebben behandelaars "instrumenten" nodig voor het optimaliseren van MTX-behandeling.

In het eerste deel van het proefschrift hebben wij een model gemaakt, dat, voor het starten van MTX, voorspelt welke patiënten een slechte respons op MTX zullen hebben. Dit model kan gebruikt worden om deze patiënten snel te kunnen behandelen met biologicals en patiënten met een goede respons dure biologicals te besparen. Verder stellen wij vast dat MTX-spiegels in rode bloedcellen (polyglutamaten) ingezet kunnen worden om te beslissen *ofwel* om MTX-dosis aan te passen *ofwel om* biologicals te geven om een goede respons te bereiken.

In het tweede deel stellen wij vast, middels een vragenlijst (MISS), dat meer dan de helft van JIA-patiënten last heeft van MTX–intolerantie: maagdarmkanaal bijwerkingen en gedragsoproblemen. Wij bevelen behandelaars aan om de MISS vragenlijst te gebruiken voor vroege opsporing van MTX-intolerantie, zodat deze tijdig behandeld kan worden. Verder vergelijken wij verschillende behandelingen voor MTX-intolerantie: MTX-pillen en gedragstherapie *versus* MTX-injecties *versus* MTX-pillen gecombineerd met een antimisselijkheidsmiddel. Het blijkt dat alle drie de behandelingen MTX-intolerantie reduceren, zelfs in de eerste behandelingsweek. Dit sugereert dat deelname in het onderzoek en een positieve instelling belangrijker zijn dan het type behandeling. Wij bevelen behandelaars daarom aan om de behandeling te kiezen waar patiënten en hun ouders het meest gemotiveerd voor zijn. Ten slotte hebben wij een model gemaakt, dat voorspelt welke patiënten MTX-intolerantie zullen ontwikkelen. Dit model kan gebruikt worden om deze patiënten vroeg na het starten van MTX-behandeling op te sporen en op deze manier het ontstaan van MTX-intolerantie te voorkomen.

In het derde deel onderzoeken wij hoe MTX het afweersysteem beïnvloedt en stellen vast dat MTX de functie van T-cellen, belangrijke spelers in het JIA-ziekteproces, bevordert. Hierdoor kunnen we constateren dat MTX het afweersysteem bij JIA niet onderdrukt, waardoor de normale functie van het afweersysteem, bijvoorbeeld om ziekteverwekkers te bestrijden, waarschijnlijk behouden blijft.

Dit proefschrift laat zien dat MTX-behandeling op maat mogelijk is en toegepast zou moeten worden om de optimale behandeling aan elke JIA-patiënt te bieden. Toekomstig onderzoek moet zich richten op het verfijnen van de bovenbeschreven instrumenten in grote groepen JIA-patiënten, en uiteindelijk op hun toepassing in de dagelijkse praktijk.

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CURRICULUM VITAE

Maja Bulatović Ćalasan was born on December 17th, 1982 in Podgorica, Montenegro. Maja completed her elementary school and started her secondary school in Podgorica, Montenegro. At the age of 16, she moved to Montezuma, New Mexico, the United States of America, after obtaining a scholarship to attend the final two years of secondary school at Armand Hammer United World College (AHUWC). In 2001, she received the International Baccalaureate diploma at AHUWC. In the same year, Maja moved to Utrecht, The Netherlands, after receiving a scholarship to attend the liberal arts University College Utrecht (UCU). In 2004, she obtained the Bachelor of Science degree *summa cum laude*. During UCU, she mastered the Dutch language and enrolled into medical school – the Selective Utrecht Medical Master (SUMMA) at the University Medical Center Utrecht (UMCU). During SUMMA, Maja completed two clinical rotations at university hospitals in Panama City, Panamá in 2006 and León, Nicaragua in 2008. Also, she undertook a research internship at the Pediatric Immunology Department at the Wilhelmina Children's Hospital (WKZ), UMCU, under supervision of Prof. dr. A.B.J. Prakken, for which she received the WKZ award. After receiving her medical degree in 2008, she started her PhD training in 2009 at the WKZ, UMCU, under supervision of Prof. dr. A.B.J. Prakken, Prof. dr. N.M. Wulffraat, dr. F. van Wijk and dr. R. de Jonge. For her PhD research, Maja received a prestigious personal Mosaic grant from the Netherlands Organisation for Scientific Research (NWO). Results of this PhD research are presented in this thesis. During this period, she completed the TULIPS PhD curriculum, a two-year programme focused on research competencies for clinician-scientists. In September 2013, she began her residency in internal medicine under supervision of dr. C.G. Schaar at the Gelre Hospital in Apeldoorn and Prof. dr. M.M.E. Schneider at the UMCU.

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