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INTRACELLULAR CONCENTRATION OF METHOTREXATE IN ERYTHROCYTES AND MTHFR POLYMORPHISMS:

Possible association with methotrexate efficacy and toxicity in patients with juvenile idiopathic arthritis ?

PhD Thesis

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ABBREVIATIONS

ACR	American College of Rheumatology
AICAR	Aminoimidazole carboxamide ribonucleotide
ALL	Acute lymfoblastic leukemia
AMP	Acute myeloid leukemia
AMP	Adenosine monophosphate
CRP	C-reactive protein
DHFR	Dihydrofolate reductase
EMTX	Concentration of methotrexate polyglutamates in erythrocyte
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FPGS	Folylpolyglutamate synthetase
FR	Folate receptor
GGH	Gammaglutamyl hydrolase
ILAR	International League of Associations for Rheumatology
JIA	Juvenile idiopathic arthritis
MRP	Multidrug resistance-associated protein
MTHFR	Methylene tetrahydrofolate reductase
MTX	Methotrexate
$MTX\text{-}_{glu1}$	Monoglutamate form of MTX (unmetabolized compound)
$MTX\text{-}_{glu2\text{-}5}$	MTX polyglutamate metabolites (with 2, 3, 4, 5 glutamyl groups)
$MTX\text{-}glu_3$	MTX metabolite with 3 glutamyl groups
NSAIDs	Non-steroidal anti-inflammatory drugs
PASI	Psoriasis area severity index
RA	Rheumatoid arthritis
RFC	Reduced folate carrier
RBC	Red blood cell
SNP	Single nucleotide polymorphism
TNF alpha	Tumour necrosis factor alpha
VAS	Visual analogue scale

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ABSTRAKT (CZ):

Východisko: Ověřit, zda stanovení polymorfismů metylentetrahydrofolát reduktázy (MTHFR) C677T a A1298C a erytrocytární koncentrace metotrexátu (EMTX) mohou sloužit k predikci terapeutické účinnosti a toxicity metotrexátu (MTX) u dětí s juvenilní idiopatickou artritidou (JIA).

Metody: Genetická analýza a vyšetření EMTX a koncentrace folátů v erytrocytech bylo provedeno u 69 pacientů s JIA léčených MTX a klasifikovaných jako respondéři (n=51, inaktivní onemocnění) či nonrespondéři (n=18, zlepšení pod 30 % dle pediatrických ACR 30 kritérií i přes podávání parenterálního MTX ≥15 mg/m^2/týden min. po dobu 3 měsíců).

Výsledky: Nonrespondéři byli léčeni vyšší dávkou MTX (medián, 17,2 vs 12,6 mg/m^2/týden, P<0.005), a dosáhli vyššího EMTX (217 nmol/l vs 106 nmol/l, P<0,02) a erytrocytárního folátu (763 nmol/l vs 592 nmol/l, P=0,052) než respondéři. Asociace mezi frekvencí alel a genotypů MTHFR a klinickým účinkem nebyla prokázána. Výskyt nežádoucích účinků byl 29,4 % u respondérů a 33,3 % u nonrespondérů (P=0,77). T alela C677T polymorfismu se vyskytovala častěji u dětí s projevy toxicity (52,4 % vs 20,9%, OR=3,88; 95% CI: 1,8–8,6; P<0,002). Signifikantně vyšší riziko toxicity bylo u dětí s genotypem 677TT ve srovnání s genotypem 677CC (OR=55,5; 95-% CI: 2,9–1080; P<0,001).

Závěry: Genotypizace MTHFR může mít prediktivní hodnotu pro toxicitu spojenou s terapií metotrexátem u dětí s JIA. I přes nedostatečný terapeutický efekt metotrexátu nonrespondéři akumulují dostatečné množství EMTX.

ABSTRACT (ENG):

Objective: To investigate whether methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphisms and erythrocyte concentration of methotrexate (EMTX) could serve as predictors of methotrexate (MTX) efficacy and toxicity in patients with juvenile idiopathic arthritis (JIA).

Methods: Genetic analyses and EMTX and folate assessment were performed in 69 JIA patients treated with MTX and classified as full responders (n=51, disease inactivity) or nonresponders (n=18, less than 30 % improvement in paediatric ACR30 criteria while on ≥ 15 mg/m^2/week parenteral MTX for at least 3 months).

Results: Nonresponders were treated with the higher median MTX dose (17.2 vs 12.6 mg/m^2/week, P<0.0001), and accumulated more EMTX (217 nmol/L vs 106 nmol/L, P<0.02) and erythrocyte folates (763 nmol/L vs 592 nmol/L, P=0.052) than responders. Analysis of MTHFR allele and genotype frequencies in relation to response failed to detect association. The frequency of any adverse effect was 29.4 % in responders and 33.3 % in nonresponders (P=0.77). The frequency of 677T allele was elevated in patients with adverse effects (52.4 % vs 20.9%, OR=3.88; 95% CI: 1.8–8.6; p<0.002). The probability of any adverse effect was significantly higher in patients with 677TT when compared to 677CC genotype (OR=55.5; 95-% CI: 2.9–1080; p<0.001).

Conclusion: MTHFR genotyping may have a predictive value for the risk of MTX associated toxicity in JIA patients. Despite the lack of therapeutic effect, nonresponders accumulate adequate concentrations of EMTX.

KLÍČOVÁ SLOVA: juvenilní idiopatická artritis, metotrexát, MTHFR, erytrocyt, klinická účinnost, toxicita

KEX INDEXING TERMS: juvenile idiopathic arthritis, methotrexate, MTHFR, erythrocyte, efficacy, toxicity

1. INTRODUCTION

Methotrexate (MTX) is a folic acid antagonist that was originally designed to inhibit proliferation of malignant cells and has been widely employed as an antineoplastic agent for the treatment of a number of solid tumours and haematologic malignancies at high dose regime.In the 1980´s, MTX in low weekly doses has been used to treat many rheumatic and other inflammatory conditions (skin, intestinal system etc.). The mechanism of action in patients with the autoimmune diseases is still not completely clarified, but MTX does not act simply as a cytotoxic agent for inflammatory cells.

Over the last two decades MTX has been commonly used as a second-line treatment of juvenile idiopathic arthritis (JIA) [1]. Although its efficacy and safety in children and adolescents with JIA have been documented in multiple clinical trials [2, 3], interpatient variability of efficacy and variety of side effects remain clinical concern. About 10 % of children fail to improve while receiving MTX [4] and about 10 to 76 % exhibit some common side effects [5]. Dose and route of administration are unpredictable and need to be tailored individually in order to achieve early and sustained therapeutic effect [4, 6]. The prolonged dose-finding process and suboptimal dosing may lead to uncontrolled progression of arthritis and development of irreversible damage of joints, whereas the higher-dose therapy increases toxicity risk.

Nowadays, the tool for MTX therapy monitoring is not available. Searching for metabolic and genetic biomarkers and their correlation with efficacy and toxicity are subject to investigation in many studies.

We studied two potential tools for individualization of therapy: intracellular concentration of MTX polyglutamates in erythrocytes (EMTX) and polymorphisms of methylene tetrahydrofolate reductase (MTHFR).

Whereas plasma concentration of the parent drug is not useful for MTX therapeutic monitoring due to its short plasma half-life, it has been postulated that long-acting polyglutamylated intracellular MTX metabolites (EMTX) mediate most of MTX anti-inflammatory effect. Polyglutamation enhances the intracellular retention of the drug [7, 8] and facilitates its affinity for several dependent enzymes. Studies evaluating possible role of EMTX for therapeutic monitoring have been inconclusive [7-9].

Growing body of evidence is now available to support important contribution of various genetic polymorphisms in MTX xenobiotic metabolic pathways to interpatient variability in therapeutic response as well as toxicity. Many pharmacogenomic studies have been nowadays exploring single nucleotide polymorphisms (SNPs) in enzymes involved in metabolism of MTX. Methylene tetrahydrofolate reductase (MTHFR) is a critical enzyme of MTX metabolism. It is associated with regeneration of reduced folates. It mediates synthesis of 5-methyltetrahydrofolate, the carbon donor required for methionine synthesis. Two relatively common single nucleotide polymorphisms (C677T, A1298C) have been studied in the MTHFR gene (9-18). Several studies in adults with MTX treated rheumatoid arthritis (RA) investigated associations between C677T and A1298C polymorphisms and clinical variables of disease outcome and/or toxicity with inconsistent results [10-18].

2. AIMS OF THE STUDY

Optimal MTX dose is highly individual and unpredictable. Metabolic or genetic biomarker for MTX therapy monitoring and prediction of MTX efficacy and toxicity could help to shorten time to remission and decrease toxicity risk in JIA patients.

The thesis study has been based on assessment of EMTX and MTHFR polymorphisms in JIA patients with clearly defined response status. MTHFR SNPs and MTX and folate polyglutamates in erythrocytes were assayed after the patients had been treated sufficiently long time with the stable, sufficiently high MTX dose. Two different groups of JIA patients from the opposite ends of the therapeutic response spectrum were recruited. Firstly, patients with excellent drug response who achieved disease inactivity while on MTX therapy were examined (full MTX responders). Secondly, patients with insufficient drug response to parenteral MTX after dose-escalating process were studied (MTX nonresponders, see definition of nonresponse bellow).

We expected lower EMTX levels in nonresponders. Furthermore, we presumed higher EMTX levels and lower folate in erythrocytes in patients with toxicity signs. We expected the correlation of TT and CT genotype of MTHFR and T allele with higher toxicity.

The specific aims of this study were:

a) to compare EMTX of MTX responders and nonresponders in order to evaluate the possible predictive value of this parameter for MTX treatment efficacy

b) to explore possible association between EMTX and toxicity signs in order to evaluate the ability of EMTX to predict toxicity

c) to analyze MTHFR C677T and A1298C polymorphisms in MTX responders and nonresponders in order to assess its usefulness for prediction of MTX efficacy in patients with different genotypes and alleles

d) to study whether MTHFR A1298C and C677T polymorphisms could serve as a predictors of MTX toxicity

e) to find out whether erythrocyte folate concentration is a useful parameter for toxicity prediction

3. OVERVIEW OF THE LITERATURE

3.1 JUVENILE IDIOPATHIC ARTHRITIS (JIA)

3.1.1 Definition

Juvenile idiopathic arthritis (JIA), a term referring to a group of disorders characterized by chronic arthritis, is the most common chronic rheumatic illness in children and is a significant cause of short- and long-term disability [19]. Juvenile idiopathic arthritis is defined as arthritis of at least 6 weeks' duration of unknown cause occurring in children less than 16 years old. Acknowledging that JIA represents a heterogeneous group, the ILAR (International League of Associations for Rheumatology) classification system calls for seven subtypes [20, 21]. These include systemic arthritis, polyarthritis with negative rheumatoid factor, polyarthritis with positive rheumatoid factor, oligoarthritis (divided to subtypes: persistent, extended), enthesitis- related arthritis, psoriatic arthritis. Unique to the ILAR classification system is a seventh “other” group for patients who do not fit into any of the other six subtypes or who fit criteria for more than one subtype. There is a distinction made between children who present with fewer than five joints in the first 6 months of disease but progress to follow a polyarticular course. The ILAR classification scheme also contains exclusion criteria for each class, with the largest number of exclusions in the oligoarthritis subtype. Family histories of psoriasis or HLA-B27 associated disease, a positive rheumatoid factor, systemic arthritis, or being an HLA-B27–positive male patient with onset of arthritis after 6 years of age exclude a patient from the oligoarthritis subtype.

3.1.2 Pathophysiology of JIA

Cytokines as well as dynamic interactions between pro-inflammatory and anti-inflammatory molecules within the joint play the main role in pathophysiology of juvenile

idiopathic arthritis. This observation resulted in successful development of new effective biologic agents, such as etanercept, that target specific inflammatory mediators [22].

A concise review of the role of cytokines in the pathogenesis of adult arthritis was recently published [23]. In brief, arthritis is characterized by destruction of bone and cartilage secondary to an increased inflammatory response. CD4+ T cells invade synovium and stimulate inflammatory cells. These inflammatory cells, such as macrophages, generate inflammatory cytokines including interleukin (IL)-1, IL-6, and tumor necrosis factor-(TNF-α). Cytokines then stimulate release of proteinases, which lead to joint damage. In addition, TNF-α can stimulate expression of adhesion molecules on endothelial cells and increase recruitment of neutrophils into joints (which subsequently release additional proteinases and cause further destruction). Cytokines are also important in pain transmission, as they can activate or sensitize C fibers. Despite improvements in understanding the cytokine profiles in patients with arthritis, the iniciating events remain unclear.

TNF- α is well known to promote inflammation and recent work supports its importance in the pathogenesis of JIA [24]. An imbalance between TNF-α and its soluble receptor might contribute to disease severity. Although TNF- α is only one of many cytokines involved in the development of arthritis, it implies the importance of dynamic interactions between a proinflammatory agent and the mechanisms used to regulate it.

3.1.3 Therapy of JIA

Non-steroidal anti-inflammatory drugs (NSAIDs) have been the mainstay treatment for this disease for decades. Their role remains important and most children with juvenile idiopathic arthritis are started on an NSAID. They are usually quite well tolerated and side-effects are less common than in adults. Intra-articular steroid injections with triamcinolone hexacetonide are frequently needed at disease onset or during disease course. In

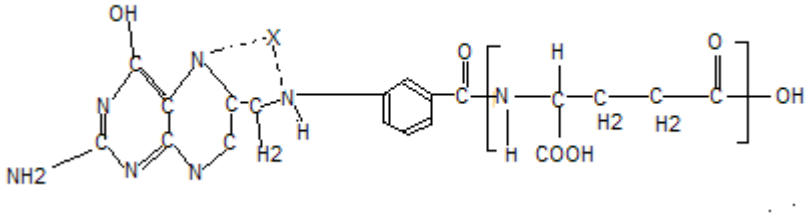
monoarticular or oligoarticular arthritis they could be used with, or substituted for, NSAIDs. Although intra-articular steroid injections are not curative, their effect can be longlasting. Methotrexate (MTX), analogue of folic acid, is the disease modifying anti-rheumatic drug of first choice for persistent, active arthritis. Despite the development of biologics MTX remains the mainstay of JIA treatment because of its effectiveness, low price and acceptable toxicity risk.

3.2 FOLATES AND ANTIFOLATES

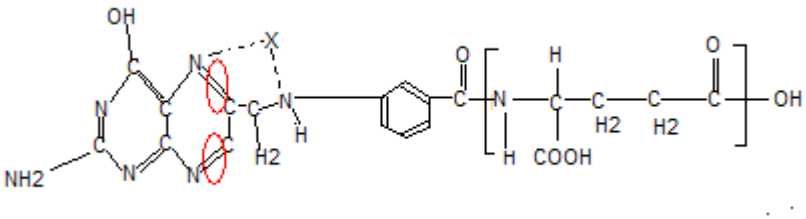
3.2.1 FOLIC ACID

3.2.1.1 Structure

Folic acid is a water soluble B vitamin. Folates are essential cofactors in metabolic pathways and are composed of three structural units: a pteridine ring, para-aminobenzoic acid, and one or more terminal glutamic acid residues (Fig. 1). They are active only in the reduced form. The enzyme dihydrofolate reductase is responsible for reducing partially oxidized folates, which are ingested or created during DNA synthesis, to reduced form [25]. Mammals may synthesize the pteridine ring but are unable to link it with other compounds. Humans depend on dietary intake and synthesis of biologically active folate [26].



A



B

Fig. 1 Reduced folate, FH_4 (A), oxidized folate, FH_2 (B)

3.2.1.2 Function

Folates facilitate methylation reactions, as well as formation and transfer of one-carbon units [26]. Tetrahydrofolate receives one-carbon fragments from donors such as

serine, glycine, and histidine and transfers them to intermediates in the synthesis of amino acids, purines, and thymidine- the characteristic pyrimidine of DNA [25].

3.2.2 METHOTREXATE

The structure of methotrexate

Methotrexate (MTX) is a folate analog with an amino group at position C4, a methyl group at position N10, and a fully oxidized pteridine ring, rendering the molecule inactive as a cofactor (Fig. 3, 4) [27].

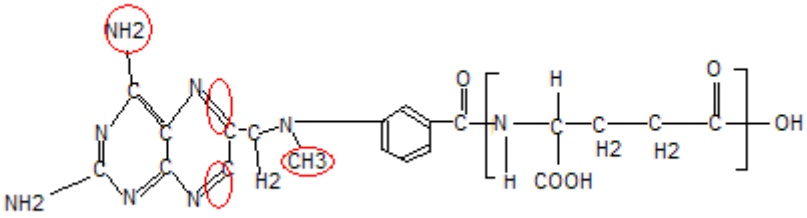


Fig.3

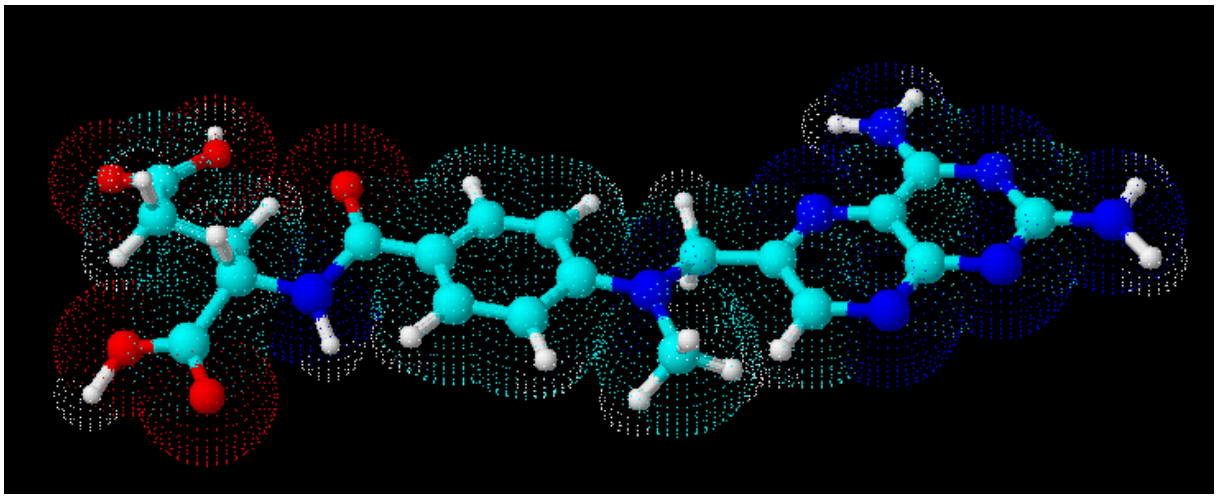


Fig. 4 Structure of methotrexate

3.2.3 ABSORPTION

3.2.3.1 Absorption of folates

Natural dietary folic acid is a small, water soluble molecule occurring mainly as polyglutamate forms. These molecules cannot cross cell membranes and therefore cannot be resorbed as such [26]. The glutamyl residues are cleaved by gamma-glutamylcarboxypetidase at the brush border membrane of the duodenum and jejunum, creating monoglutamate folate that is then easily resorbable in the proximal small intestine. The transmembranous transport at the enteral cell is specific and depends on pH. The conjugase of the jejunal brush border membrane is a lysosomal exopeptidase which cleaves the terminal gamma-glutamate-residue. A second enzyme with endopeptidase activity is located intracellularly in the jejunal mucosa [28]. High doses of folic acid (several miligrams) are resorbed almost quantitatively and exit the circulation within minutes from uptake in the liver and other tissues. After oral administration the maximum plasma concentration is normally reached after 1-2 hours. Monoglutamates will be conjugated into polyglutamates intracellularly by gamma-glutamate synthase and can be stored and metabolized in this configuration [26].

3.2.3.2 Absorption of methotrexate

Similarly, after oral administration methotrexate is absorbed in the proximal intestine by active transport process intended for folates [27]. This process is influenced by many factors, such as food, state of intestinal mucosa, and leads to great variability of absorption [29]. MTX shares mechanisms for active, carrier-mediated, saturable intestinal absorption with folic acid and reduced folates. In many individuals, the intestinal folate transport system saturates at the higher end of usual oral dose (above 10 – 15 mg/m^2) [6].

3.2.4 TRANSMEMBRANE TRANSPORT

3.2.4.1 Intracellular uptake of folates and methotrexate

The cells are provided with different type and number of folate transport systems. Receptors and specific carriers are active in transmembranous folate transport [28].

MTX and leucovorin (5-formyltetrahydrofolate) enter the cell through active transport by RFC, which has a ubiquitous distribution [30]. Reduced folate carrier (RFC1), a bidirectional anion transporter, is a carrier system with low affinity but great capacity for reduced folate.

Members of another membrane transport group, termed folate receptors (FR), are variably expressed. The folic acid enters the cell preferentially via these human FR. The FR has a significantly higher affinity for transport of folate than does the RFC. They are capable of transporting MTX in cells in the physiologically and metabolically up-regulated state with increased metabolic activity, including malignant tissues and synovial macrophages [31]. Normal tissues express low or undetectable levels of FRs.

RFCs and FRs can be expressed either separately or simultaneously in the same cell. At very high concentration of methotrexate around cell, non-specific uptake mechanisms become important [27].

Recently, the proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1) has been suggested to be the possible molecular entity of the intestinal carrier for folates and antifolates [32]. The key role for this protein has been confirmed by the demonstration of a mutation in this gene in hereditary folate malabsorption [33].

3.2.4.2 Efflux of folates and MTX

The monoglutamate form of MTX and folate can easily efflux from the cell, which is not possible for polyglutamated drug. γ-glutamyl hydrolase (GGH) facilitates MTX efflux from the cell by catalyzing the removal of γ-linked polyglutamates [28]. MTX is effluxed from the cell by members of the ATP-binding cassette (ABC) family of transporters. The ABC family includes 48 proteins classified into seven distinct subfamilies ABC A-G [30]. They range among multidrug resistance-associated proteins (MRPs), which transport MTX, folic acid and leucovorin out of cell [28].

3.2.5 BASIC PHARMACOKINETICS OF METHOTREXATE

3.2.5.1 Dose and absorption

MTX is administered once weekly orally or subcutaneously in low dose regime. The effective recommended dose in children is generally higher than in adults. It ranges between 7, 5 to 15 mg/m^2 [1].

At doses more than 10 mg/m^2 weekly the parenteral route may be preferred because oral bioavailability of the drug decreases at higher doses [34].

The benefit of giving MTX in the fasting state is ambiquous. Despite the shortened absorption phase and increased peak concentration no significant change in the area under the curve (AUC) was observed [35]. Folate supplements directly compete with MTX for uptake, they are administered at least 24 – 48 after MTX dose.

3.2.5.2 Distribution

At low doses, MTX is only moderately bound to proteins, with a reported range of 11% to 57%. Displacement of protein-bound MTX by other drugs, therefore, is unlikely to have an appreciable clinical effect.

3.2.5.3 Elimination

After its application, MTX is rapidly cleared unchanged via glomerular filtration and tubular secretion within about 48 hours [27]. The maximal plasma level of methotrexate was observed 1 hour after administration [29]. Most of MTX is eliminated unmetabolized, 3-11 % is hydroxylated in liver and circulates as 7- OH-MTX [27]. Rapid renal clearance [36] in children explains the need of relatively higher doses in children in comparison with adults. Unrecognized alteration in renal clearance is a major risk factor for MTX severe acute toxicity.

3.2.6 INTRACELLULAR PHARMACOLOGY OF FOLATES AND METHOTREXATE

Inside the cell, extra glutamyl residues may be added folate or methotrexate molecule by the enzyme folylpolyglutamate synthetase, creating polyglutamates. Polyglutamates are often more efficient cofactors than their parent compounds [28]. Folylpolyglutamate synthetase may add up to 6 glutamyl groups in a gamma peptide linkage to the folate substrate. This gamma-linked sequential addition of glutamic acid serves three purposes: 1. It facilitates the accumulation of intracellular folates and folate analogues in vast excess of the monoglutamate pool, which is freely transportable into and out of cells. 2. It enhances the intracellular retention of folates and methotrexate. 3. It greatly enhances folate cofactor affinity for several folate-dependent enzymes.

3.2.7 MECHANISMS OF ACTION

3.2.7.1 Folates

There are three specific tetrahydrofolates that play essential roles as 1- carbon carriers involved in the synthesis of DNA precursors. The first, *10-formyltetrahydrofolate (10-formyl-THF)*, provides its one-carbon group for the synthesis of purines in reactions mediated by glycinamide ribonucleotide (GAR) transformylase and aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase. A second cofactor, *5,10 – methylenetetrahydrofolate* (CH2-THF), donates its 1- carbon group to the reductive methylation reaction converting dUMP to thymidylate dTMP. A third, *5-methyltetrahydrofolate* (5-CH3-THF), donates a methyl group in the conversion of homocysteine to methionine. This metabolite is oxidized to dihydrofolate (DHF), which can in turn be reduced back to THF by dihydrofolate reductase (DHFR) [31].

3.2.7.2 Methotrexate

Two mechanisms exist by which MTX and its derivatives interfere with synthetic pathways that require folate cofactors. The first is indirect, through inhibition of dihydrofolate reductase. The second mechanism is direct competition for cofactor binding sites.

3.2.7.2.1 Different mechanisms of action between high dose and low dose MTX regimes

The mechanism of action in low dose weekly MTX differ from antiproliferative effect of high dose regime. Several lines of evidence are available: First, folate supplements are commonly administered to patients taking low-dose methotrexate to avoid methotrexate-induced toxicity; folate supplementation does not diminish the therapeutic efficacy of methotrexate [37]. Second, two common methotrexate- mediated toxicities, marrow suppression and stomatitis, are most likely caused by inhibition of cellular proliferation but do not correlate with the therapeutic effects of the drug [38].

3.2.7.2.2 Antiproliferative effect of high-dose MTX

MTX, a folate analogue, is a competitive inhibitor of the enzyme dihydrofolate reductase (DHFR). This indirect mechanism depends on thymidylate synthesis, which is a step in the construction of DNA and is limited to actively dividing cells. During thymidylate synthesis, methylenetetrahydrofolate donates a methyl group and is oxidized to dihydrofolate. To function again as a cofactor, dihydrofolate must then be reduced to tetrahydrofolate by dihydrofolate reductase. MTX inhibits dihydrofolate reductase with high affinity, leading to accumulation of dihydrofolates and relative depletion of tetrahydrofolates. Although unable to donate one-carbon groups, the partially oxidized folates retain their affinity for enzymes that require folate cofactors and are effective competitive inhibitors of the remaining tetrahydrofolates. Dividing cells are thereby deprived of reduced folates, which are essential cofactors in the DNA-, RNA – and proteosynthesis [39]. MTX has an unusual strong affinity for DHFR. Its inhibition can only

be reversed by a thousand-fold excess of the natural substrate, dihydrofolate or by administration of leucovorin, which bypasses the blocked enzyme and replenishes the folate pool [40].

3.2.7.2.3 Antiinflammatory effect of low-dose MTX

Metabolism of tetrahydrofolates is affected by MTX which leads to MTX effects on purine metabolism and de novo synthesis of DNA (see Fig. 5).

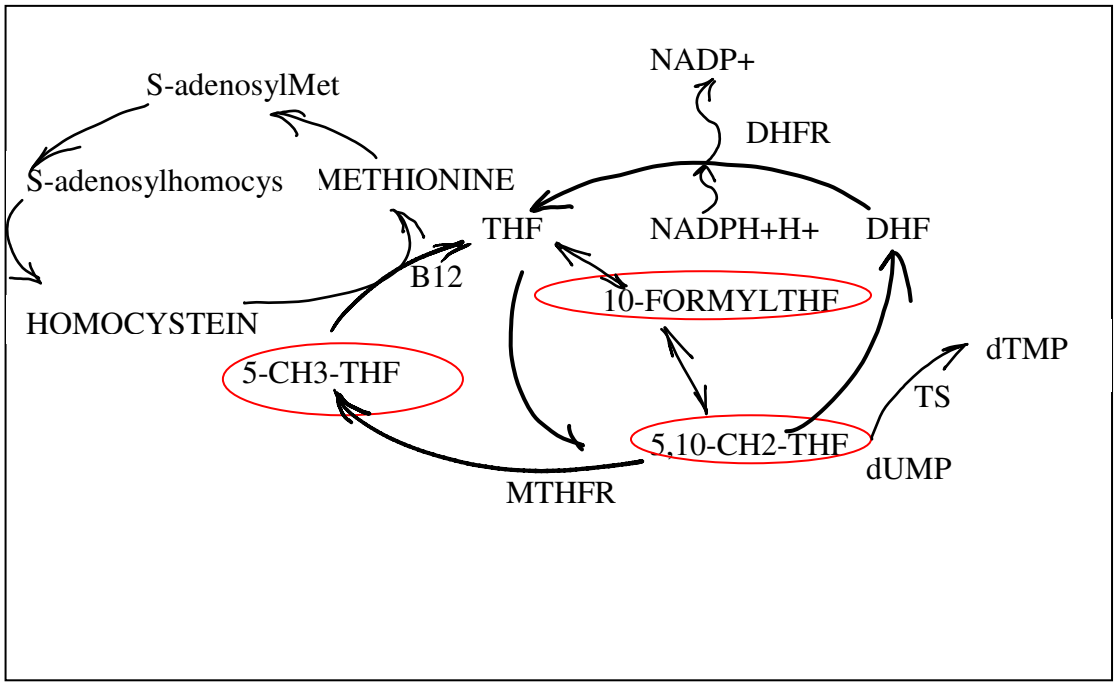


Fig. 5 Three tetrahydrofolates affected by methotrexate

This second mechanism, direct binding of drug to enzymes that require cofactors, is significant only in cells in which high levels of MTX polyglutamates are present. MTX and 7-OH-MTX are not particularly good inhibitors of the folate-dependent biosynthetic enzymes. Long chain polyglutamates are better inhibitors of other folate-requiring enzymes that are not substantially inhibited by MTX, including thymidylate synthase and the transformylases required for de novo purine synthesis [39]. Direct inhibition is independent of thymidylate synthesis and so is not limited to dividing cells.

MTX polyglutamates are potent inhibitors of enzymatic systems including phosphoribosylaminoimidazolecarboxamide (AICAR) transformylase. Consequent intracellular accumulation of AICAR and its metabolites inhibit two enzymes important in adenosine metabolism, adenosine deaminase and AMP deaminase. It has been associated with increased release of adenosine [38, 41, 42]. Endogenous adenosine has been shown to regulate many physiological processes. It acts via specific membrane receptors present on the surface of different cells including neutrophiles, lymphocytes, monocytes/macrophages and endothelial cells. Their occupancy leads to various events, majority of which have an anti-inflammatory impact [43, 44].

3.2.8 Polymorphisms of methylene tetrahydrofolate reductase

Methylene tetrahydrofolate reductase (MTHFR) is associated with regeneration of reduced folates. It mediates synthesis of 5-methyltetrahydrofolate, the carbon donor required for methionine synthesis. Two relatively common single nucleotide polymorphisms (C677T, A1298C) have been studied in the MTHFR gene [9-18]. The C677T polymorphism consists of a C>T change resulting in an alanine to valine substitution that renders the enzyme more thermolabile and decreases its enzymatic activity. The occurrence of T allele is connected with smaller pool of methyltetrahydrofolate and higher levels of homocysteine [45]. In the A1298C polymorphism, the A>C change causes a glutamine to alanine substitution and leads to reduced enzyme activity [46]. Several studies in adults with MTX treated rheumatoid arthritis (RA) investigated associations between C677T and A1298C polymorphisms and clinical variables of disease outcome and/or toxicity with inconsistent results [9, 11, 14, 16-19].

3.3 INTRACELLULAR CONCENTRATION OF MTX POLYGLUTAMATES IN ERYTHROCYTES

3.3.1 Background

MTX exists in erythrocytes both as unmetabolized compound (monoglutamate form of MTX, MTX – glu_1) and as polyglutamate metabolites (with 2, 3, 4, 5 glutamyl groups, MTX – glu_{2-5}). The principal metabolite is MTX with three glutamyl groups (MTX-glu_3) accounting for 37 % of the MTX in the red blood cells. The longer intracellular retention of the MTX polyglutamate may be caused by stronger binding to hemoglobin by the longer chain polyglutamates compared with the monoglutamate form of MTX [47].

After repeated MTX application (once a week) intracellular concentration of methotrexate increases slowly and steady state is achieved within 4-8 weeks [36, 39, 47] (Fig.6).

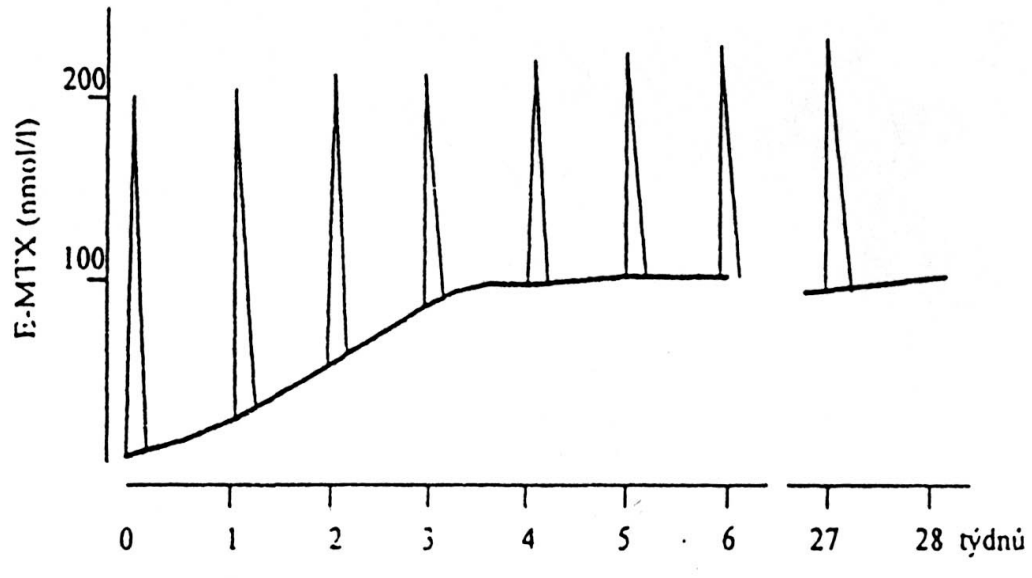


Fig 6 Concetration of methotrexate plyglutamates in erythrocytes after repeated application of drug, according to Hendel and Nyfors [47]

Fig. 7 shows the decline of the individual MTX- polyglutamates in erythrocytes after discontinuation of MTX therapy [48].

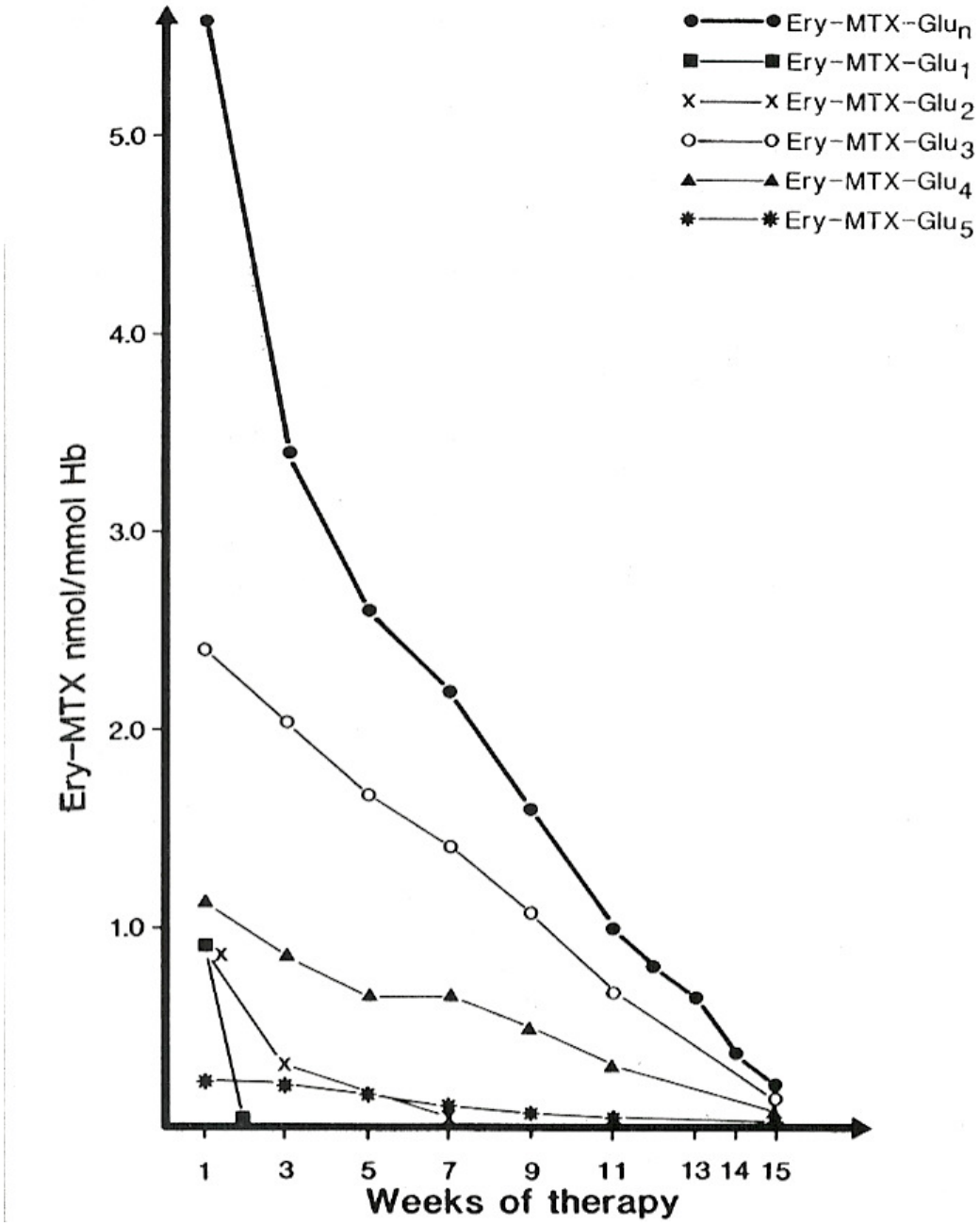


Fig. 7 Decline of the erythrocyte MTX concentration (ery-MTX glu$_n$) and the individual polyglutamates (mtx glu1-5) up to 15 weeks after discontinuation of MTX maintenance treatment according to Schroder and Fogh [48].

MTX polyglutamates do not have increased affinity for enzymes compared with monoglutamate form, but seem to dissociate at a slower rate [39]. Pharmacokinetics of

intracellular methotrexate may explain the delay in the onset and end of the drug therapeutic effect.

Since the concentration of MTX in erythrocytes (EMTX) may reflect bone marrow exposure to MTX, this may turn out to be a useful parameter for monitoring and adjusting the dose of MTX, paralleling what is incorporated into and retained by the cells [8].

3.3.2 MTX accumulation in mature erythrocytes

MTX transport and accumulation differ in mature erythrocytes and eryhroid precursors of the bone marrow. After administration MTX enters erythrocytes probably by passive diffusion and shows a concentration profile paralleling that of the serum. Twenty-four hours after the first dose of MTX, when the serum MTX concentration drops, the EMTX returns to zero. These kinetic studies indicate that MTX, which enters circulating mature erythrocytes, is not bound to proteins (hemoglobin, DHFR) or metabolized to intracellular retainable polyglutamates to any significant degree [48].

3.3.3 MTX incorporation into erythroid precursors

After drug administration MTX is thought to be incorporated in the red cell precursors of the bone marrow [49-51]. The evidence for this is indirect, being based on the pharmacokinetic observation that the EMTX starts to rise 3-5 days after MTX administration, corresponding roughly to the intramedullary erythrocyte maturation time. The EMTX continues to increase up to 10-14 days after an MTX administration. Schroder demonstrated that reticulocyte enriched erythrocyte fractions had the highest MTX content about 7 days after 14 hour MTX infusions. This provided more direct evidence for the MTX incorporation into red blood cell precursors of the bone marrow.

MTX uptake in reticulocyte is mediated by a carrier mechanism which seems to disappear with reticulocyte maturation. This is compatible with a much greater capacity of these cells for uptake and retention of MTX compared with mature erythrocytes.

Quantitative aspects of MTX uptake into the various intramedullary red blood cell precursors, however, are not known. Reticulocytes are able to metabolize MTX to polyglutamate forms [49-51].

3.3.4 MTX polyglutamates in erythrocytes of different ages

During erythrocyte ageing MTX disappeared from the cells, probably through efflux. The change of the MTX polyglutamate distribution in red blood cells during the weeks after discontinuation of maintenance therapy showed that the erythrocyte half lives of MTX-glu_1 and $-glu_2$ were roughly 2-3 days and 4-15 days respectively, whereas MTX-glu_{3-5} seemed to remain in the erythrocytes in approximately unchanged concentrations until their age dependent destruction [39].

At steady state EMTX shows only minor variations during the week between two MTX administrations. The EMTX does not increase with increasing duration of treatment and does not correlate with the cumulative dose of MTX [51].

3.3.5 MTX polyglutamates in white blood cells

Since intravascular circulation time of the neutrophils is much shorter (<24 hours) than that of erythrocytes MTX concentration in circulating neutrophils might reflect the amount of MTX incorporated into the myeloid precursors of the bone marrow. Cells belonging to the proliferating myeloid pool accumulated MTX to a much greater extent than the more mature myeloid cells and circulating neutrophils. These myeloid precursors matured and reached the circulation as neutrophils in 6-7 days [39].

3.3.6 EMTX and clinical efficacy and toxicity of methotrexate

3.3.6.1 EMTX and correlation with MTX dose

Different results were reported by several investigators. Whereas, steady-state EMTX and weekly dose of MTX correlated in study of Schroder and Dervieux [52, 53], weak correlation [7, 9] or no correlation was observed by others [8, 48].

3.3.6.2 EMTX and MTX clinical efficacy

Several studies in adults and children with various diagnoses have demonstrated correlation between EMTX and MTX efficacy, although conflicting data have been published.

Adults with rheumatoid arthritis and EMTX

Angelis-Stoforidis et al. observed significantly higher EMTX in responders and partial responders with RA. Clinical effect in this study was evaluated by accidental one-time assessment of response independently from the MTX dose and duration of treatment [8]. Classification of patients was based on the treating rheumatologist´s decision which resulted from the patient history, joint examination and comparison of disease course in time. The single evaluation of clinical response did not respect spontaneous fluctuation of disease activity in patients with chronic arthritis. Moreover, the difference between MTX weekly dose in responders, partial responders and nonresponders was not statistically significant. Nonresponders may have been found to be MTX responders if treated with higher MTX dose. No correlation between EMTX and side effects or weekly dose of MTX was found [8].

Hornung et al. monitored clinical effect during the followup period (52 weeks) and evaluation was according to preliminary ACR core set criteria (none response, 20%, 50%). However, response was assessed independtly from the MTX dose. Patients with RA who were classified as responders had significantly higher mean steady-state EMTX, but significantly higher dose at the same time. Since the correlation between dose and EMTX

was statistically significant, the MTX dose may have affected EMTX in nonresponders who were underdosed [7].

Dervieux et al revealed that increased EMTX was associated with lower number of tender and swollen joints. These investigators identified a therapeutic threshold of 60 nmol/liter EMTX to be associated with a 14- fold higher likelihood of a VAS score of less than 2 cm [54].

Psoriasis

Chládek et al. found a good correlation between clinical efiicacy in patients with psoriasis (the PASI score) and EMTX [36].

Children with acute leukemia and EMTX

Schmiegelow et al showed that MTX polyglutamates correlate with the clinical response and prognosis in children with ALL. EMTX was found to be a predictor of relapse, with higher concentrations being associated with better prognosis [53, 55].

Children with JIA and EMTX

Only one study was conducted to explore the relation between EMTX and clinical and laboratory status in children with JIA. All patients received methotrexate orally, none received folic acid supplementation. The study was longitudinal; the patients served as their own controls, the design of the study therefore did not allow a crossectional analysis of the relation between EMTX and disease parameters. There was a weak, but not significant correlation between the dose of MTX (related to body surface) and EMTX. No significant differences in disease parameters values (VAS, CRP, affected joints) were found in patients with the lowest and the highest EMTX. Spontaneous fluctuation in disease activity in patients did not reflect an inverse fluctuation in EMTX [9].

EMTX appeared higher in nonresponders with JIA in our previous study when compared with responders, but the difference did not reach statistical significance [34].

3.3.6.3 Toxicity and EMTX

EMTX was found significantly higher in the combined group of patients with progressive hepatic changes in adults with psoriasis. The critical EMTX concentration was not defined. The cumulative dose and the length of treatment were stronger predictors of progressive hepatic disease than EMTX. [56].

3.3.7 Concentration of folates in erythrocytes

Measurement of RBC folate levels reflects long term distribution better than serum levels, because mature erythrocytes are no longer permeable for folates and do not metabolize but only store them [26].

3.4 MTHFR POLYMORPHISMS AND CLINICAL RESPONSE AND TOXICITY

The only one previous study has investigated impact of MTHFR polymorphisms on treatment outcome and toxicity in children with JIA receiving MTX [57].

3.4.1 MTHFR polymorphisms in juvenile idiopathic arthritis

3.4.1.1 Clinical efficacy and A1298C in JIA

This retrospective study in JIA patients suggested better clinical efficacy of MTX in children with 1298C allele. Clinical improvement in child was not evaluated generally by core set criteria, but association between constituent sings of clinical response (ESR, C-reactive protein, the number of tender and swollen joints) and polymorphisms were evaluated separately.

3.4.1,2 Clinical efficacy and C677T in JIA

No significant difference in MTX efficacy was found in patients with different genotype and haplotype of C677T polymorphism.

3.4.1.3 Toxicity and A1298C in JIA

Similarly, the A1298C polymorphism was not associated with the occurrence of adverse events in our study and in study of JIA patients [57].

3.4.1.4 Toxicity and C677T in JIA

In JIA patient MTHFR 677 CC genotype was associated with higher tolerability of MTX [57].

3.4.2 MTHFR polymorphisms in adults with rheumatoid arthritis

The results of previous studies on the association of MTHFR polymorphisms with toxicity and clinical efficacy in patients with rheumatoid arthritis (RA) are very inconsistent and often contrary. These discrepancies may be in part due to ethnic differences, different criteria of the disease outcome evaluation and co-administered drugs, especially folic acid supplementation.

3.4.2.1 Clinical efficacy and A1298C

Berkun Y et al observed no correlation of disease activity variables (EULAR criteria) with A1298 C and C677T polymorphisms [13] and Dervieux et al. showed the same result with A1298C polymorphism [11]. Similarly, Hughes et al found no association between SNP alleles or haplotypes (A1298C, C677T) and efficacy (as defined by the mean change in Disease Activity Score 28 or achievement of ACR20, ACR50 or ACR70 response) [18].

Wessels et al found significant association of MTHFR 1298AA and 677 CC genotypes with greater clinical improvement (Disease Activity Score in 44 joints) [14], whereas C at the A1298C polymorphism rendered patients more sensitive to MTX treatment in study of Urano et al. and Kurzawski et al. [10, 12].

3.4.2.2 Clinical efficacy and C677T

Three investigators reported no significant difference in MTX efficacy in patients with different genotype and haplotype of C677T polymorphism [13, 17, 18].

On the other hand, Dervieux et al. 2006 observed that carriers of MTHFR 677TT genotype were less likely to respond to MTX (EULAR response criteria) [11], whereas Kurzawski et al. reported that the presence of MTHFR 677 T allele was associated with higher frequency of remission rate [10].

3.4.2.3 Toxicity and A1298C

No association of A1298C polymorphism and toxicity signs was reported by Urano et al. [12].

Hughes et al. 2006 found association between the 1298 A allele and MTX – related adverse events [18] and Berkun et al. showed similar association between 1298 CC

genotype and low rate of MTX related side effects [13], whereas MTHFR 1298 C allele was associated with an increased risk of toxicity in other studies [11, 14].

3.4.2.4 Toxicity and C677T

No effect of C677T polymorphism on toxicity was reported by several studies in RA patients [13, 14, 17].

Overall MTX- related toxicity was more frequent in patients with the 677T allele in study of Urano et al. [12], whereas association between individual adverse effect and C677T polymorphism was shown by other studies (association of 677 T allele with alopecia [16], 677 CT and TT genotypes with an increased risk of elevated liver enzyme levels [58] and 677 TT genotype with increased occurrence of side effects in central nervous system [15]).

4. PATIENTS AND METHODS

Patients with JIA were consecutively recruited from the paediatric rheumatology out-patient clinic population at the Department of Paediatrics and Adolescent Medicine, 1st Medical School, Charles University in Prague, between 2005-2008. All patients met proposed ILAR classification criteria [20, 21] (Tab. 1).

ILAR subtype	N	
	Responders	Nonresponders
Polyarthritis RF negative	26	13
Oligoarthritis persistent	15	1
Oligoarthritis extended	7	2
Systemic onset arthritis	1	2
Enthesopathic arthritis	1	0
Psoriatic arthritis	1	0

Tab.1 The onset JIA subtype distribution of responders and nonresponders

The study was approved by the Local Research Ethics Committee and informed consent was obtained from the patients and/or their legal guardians according to the Declaration of Helsinki (Fifth revision, 2000, Edinburgh, Scotland).

To become eligible patients must have had a definitive diagnosis of JIA and fully documented disease activity with MTX therapy for at least 3 months prior to recruitment. Only patients within extreme ends of the response spectrum (full responders and nonresponders) were enrolled (Tab. 2).

For the purpose of this study, responders were defined as having achieved disease inactivity on medication with MTX monotherapy according to Wallace et al. Criteria for

inactive disease included: no active arthritis; no fever, rash, serositis, splenomegaly, or generalized lymphadenopathy attributable to JIA; no active uveitis; normal ESR or CRP; physician's global assessment of disease activity indicating clinical disease quiescence [59].

Using pediatric ACR 30 definition of improvement [59-62] therapeutic efficacy was assessed monthly during the dose escalation and at 3 monthly intervals while on a stable dose. In nonresponders at least 3 of any 6 JIA core set variables did not improve by a minimum of 30 % and no more than 1 of the remaining variables improved by more than 30 % [59]. Core set outcome variables include numbers of joints with active arthritis, joints with limited range of motion, physician's global assessment of disease activity (10 cm VAS), parent's global assessment of the child's overall well-being (10 cm VAS), disability index of the Childhood Health Assessment Questionnaire (CHAQ), and ESR [63-65]. To qualify as a nonresponder, patient must have been treated with a minimum of 15 mg/m^2/week MTX subcutaneously for at least 3 months [4].

Before entering the study, patients were treated with MTX using a standard dose-escalation Department protocol. Over the first 3-6 months initial weekly MTX dose of 7.5 - 10 mg/m^2 orally was titrated according to both efficacy and toxicity evaluations up to the weekly dose of around 15 mg/m^2 (max.20-25 mg). Patients with persistent disease activity on oral MTX requiring more than 10 mg/m^2 were switched to subcutaneous administration [6]. MTX injections were chosen as an initial treatment modality in polyarthritis patients with high disease activity and in small children (usually below 4 years of age). On top of MTX, most patients received once-weekly folic acid (5-10 mg/week, 24-48 hours after MTX), and were allowed to take one non-steroidal anti-inflammatory drug, usually ibuprofen.

Adverse effects of MTX (obtained by direct questioning) and laboratory signs of toxicity (routine clinical chemistry and hematology investigations) were prospectively evaluated according to the usual practice with one to three-monthly visits. MTX adverse effects were defined as those affecting the gastrointestinal tract (stomatitis, nausea, vomiting, abdominal pain/discomfort), disturbed liver function test (alanine and/or aspartate aminotransferase above twice the upper limit of normal range), alopecia and other (marrow suppression, behavioral changes, headache, nodulosis).

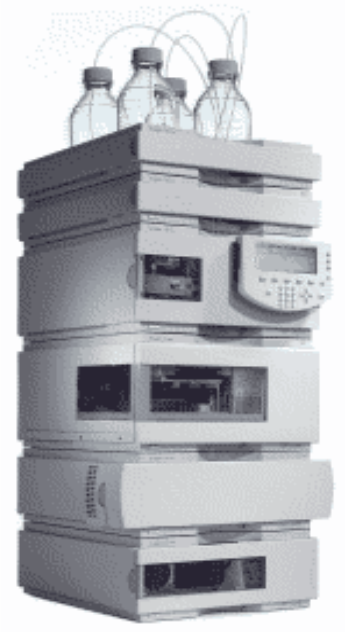
	Response to MTX therapy:		
	Responders	Nonresponders	P
N	51	18	-
Gender (male/female)	20/31	10/8	0.28
Age, yrs*	8.2 (2.8-19.6)	9.8 (2.5-17.1)	0.54
Body surface area, m^2 *	0.96 (0.57-1.93)	1.06 (0.57-1.79)	0.55
Disease duration&, yrs*	2.2 (0.5-17.0)	1.9 (0.7-4.2)	0.31
MTX therapy duration&, yrs*	1.4 (0.3-11.5)	1.3 (0.5-4.1)	0.93
ESR°, mm/h*	37 (3-116)	28 (2-140)	0.034
Active joints°, n*	6 (2-40)	6 (2-59)	0.87
Joints with limited motion°, n*	6 (2-40)	7 (2-59)	0.63
Route of MTX administration& (p.o./s.c.)	24/27	0/18	<0.0001
Weekly MTX dose&, mg/m^2 *	12.6 (7.4-20.0)	17.2 (11.8-24.2)	<0.0001
Folic acid supplementation, n (%)	44 (82.3)	17 (94.4)	0.67
Weekly dose of folic acid, mg*	10 (5-10)	10 (10-10)	0.12

*& at the time of sampling, ° before MTX initiation, * median (range)*

Tab. 2 Patient and treatment characteristics

MTX concentration in erythrocytes

In patients who had been on a stable MTX dose for at least 8 weeks, venous blood samples were drawn just before MTX administration. Samples were collected into standard EDTA-coated tubes and processed within 1 hour. Erythrocytes were separated, washed in ice-cold 0.9% NaCl and haemolyzed as described elsewhere [66]. Haematocrite of the suspension of washed erythrocytes was measured by a hematology analyzer(Nihon Kohden Celltac E) and used for calculation of EMTX as follows: EMTX= (MTX concentration in the hemolysate)/haematocrite. Plasma samples and erythrocyte suspensions were stored for no longer than 1 month at -20°C before analysis. Plasma MTX and EMTX were determined by HPLC methods using fluorimetric detection after post-column derivatization in a photochemical reactor as described previously (Fig. 8) [36, 68, 69]. During sample preparation, all MTX polyglutamates are hydrolyzed to MTX. Thus, EMTX concentration represents the sum of all polyglutamates in the erythrocyte. A simple and elective isocratic reversed phase chromatographic method with fluorescence detection was developed [36, 66, 67]. Separation was carried out on a Phenomenex GEMINI C18 column. The chromatograph Agilent 1100 series HPLC was used for all separations.



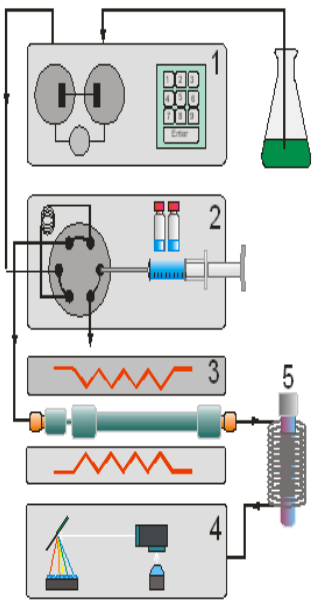


Fig. 8 Chromatograph Agilent 1100 series HPLC

Folate concentration in erythrocytes

Erythrocyte concentration of folates was measured on the Elecsys analyzer using an automated electrochemiluminescence immunoassay (ECLIA), including RBC Folate Hemolyzing Reagent and Elecsys Folate II kit (Roche, Prague, Czech Republic).

Genotype analysis

Genomic DNA was extracted from the white blood cells. The A1298C and C677T polymorphisms of the MTHFR gene were analysed by polymerase chain reaction restriction fragment length polymorphism method as described elsewhere [45, 46]. The rheumatologist evaluating the efficacy and safety of MTX was blinded to the results of genotyping and MTX and folate analysis.

Statistical analysis

Differences between responders and nonresponders were assessed using Mann-Whitney U test or chi-square test. Allele and genotype frequencies were compared using two-sided Fisher exact test. Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated for the chance of response and the risk of overall adverse effects of MTX therapy. Univariate and multivariate logistic regression models were used to analyze the influence of 677C>T and 1298A>C polymorphisms on the frequencies of response and adverse effects. In these models, the numbers of 677T and 1298C alleles (0, 1 or 2) for each patient served as independent variables. A p-level of less than 0.05 was considered statistically significant. Calculations were performed using Statistica 8.0 software (StatSoft, Inc., Tulsa, OK, USA).

5. RESULTS

5.1 Stratification of patients according to treatment response

A total of 69 children aged 2.5-19.6 years (30 males, 39 females, all Caucasians), who had been treated with MTX for at least 4 months (median duration of therapy: 1.4 years) were enrolled. Based on the treatment efficacy assessment, 51 of 69 patients (74%) were classified as full responders and 18 (26%) as non-responders. Their demographic and disease characteristics were similar (Table 1). Only 4 patients with persistent oligoarthritis had a history of chronic uveitis (all responders).

In nonresponders disease activity persisted despite treatment with subcutaneous MTX at a 37% higher dose than in responders ($P<0.0001$), who received the drug orally (n=24) or subcutaneously (n=27). The majority of patients from both groups took folic acid and its weekly dose did not differ between the groups (Table 1). Only 12 patients (all nonresponders) received other medication at the time of the study (corticosteroids n=11, sulphasalazine n=1, etanercept n=1, cyclosporine n=1).

5.2 MTX toxicity

Signs of toxicity were noticed in 21 patients (30.4 %). Gastrointestinal complaints (mucosal, nausea, vomitting, abdominal pain) were found in 16, hepatopathy (activity of transaminases ≥2-fold the upper limit) in 3, and alopecia in 2 patients. Other adverse effects (bone marrow suppression, behaviour changes, nodulosis) were not detected. The frequency of overall adverse effects was 29.4 % in MTX responders (15 out of 51 patients) and 33.3 % in nonresponders (6 out of 18 patients), respectively ($P=0.77$).

5.3 EMTX and folate concentration in erythrocytes

Results of EMTX and erythrocyte folate measurements were available for 51 responders and 13 nonresponders and for 40 responders and 14 nonresponders, respectively. The median (inter-quartile range) EMTX concentrations in nonresponders

(217, 91.4-354 nmol/L) were two-fold higher than those in responders (106, 65.3-168 nmol/L) (P<0.02, Fig. 9). Nonresponders tended to have higher concentrations of erythrocyte folates compared to responders (763, 583-935 nmol/L vs 592, 487-751 nmol/L, P=0.052, Fig. 9). No differences were observed in either MTX or folate concentrations in erythrocytes of patients stratified according to the MTHFR 677/1298 genotypes.

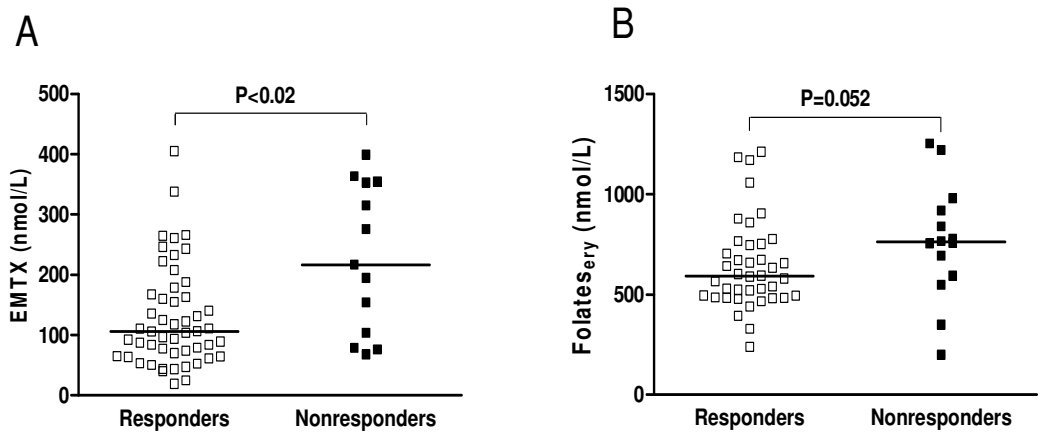


Figure 9. EMTX (A) and erythrocyte folates (B) in JIA patients according to the therapy response. Horizontal lines are medians.

5.4 MTHFR polymorphisms

Results of the MTHFR SNP analysis were available in 18 nonresponders (100%) and in 46 out of 51 (90.2%) responders to MTX. The following estimates for allele frequencies were obtained: allele C677T 36.3% (95-% CI: 26.6-47.2), and allele A1298C 29.4% (95-% CI: 22.1-37.8), respectively. Prevalence of MTHFR C677T and A1298C genotypes and dosing of MTX in JIA patients stratified according to the genotypes is summarized in Table 2. The dose-escalation protocol guided by efficacy resulted in a higher final dose administered to patients with homozygous 677TT genotype compared to the wild-type 677CC genotype (p<0.05, Table 2).

5.5 EMTX and folate concentration in erythrocytes and MTHFR

No differences were observed in either MTX or folate concentrations in erythrocytes of patients stratified according to the MTHFR 677/1298 genotypes.

5.6 Association of MTX efficacy and MTHFR polymorphisms

Full clinical response was reached in four out of eight 677TT genotype carriers (50%), as compared to 19 out of 25 carriers of 677CT (76%), and to 23 out of 31 carriers of 677CC (74.2%). The probability of response was 2.9-fold less in patients with 677TT genotype compared to the reference genotype 677CC. However, this difference did not reach statistical significance (OR: 0.35; 95-% CI: 0.07–1.73; p = 0.22) (Table 3). The frequency of T allele among MTX responders was 29.3 % compared to 38.9% in nonresponders (OR: 0.65; 95% CI: 0.29–1.46; p = 0.30; Table 3).

The response rates in relation to the A1298C polymorphism were as follows: four out of six of 1298CC genotype carriers (66.7%), 22 out of 29 1298AC genotype carriers (75.9 %), and 20 out of 29 1298AA genotype carriers (70%), respectively. The probability of response was similar in all genotypes (Table 3). The frequency of C allele among MTX responders was 30.4% and that in a group of nonresponders achieved 30.6% (OR: 0.99; 95-% CI: 0.43–2.30; p = 1.0; Table 3).

In univariate and multivariate regression analyses, the presence of neither 677T nor 1298C alleles was associated with an altered frequency of response (data not shown).

Regarding the two common MTHFR polymorphisms, six combined genotypes were similar as to the response rate. However, statistical analysis could not be performed due to small numbers of patients in the respective groups.

MTHFR polymorphism	All patients n (%)	Response to MTX therapy:		OR (95% CI)
		Responders n (%)	Nonresponders n (%)	
677C>T alleles				
T	41 (32.0)	27 (29.3)	14 (38.9)	0.65 (0.29-1.46)
C	87 (68.0)	65 (70.7)	22 (61.1)	-
1298A>C alleles				
C	39 (30.5)	28 (30.4)	11 (30.6)	0.99 (0.43-2.30)
A	89 (69.6)	64 (69.6)	25 (69.4)	-
677C>T genotype				
TT	8 (12.5)	4 (8.7)	4 (22.2)	0.35 (0.07-1.73)
CT	25 (39.1)	19 (41.3)	6 (33.3)	1.10 (0.32-3.73)
CC	31 (48.4)	23 (50.0)	8 (44.4)	-
1298A>C genotype				
CC	6 (9.4)	4 (8.7)	2 (11.1)	0.90 (0.14-5.85)
AC	29 (45.3)	22 (47.8)	7 (38.9)	1.41 (0.44-4.51)
AA	29 (45.3)	20 (43.5)	9 (50.0)	-

Fisher exact test: [a]… p<0.002, [b]...p<0.001 compared with CC (or C) as reference genotype (allele), OR…odds ratio

Table 3. The MTHFR polymorphisms and genotypes in relation to patients` response to methotrexate

5.7 Association of MTX adverse effects and MTHFR polymorphisms

The occurrence of any adverse effect in relation to the 677C>T SNP was the following: in eight of eight (100%) carriers of homozygous 677TT genotype, in six out of 25 (24%) carriers of 677CT, and in seven out of 31 (22.6%) carriers of 677CC.

The probability of any adverse effect was significantly and markedly elevated in patients with 677TT genotype as compared to the reference genotype 677CC (OR=55.5; 95-% CI: 2.9–1080; p<0.001) (Table 4). The frequency of T allele was significantly higher in patients with adverse effects than without (52.4 % vs 20.9%, OR=3.88; 95% CI: 1.8–8.6; p<0.002 (Table 4).

Contrary to the MTHFR C677T genotype, the association between the A1298C genotype and occurrence of adverse effects was much less. Adverse effects were detected in two out of six of 1298CC genotype carriers (33.3%), in six out of 29 1298AC genotype carriers (20.7 %), and in 13 out of 29 1298AA genotype carriers (44.8%), respectively. The probability of any adverse effect tended to be less in homozygotes or heterozygotes for 1298C allele as compared to homozygous carriers of 1298A allele, but the differences did not reach statistical significance (Table 4). The frequency of C allele among patients with and without adverse effects was 21.4% and 30.9%, respectively (OR: 0.51; 95-% CI: 0.22–1.20; p = 0.15; Table 4).

Both in univariate (OR= 1.46, 1.15-1.74, P<0.005) and multivariate (OR=1.46, 1.10-1.96, P<0.01) regression analyses, only MTHFR 677T allele was confirmed as a factor strongly associated with an increased frequency of overall adverse effects while the 1298C allele was not associated with an altered frequency.

MTHFR polymorphism	All patients	Overall side effects:		OR (95% CI)
	n (%)	YES	NO	
		n (%)	n (%)	
677C>T alleles				
T	41 (32.0)	22 (52.4)	19 (20.9)	3.88[a] (1.8-8.6)
C	87 (68.0)	20 (47.6)	67 (79.1)	-
1298A>C alleles				
C	39 (30.5)	9 (21.4)	30 (34.9)	0.51 (0.22-1.20)
A	89 (69.6)	33 (78.6)	56 (65.1)	-
677C>T genotype				
TT	8 (12.5)	8 (38.1)	0 (0)	55.5[b] (2.9-1080)
CT	25 (39.1)	6 (28.6)	19 (44.2)	1.08 (0.31-3.76)
CC	31 (48.4)	7 (33.3)	24 (55.8)	-
1298A>C genotype				
CC	6 (9.4)	2 (9.5)	4 (9.3)	0.62 (0.097-3.9)
AC	29 (45.3)	6 (28.6)	23 (53.5)	0.32 (0.10-1.02)
AA	29 (45.3)	13 (61.9)	16 (37.2)	-

Fisher exact test: [a]… p<0.002, [b]...p<0.001 compared with CC (or C) as reference genotype (allele), OR…odds ratio

TABLE 4. The MTHFR polymorphisms and genotypes in relation to patients` occurrence of side effects

All eight carriers of homozygous 677TT genotype had the combined genotype 677TT/1298CC and experienced adverse effects as compared with only one out of six carriers of the reference 677CC/1298AA genotype (Table 3).

6. DISCUSSION

This PhD thesis investigated value of two common SNPs of the MTHFR gene (C677T and A1298C) and EMTX for prediction of MTX efficacy and safety in children and adolescents with JIA. The pedACR30 nonresponders and responders with inactive disease were selected among MTX treated patients. MTHFR SNPs were detected and MTX and folate polyglutamates in erythrocytes were assayed after the patients had been treated sufficiently long time with the stable, sufficiently high MTX dose. This is the first study in JIA children simultaneously evaluating contribution of pharmacogenetic and metabolic markers to MTX efficacy and toxicity.

Significantly increased risk of overall MTX side effects was found in carriers of the 677T allele. Furthermore, all homozygotes for this variant, which is associated with a decreased activity of MTHFR, experienced adverse effects as compared to 22.6% homozygotes for the wild type 677C allele. This represents a 55-fold elevated risk for adverse effects in homozygotes for the mutated allele. Observed adverse effects included gastrointestinal complaints, elevation of aminotransferases and alopecia. In addition, when compared to the carriers of 677TT genotype, patients with the reference genotype 677CC had a 3-fold higher chance to achieve the full response. However, this difference was not statistically significant. Separate examination of A1298C polymorphism in relation to MTX effects showed no association.

Evaluating possible false positivity of this finding due to the limited number of patients we applied the concept of false-positive report probability (FPRP) as suggested by Wacholder et al [68]. and introduced to the field of MTX genetic association studies by Lee and colleagues [69]. The high to moderate probability level set-up appears justified as in the metaanalysis of eight studies in adults an increased risk of MTX toxicity was found in carriers of MTHFR C677T SNP (OR 1.71, 95%CI: 1.32-2.21) [70].. Our estimates of the FPRP are similar to those retrospectively determined by Dervieux [71] for the study of

Wessels et al [14] and lower as compared to the studies of Dervieux et al [10]. Therefore, our finding of association of C677T SNP and MTX adverse effects in JIA patients deserves attention being at least as noteworthy as conclusions of the three recent studies focusing on different SNPs in MTX treated adults with RA [11, 14, 71].

One previous study in MTX-treated JIA children investigated an impact of MTHFR SNPs on the treatment outcome and toxicity [57]. In this retrospective study, the probability of improvement was higher in carriers of the 1298C allele while no association was found for the C677T SNP polymorphism. In agreement with our findings, heterozygotes for the 677T genotype exhibited adverse effects more frequently than homozygotes for the wild-type allele. Pharmacogenetic studies in adult patients with RA provided inconsistent conclusions. This may be in part explained by different study design and outcome measures, co-administered drugs, ethnicity etc. According to the recent reviews [70, 72], clinical data in adults support possible association between the C677T variant and increased MTX toxicity.

During the treatment pharmacologically active MTX polyglutamates with up to 5 glutamic acid residues accumulate in cells [73]. Polyglutamylation enhances the intracellular retention of the drug and facilitates its affinity for several folate-dependent enzymes [27]. These metabolites have longer half-live than MTX itself enabling once weekly dosing. The steady-state EMTX level could be an indicator of the long-term MTX exposure. It is influenced by several factors including MTX bioavailability, elimination kinetics and patient compliance. Under well-controlled conditions of prospective clinical studies, the steady-state concentration of EMTX correlated strongly with the area under the concentration-time curve (AUC) of plasma MTX reflecting its bioavailability, as shown in patients with RA [7] as well as psoriasis [36]. Nevertheless, weak to moderate correlation of EMTX with MTX dose was found [34, 74]. Circulating erythrocytes lack folylpolyglutamate synthetase and MTX polyglutamates are mainly formed in bone

marrow progenitor cells. Therefore, EMTX may reflect MTX polyglutamate concentrations in immunocompetent cells, e.g. lymphocytes, and work as a bioindicator of the effect [39].

Most studies with adult RA patients suggest that higher EMTX concentration is associated with better response [7, 8, 10, 11, 54]. Hornung et al. monitored clinical effect during the follow-up period (52 weeks) using preliminary ACR core criteria (no response, 20%, 50% improvement). Patients with RA classified as responders had a significantly higher mean steady-state EMTX, but significantly higher dose at the same time. Since the correlation between the dose and EMTX was found, the MTX dose might have affected EMTX concentration in nonresponders who were under-dosed [7]. In adults with RA, two- to three-fold higher concentrations of EMTX were found in responders and partial responders as compared to nonresponders [8]. However, MTX was administered orally and mean doses used in all three groups were similar and low (≤11.2 mg/week). Nonresponders might have reached response if treated parenterally with a higher MTX dose [8]. Dervieux et al used a dose-escalation protocol in adults with RA on oral MTX and found approximately 20% lower accumulation of EMTX in patients with the lower than median improvement in the DAS28 score as compared to the better responders [11]. Moreover, three nonresponders had 33% lower EMTX concentrations when compared to responders. In another study with adult patients, the probability of a good response was 20 to 30 % lower in patients with EMTX level below 60 nmol/l [11, 54].

Kristensen et al. explored relationship between EMTX and clinical and laboratory parameters in children with JIA. The design of the study did not allow a cross-sectional analysis of the relation between EMTX and disease parameters, patients served as their own controls. Within the 3-month interval, spontaneous fluctuation in disease activity did not reflect intra-individual changes in EMTX [9]. In the present study, the dose-escalation protocol allowed switching from oral to subcutaneous administration which resulted in an

exclusive use of subcutaneous route in nonresponders who received 37% higher MTX dose than responders. Nonresponders also presented higher EMTX levels suggesting that exposure to MTX was maximized and that the lack of response was associated with pharmacodynamic factors rather than pharmacokinetics. Our assay for EMTX did not allow to separately quantify concentrations of individual polyglutamates. Therefore we have not been able to evaluate the proportion of longer chain polyglutamates that might better reflect MTX efficacy as shown in adult patients [11]. Moreover, route of administration contributed to the pattern of polyglutamation of MTX in JIA children in a recent study. Higher proportion of long-chain (3 to 5) polyglutamates was observed in patients treated subcutaneously, and, conversely, a higher proportion of short-chain (1+2) derivatives was found in orally treated patients [75].

Concentration of erythrocyte folates is one of important factors of MTX therapeutic response. In our study patients classified as nonresponders tended to have higher concentrations of erythrocyte folates as compared to full responders. Importantly, results of large cross sectional studies in adults also established that increased erythrocyte folates were associated with high disease activity [11,76].

7. SUMMARY

In conclusion, nonresponders to the dose-escalation protocol had twice as high EMTX concentrations as compared to responders and similar rate of adverse effects. No significant relationship between EMTX and treatment efficacy was found. The evaluation of reationships between MTHFR gene SNPs (C677T and A1298C) and MTX efficacy and safety in children with JIA under simultaneous control for both antifolate and folate status revealed that carriers of the 677T allele have a 3.9- fold increased risk for MTX adverse effects. This elevation may be attributed to a 55-fold augmented risk in patients carrying 677TT, which almost exclusively combines with 1298AA genotype. Analysis of MTHFR allele and genotype frequencies in relation to response failed to detect any significant association. Significance of these results is limited by small patient numbers, due to which estimation of MTHFR 677/1298 haplotype distributions and statistical evaluation of their influence could not have been done. This area definitely deserves further exploration.

8. REFERENCES

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9. RELEVANT AUTHORS PUBLICATIONS RELATED TO PhD THESIS

Original articles in journals with impact factor

1) Hroch M, Tuková J, Dolezalová P, Chládek J. An improved high-performance liquid chromatography method for quantification of methotrexate polyglutamates in red blood cells of children with juvenile idiopathic arthritis. Biopharm Drug Dispos, 2009. **30**(3): p. 138-48.

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2) Tuková J, Chládek J, Nemcová D, Chládková J, Dolezalová P. Methotrexate bioavailability after oral and subcutaneous administration in children with juvenile idiopathic arthritis. Clin Exp Rheumatol. 2009 Nov-Dec;27(6):1047-53.

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3) Tuková J, Chládek J, Hroch M, Nemcová D, Hoza J, Dolezalova P. The 677TT genotype is associated with elevated risk of methotrexate toxicity in juvenile idiopathic arthritis patients: Treatment outcome, erythrocyte concentrations of methotrexate and folates and methylenetetrahydrofolate reductase polymorphisms relationships. J Rheum, 2010, "in press".

   IF= 3.282

Articles in journals without impact factor

Tuková J, Němcová D, Hoza J, Doležalová P. Metotrexát u dětí s juvenilní idiopatickou artritidou. Cesk pediatr, 2010, „in press".

10. APPENDICES

Article No. 1

Hroch M, Tuková J, Dolezalová P, Chládek J. An improved high-performance liquid chromatography method for quantification of methotrexate polyglutamates in red blood cells of children with juvenile idiopathic arthritis. Biopharm Drug Dispos, 2009. **30**(3): p. 138-48.

Article No. 2

Tuková J, Chládek J, Nemcová D, Chládková J, Dolezalová P. Methotrexate bioavailability after oral and subcutaneous administration in children with juvenile idiopathic arthritis. Clin Exp Rheumatol. 2009 Nov-Dec;27(6):1047-53.

Article No. 3

Tuková J, Chládek J, Hroch M, Nemcová D, Hoza J, Dolezalova P. The 677TT genotype is associated with elevated risk of methotrexate toxicity in juvenile idiopathic arthritis patients: Treatment outcome, erythrocyte concentrations of methotrexate and folates and methylenetetrahydrofolate reductase polymorphisms relationships. J Rheum, 2010, “in press”.