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PhD thesis summary

INTRACELLULAR CONCENTRATION OF METHOTREXATE IN ERYTHROCYTES AND MTHFR POLYMORPHISMS : Possible association with of methotrexate efficacy and toxicity in patients with juvenile idiopathic arthritis ?

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Content:

Souhrn

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 folátů 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 meotrexátem navozenou toxicitu u dětí s JIA. I přes nedostatečný terapeutický efekt metotrexátu nonrespondéři akumulují dostatečné množství EMTX.

Summary

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.

1. Introduction

Over the last two decades methotrexate (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 is 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 to efficacy and toxicity is subject of investigation in many studies.

Two potential tools for individualization of therapy have been studied in our study: 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 [7-8].

Methylene tetrahydrofolate reductase (MTHFR) is a critical enzyme of MTX metabolism. It is associated with regeneration of reduced folates. Two relatively common single nucleotide polymorphisms (C677T, A1298C) have been studied in the MTHFR gene and association between these polymorphisms and clinical variables of disease outcome and/or toxicity in adult patients revealed inconsistent results [10-19].

2. Hypothesis and aims

The thesis study is 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 borders of the therapeutic response spectrum were recruited. Firstly, patients with excellent drug response who achieve disease inactivity while on MTX therapy were examined (full MTX responders). Secondly, patients with insignificant 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 erythrocyte 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 folate erythrocyte is usable parameter for prediction of toxicity

3. Material and methods

Patients with JIA were consecutively recruited from the paediatric rheumatology out-patient clinic population. To become eligible patients must have had a definitive diagnosis of JIA [20] 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.

Responders were defined as having achieved disease inactivity on medication with MTX monotherapy according to Wallace et al. [21]. Using pediatric ACR 30 definition of improvement [21-24] 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 % [22-24]. 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].

On top of MTX, most patients received once-weekly folic acid (5-10 mg/week, 24-48 hours after MTX.

Toxicity was prospectively monitored at each visit by routine questioning and laboratory tests. MTX toxic effects of any grade were defined as those related to the gastrointestinal tract, liver function, bone marrow suppression and other (alopecia, behavioral changes, headache, nodulosis).

MTX concentration in erythrocytes

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 [25]. 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 MTX and EMTX were determined by HPLC methods using fluorimetric detection after post-column derivatization in a photochemical reactor as described previously [26,27].

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, CzechRepublic).

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 [28,29].

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).

4. Results

4.1 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. 51 of 69 patients (74%) were classified as full responders and 18 (26%) as non-responders. Their demographic and disease characteristics were similar. 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).

4.2 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 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).

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

4.4 MTHFR polymorphisms

Results of the MTHFR SNP analysis were available in 18 nonresponders (100%) and in 46 out of 51 (90.2%) responders. 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. 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 ($p<0.05$).

4.5 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.

4.6 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; $p = 0.22$). 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; $p = 0.30$).

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. 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; $p = 1.0$).

In univariate and multivariate regression analyses, the presence of neither 677T nor 1298C alleles was associated with an altered frequency of response.

4.7 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; $p<0.001$). 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; $p<0.002$).

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%). 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.

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.

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.

5. 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 the concept of false-positive report probability (FPRP) was applied as suggested by Wacholder et al [30] and introduced to the field of MTX genetic association studies by Lee and colleagues [31]. 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) [32]. Our estimates of the FPRP are similar to those retrospectively determined by Dervieux [33] 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 [10,14,32].

One previous study in MTX-treated JIA children investigated an impact of MTHFR SNPs on the treatment outcome and toxicity [34]. 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 [32, 35], 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 [36]. Polyglutamylation enhances the intracellular retention of the drug and facilitates its affinity for several folate-dependent enzymes [37]. 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 [38]. Nevertheless, weak to moderate correlation of EMTX with MTX dose was found [39,40]. 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 [41].

Most studies with adult RA patients suggest that higher EMTX concentration is associated with better response [7,8,10,11,42]. 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 [10]. 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 [10,11,42].

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 [43]. 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. The 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 [10].

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 [10,44].

6. Conclusions

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.

7. References

1. Cassidy JT, Petty RE. Juvenile Rheumatoid Arthritis. In: Cassidy JT, Petty RE, editors. Textbook of Pediatric Rheumatology. Philadelphia: WB Saunders; 2001:218-321.
2. Woo P, Southwood TR, Prieur AM, Doré CJ, Grainger J, David J, et al. Randomized, placebo- controlled, crossover trial of low- dose oral methotrexate in children with extended oligoatricular or systemic arthritis. Arthritis Rheum 2000;43:1849-57.
3. Giannini EH, Brewer EJ, Kuzmina N, Shaikov A, Maximov A, Vorontsov I, et al. Methotrexate in resistant juvenile rheumatoid arthritis: Results of the U.S.A.-U.S.S.R. double-blind, placebo-controlled trial. N Engl J Med 1992;326:1043-9.
4. Ruperto N, Murray KJ, Gerloni V, Wulffraat N, de Oliveira SK, Falcini F, et al. A randomized trial of parenteral methotrexate comparing an intermediate dose with a higher dose in children with juvenile idiopathic arthritis who failed to respond to standard doses of methotrexate. Arthritis Rheum 2004;50:2191-201.
5. Wallace CA. The use of methotrexate in childhood rheumatic diseases. Arthritis Rheum 1998;3:381-91.
6. Tuková J, Chládek J, Němcová D, Chládková J, Doležalová P. Methotrexate bioavailability after oral and subcutaneous administration in children with juvenile arthritis. Clin Exp Rheumatol 2009;27:1047-53.
7. Hornung N, Ellingsen T, Attermann J, Stengaard-Pedersen K, Poulsen JH. Patients with rheumatoid arthritis treated with methotrexate (MTX): concentrations of steady-state erythrocyte MTX correlate to plasma concentrations and clinical efficacy. J Rheumatol 2008;35:1709-15.
8. Angelis-Stoforidis P, Vajda FJE, Christophidis N. Methotrexate polyglutamate levels in circulating erythrocytes and polymorphs correlate with clinical efficacy in rheumatoid arthritis. Clin Exp Rheumatol 1999;17:313-20.
9. Kurzawski M, Pawlik A, Safranow K, Herczynska M, Drozdzik M. 677C>T and 1298A>C MTHFR polymorphisms affect methotrexate treatment outcome in rheumatoid arthritis. Pharmacogenomics 2007;8:1551-9.
10. Dervieux T, Furst D, Lein DO, Capps R, Smith K, Caldwell J, et al. Pharmacogenetic and metabolite measurements are associated with clinical status in rheumatoid arthritis patients treated with methotrexate: results of a multicentered cross sectional observational study. Ann Rheum Dis 2005;64:1180-5.
11. Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum 2006;54:3095-103.

12. Urano W, Taniguchi A, Yamanaka H, Tanaka E, Nakajima H, Matsuda Y, et al. Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. Pharmacogenetics 2002;12:183-90.
13. Berkun Y, Levartovsky D, Rubinow A, Orbach H, Aamar S, Grenader T, et al. Methotrexate related adverse effects in patients with rheumatoid arthritis are associated with the A1298C polymorphism of the MTHFR gene. Ann Rheum Dis 2004;63:1227-31.
14. Wessels JA, de Vries-Bouwstra JK, Heijmans BT, Slagboom PE, Goekoop-Ruiterman YP, Allaart CF, et al. Efficacy and toxicity of methotrexate in early rheumatoid arthritis are associated with single-nucleotide polymorphisms in genes coding for folate pathway enzymes. Arthritis Rheum 2006;54:1087-95.
15. Ede AE, Laan RFJM, Blom HJ, Huizinga TWJ, Haagsma CJ, Giesendorf BAJ, et al. The C677T mutation in the methylentetrahydrofolate reductase gene. Arthritis Rheum 2001;44:2525-30.
16. Weisman MH, Furst D, Park GS, Kremer J, Smith K, Wallace DJ, et al. Risk genotypes in folate-dependent enzymes and their association with methotrexate-related side effects in rheumatoid arthritis. Arthritis Rheum 2006;54:607-12.
17. Ranganathan P, Culverhouse R, Marsh S, Mody A, Scott-Horton TJ, Brasington R, et al. Methotrexate (MTX) pathway gene polymorphisms and their effects on MTX toxicity in Caucasian and African American patients with rheumatoid arthritis. J Rheumatol 2008;35:572-9.
18. Aggarwal P, Naik S, Mishra KP, Aggarwal A, Misra R. Correlation between methotrexate efficacy & toxicity with C677T polymorphism of the methylenetetrahydrofolate gene in rheumatoid arthritis patients on folate supplementation. Indian J Med Res 2006;124:521-6.
19. Hughes LB, Beasley TM, Patel H, Tiwari HK, Morgan SL, Baggott JE, et al. Racial or ethnic differences in allele frequencies of single-nucleotide polymorphisms in the methylenetetrahydrofolate reductase gene and their influence on response to methotrexate in rheumatoid arthritis. Ann Rheum Dis 2006;65:1213-8.
20. International League of Associations for Rheumatology Classification of Juvenile Idiopathic Arthritis: Second Revision, Edmonton. J Rheumatol 2001;31:390-2.
21. Wallace CA, Ruperto N, Giannini EH. Preliminary Criteria for Clinical Remission for Select Categories of Juvenile Idiopathic Arthritis. J Rheumatol 2004;31:2290-4.
22. Ruperto N, Ravelli A, Falcini F, Lepore L, De Sanctis R, Zulian F, et al. Performance of the preliminary definition of improvement in juvenile

chronic arthritis patients treated with methotrexate. Italian Pediatric Rheumatology Study Group. Ann Rheum Dis 1998;57:38-41.
23. Albornoz MA. ACR formally adopts improvement criteria for juvenile arthritis (ACR Pediatric 30). ACR News 2002;21:3.
24. Giannini EH, Ruperto N, Ravelli A, Lovell DJ, Felson DT, Martini A. Preliminary definition of improvement in juvenile arthritis. Arthritis Rheum 1997;40:1202-9.
25. Schroder H, Heinsvig E-M. Enzymatic assay for methotrexate in erythrocytes. Scand J Clin Lab Invest 1985;45:657-9.
26. Chládek J, Martínková J, Šimková M, Vaněčková J. Pharmacokinetics of low doses of methotrexate in patients with psoriasis over the early period of treatment. Eur J Clin Pharmacol 1998;53:437-44.
27. Hroch M, Tukova J, Dolezalova P, Chladek J. An improved high-performance liquid chromatography method for quantification of methotrexate polyglutamates in red blood cells of children with juvenile idiopathic arthritis. Biopharm Drug Dispos 2009;30:138-48.
28. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-3.
29. Van der Put NM, Gabreels F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 1998;62:1044-51.
30. Wacholder S, Chanock S, Garcia-Closas M, El Ghormli L, Rothman N. Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. J Natl Cancer Inst 2004;96:434-42.
31. Lee YC, Cui J, Costenbader KH, Shadick NA, Weinblatt ME, Karlson EW. Investigation of candidate polymorphisms and disease activity in rheumatoid arthritis patients on methotrexate. Rheumatology (Oxford) 2009;48:613-7.
32. Fisher MC, Cronstein BN. Metaanalysis of methylenetetrahydrofolate reductase (MTHFR) polymorphisms affecting methotrexate toxicity. J Rheumatol 2009;36:539-45.
33. Dervieux T. Methotrexate pharmacogenomics in rheumatoid arthritis: introducing false-positive report probability. Rheumatology (Oxford) 2009;48:597-8.
34. Schmeling H, Biber D, Heins S, Horneff G. Influence of methylentetrahydrofolate reductase polymorphisms on efficacy and toxicity of methotrexate in patients with juvenile idiopathic arthritis. J Rheumatol 2005;32:1832-6.

35. De Mattia E, Toffoli G. C677T and A1298C MTHFR polymorphisms, a challenge for antifolate and fluoropyrimidine-based therapy personalisation. Eur J Cancer 2009;45:1333-51.
36. Dalrymple JM, Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Barclay ML. Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. Arthritis Rheum 2008;58:3299-308.
37. Hillson JL, Furst DE. Pharmacology and pharmacokinetics of methotrexate in rheumatic disease. Rheum Dis Clin North Am 1997;23:757-78.
38. Chladek J, Grim J, Martinkova J, Simkova M, Vaneckova J. Low-dose methotrexate pharmacokinetics and pharmacodynamics in the therapy of severe psoriasis. Basic Clin Pharmacol Toxicol 2005;96:247-8.
39. Dolezalova P, Krijt J, Chladek J, Nemcova D, Hoza J. Adenosine and methotrexate polyglutamate concentrations in patients with juvenile arthritis. Rheumatology (Oxford) 2005;44:74-9.
40. Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Frampton C, James J, et al. Determinants of red blood cell methotrexate polyglutamate concentrations in rheumatoid arthritis patients receiving long-term methotrexate treatment. Arthritis Rheum 2009;60:2248-56.
41. Schroder H. In vivo methotrexate kinetics and metabolism in human hematopoietic cells. Clinical significance of methotrexate concentrations in erythrocytes. Dan Med Bull 1990;37:22-40.
42. Dervieux T, Furst D, Lein DO, Capps R, Smith K, Walsh M, et al. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum 2004;50:2766-74.
43. Kristensen, Nielsen S, Pedersen FK, Marek Z. Erythrocyte - methotrexate and disease activity in children treated with oral methotrexate for juvenile chronic arthritis. Scand J Rheumatol 2000;29:187-9.
44. Stamp LK, O'Donnell JL, Chapman PT, Zhang M, James J, Frampton C, Barclay ML. Methotrexate polyglutamate concentrations are not associated with disease control in rheumatoid arthritis patients receiving long-term methotrexate therapy. Arthritis Rheum 2010;62:359-368.

Author´s articles*:*

1. original articles related to PhD thesis in journals with impact factor

a)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. IF=1.542

b)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. IF=2.364

c)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

2. article related to PhD thesis without impact factor

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

3. articles with different theme in journal with impact factor – 0

4. without impact factor

Rotreklová J, Molinský J, Tuka V, Malík J. Wall shear stress and endothelium. Cas Lek Cesk. 2004;143(7):467-70.