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CLINICAL TRIAL

The role and utility of measuring red blood cell methotrexate polyglutamate concentrations in inflammatory arthropathies—a systematic review

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Abstract
Purpose Evidence regarding the relationship between red blood cell methotrexate polyglutamate concentration and response to treatment and adverse drug reactions in patients using methotrexate for inflammatory arthropathies is complex and in some respects appears conflicting. Accordingly, we undertook a systematic analysis of available evidence to determine the clinical utility of dosing methotrexate to a target red blood cell methotrexate polyglutamate concentration.

Methods A systematic literature review was conducted to identify all studies that had reported an association between red blood cell methotrexate polyglutamate concentration and disease activity or adverse drug reactions in users of methotrexate for the treatment of rheumatoid arthritis, juvenile idiopathic arthritis or psoriatic arthritis.

Results No randomised controlled trials were identified. Thirteen studies (ten in patients with rheumatoid arthritis and three in patients with juvenile idiopathic arthritis) were identified. All studies evaluated an association between red blood cell methotrexate polyglutamate concentration and response to treatment, and eight evaluated an association with toxicity. Eight studies identified lower disease activity with at least one higher red blood cell methotrexate polyglutamate concentration, although there was at least moderate potential for bias in all of these studies. Relatively large increases in concentration appeared to be required to produce a meaningful reduction in disease activity. Only one study identified an association between red blood cell methotrexate polyglutamate concentration and methotrexate-induced side effects, although studies were likely underpowered to detect this type of association.

Conclusions The manner in which data were presented in the included studies had many limitations that hampered its conclusive assessment, but red blood cell methotrexate polyglutamate concentrations appear to be a potentially useful guide to treatment in patients with inflammatory arthropathies, but the specific polyglutamate that should be monitored and how monitoring could be integrated into treat-to-target approaches should be clarified before it can be routinely implemented.

Keywords Methotrexate polyglutamates · Rheumatoid arthritis · Juvenile idiopathic arthritis · Concentration-targeted dosing · Intracellular drug concentration · Personalised medicine

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Abbreviations

MTX	Methotrexate
DMARD	Disease-modifying anti-rheumatic drug
RA	Rheumatoid arthritis
JIA	Juvenile idiopathic arthritis
MTX_{Glu-n}	Methotrexate polyglutamate
RBC	Red blood cell
NSAIDs	Non-steroidal anti-inflammatory drugs
DAS28	28-Joint Disease Activity Score
JADAS-27	27-Joint Juvenile Arthritis Disease Activity Score
QuIPS	Quality in prognostic studies
RCT	Randomised controlled trial
ALT	Alanine transferase
AST	Aspartate transferase
LFTs	Liver function tests
ACR	American College of Rheumatology
EULAR	European League Against Rheumatism

Introduction

Methotrexate (MTX) is one of the most widely used disease-modifying anti-rheumatic drugs (DMARDs) for the treatment of rheumatoid arthritis (RA). MTX is also used for several other inflammatory diseases, including juvenile idiopathic arthritis (JIA), psoriatic arthritis, polymyositis, systemic lupus erythematosus, and ankylosing spondylitis [1, 2].

MTX is actively transported into cells by the reduced folate carrier. Glutamic acid residues are added to MTX by the enzyme folyl-polyglutamate synthetase to form MTX polyglutamates (MTX_{Glu-n}) [3]. There are several subtypes that are collectively referred to as MTX_{Glu-n}, where n represents the number of glutamic acid residues that have been covalently attached to the MTX, noting that MTX itself contains one glutamic acid residue. Throughout this manuscript, MTX_{Glu-n} is used to refer generically to MTX polyglutamates. With low-dose MTX therapy as used in inflammatory arthropathies, metabolites up to MTX_{Glu5} are detected in most patients [4].

One of the purported mechanisms of action of MTX in inflammatory arthropathies is inhibition of dihydrofolate reductase. Compared to MTX, longer-chained MTX polyglutamates (i.e. $MTX_{Glu3–5}$) are more potent inhibitors since they have a greater affinity for and slower dissociation from dihydrofolate reductase [5–7]. MTX_{Glu-n} also inhibit methylene-tetrahydrofolate reductase, an enzyme responsible for converting 5-10-methylene-tetrahydrofolate reductase to 5-methyl-tetrahydrofolate, the primary circulating precursor of the active folate cofactors required for synthesis of purine bases [3]. Whereas unchanged MTX exits cells relatively rapidly, the polyglutamate metabolites are retained within cells for a longer period of time. In red blood cells (RBC), MTX_{Glu-n} accumulate with half-lives ranging from 1.9 to 45.2 weeks, and with a constant dose of MTX, steady state is reached after 6 to 149 weeks [4, 8]. These characteristics lead to a prolongation of MTX effect beyond the time that parent MTX has been lost from intracellular and extracellular milieu [7, 9].

Given the above characteristics, it has been proposed that $RBCMTX_{Glu-n}$ concentrations may be a useful parameter for monitoring and adjusting MTX dose [10], although the primary site of action of MTX_{Glu-n} is more likely to be within white blood cells [11]. Concentrations within RBC have been more commonly investigated as RBC are more abundant and therefore suited to measurement of intracellular drug concentrations, especially in circumstances where sensitivity may be limiting. Data are conflicting regarding the relationship between RBC MTX_{Glu-n} concentration and MTX efficacy and/or toxicity [8], and the applicability of using these concentrations to personalise MTX therapy is therefore uncertain.

To investigate the utility and limitations of guiding MTX dosing with RBC MTX_{Glu-n} concentrations, we performed a systematic literature review of the evidence supporting personalised MTX dosing based upon RBC MTX_{Glu-n} concentrations in patients with inflammatory arthropathies.

Methods

Studies involving participants with inflammatory arthropathies such as RA, JIA or psoriatic arthritis were considered eligible. There were no restrictions regarding the age or gender of study participants, the dose, duration or route of MTX administration, concurrent therapy (e.g. DMARDs, corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs) or folic acid), efficacy or toxicity criteria nor the language or geographical location of the primary study.

Inclusion criteria

1. Studies including patients treated with MTX for RA, JIA or psoriatic arthritis as confirmed by standard diagnostic criteria.
2. At least one measurement of $RBCMTX_{Glu-n}$ concentration whilst taking MTX.
3. Exposure or concentration of $RBCMTX_{Glu-n}$ correlated with a change in one or more disease activity measures or side effects.

Exclusion criteria

1. Inaccessible abstract or insufficient data within the abstract.
2. Inaccessible full conference presentations.

Medline, Embase and Web of Science were searched for relevant studies. To investigate publication bias, we also searched the Australian and New Zealand, US National Institutes of Health and the EU Clinical Trials registries.

The search strategy was as follows: (Rheumatoid Arthritis OR Arthritis OR Arthriti*) AND (Polyglutamate OR polyglutamic acid OR methotrexate polyglutamate OR MTX polyglutamate OR RBC methotrexate) AND (Role* OR Utilit* OR efficacy OR Response OR DAS28 OR disease activity OR ACR20 OR ACR50 OR ACR70 OR EULAR Response OR toxicity OR adverse drug reaction OR side effect OR liver toxicity). MeSH and Emtree terms were utilised where possible, and references of selected studies were hand searched to identify any further relevant studies.

Eligibility assessment was performed by the lead author (HJM) under the supervision of the senior author (MW). Assessment of relevance was initially conducted using study titles, then abstract and full-text reports. Disagreements were resolved by consensus. References of selected studies were hand searched to identify any further relevant studies. Data were extracted according to a standardised list of items.

The risk of bias for each identified study was formally assessed by MH and MW with the Quality in Prognostic Studies (QuIPS) tool [12], and identified studies were stratified according to study population, quality and design. As the primary evidence of clinical utility would be provided from randomised controlled trials (RCTs) that compare RBC $MTX_{Glu\text{-}n}$ concentration guided MTX dosing with conventional MTX dosing, these were considered to be the most preferable source of evidence. Secondary sources of evidence were observational studies (prospective cohort studies in preference to cross-sectional or case-control studies) that examined the relationship between RBC $MTX_{Glu\text{-}n}$ concentration and MTX efficacy and/or toxicity.

Where possible, we estimated the impact of changing RBC $MTX_{Glu\text{-}n}$ concentration(s) on disease activity measures by calculating the effect of doubling the median concentration of RBC $MTX_{Glu\text{-}n}$ using the median concentrations and regression coefficients (and corresponding 95 % confidence intervals (CIs)) that were reported in the identified studies.

Results

Identification of studies for the systematic review is outlined in Fig. 1.

Study characteristics and assessment of bias

Of the 13 studies included, 10 included patients with RA and 3 included patients with JIA (Table 1). No RCTs were identified which compared $RBCMTX_{Glu\text{-}n}$ guided MTX dosing to standard dosing, and direct estimation of the clinical utility of a targeted $RBCMTX_{Glu\text{-}n}$ approach was therefore not possible. All 13 observational studies investigated a correlation between $RBCMTX_{Glu\text{-}n}$ and efficacy and, 8 investigated a correlation between $RBCMTX_{Glu\text{-}n}$ and toxicity.

Assessment of risk of bias (Table 2) identified confounding variables and statistical analysis and reporting as the main areas where bias was potentially introduced. There was significant heterogeneity with regard to baseline patient, disease and treatment characteristics, and outcome measures between studies (Supplementary Table 1).

Efficacy

Four of the prospective cohort studies reported point estimates (3 linear and 1 logistic regression) for the association between one or more RBC $MTX_{Glu\text{-}n}$ concentration and a composite measure of disease activity at one or more time points, and 2 studies reported results using Spearman's rho coefficients. The linear regression coefficients and corresponding 95 % confidence intervals associated with disease activity measures (DAS28 in the R-MTX and treatment in Rotterdam studies [13] and $\log_{10}$ JADAS-27 in Bulatovic Calasan et al. [14]) are presented in Fig. 2. The R-MTX and treatment in Rotterdam studies included a longitudinal analysis which considered $RBCMTX_{Glu\text{-}n}$ concentration and DAS28 at the corresponding time [13], and in Bulatovic Calasan et al. [14], the longitudinal analysis determined the association between the single $RBCMTX_{Glu\text{-}n}$ concentration and the JADAS-27 across the first year of treatment (Fig. 2). In Dervieux et al. (2006), logistic regression analysis identified a trend for individuals with lower RBC MTX_{Glu3} concentrations to be less likely to achieve moderate or good EULAR response after 4 months of MTX treatment ($\beta = 0.034$, 95 % CI −0.006 to 0.074, $p = 0.095$) [15].

In the studies that reported associations with Spearmans rho coefficients, Hobl et al. reported that maximum concentration of $RBCMTX_{Glu2}$ after 5 and 10 weeks of therapy was significantly associated with the change in DAS28 over 16 weeks (Table 3) [16]. In this study, the association between maximal concentration of $RBCMTX_{Glu1}$ and $RBCMTX_{Glu3}$ was not significant—no quantitative estimate of the effect size was available (personal communication, E Hobl). In Stamp et al., 24 weeks after changing from oral to subcutaneous MTX, the change in DAS28 was significantly associated with $RBCMTX_{Glu5}$ and $RBCMTX_{Glu3–5}$ concentration, but not other shorter chain RBC $MTX_{Glu\text{-}n}$ concentrations (Table 3) [17].

Of the seven cross-sectional studies identified, three (all in patients with RA) reported an association between $RBCMTX_{Glu\text{-}n}$ concentration and response to treatment and/or at least one measure of disease activity [10, 18, 19]. Angelis-Stoforidis et al. reported that $RBCMTX_{Glu\text{-}total}$ concentration (mean ± standard deviation) was significantly

higher in responders (60.9±19.1 nmol/L) and partial responders (50.9±23.4 nmol/L, as determined by physicians' global clinical assessment) compared to non-responders (21.5±10.7 nmol/L, $p<0.001$) [10]. In studies by Dervieux et al. (2004 and 2005), linear regression analysis identified that RBC MTX_{Glu3} concentration was associated with reduced counts of 22 joints for tenderness (β=−0.048 (95 % CI −0.094 to −0.002) and β=−0.050 (95 % CI −0.080 to −0.020)) and swelling (β=−0.045 (95 % CI −0.083 to −0.007) and β=−0.022 (95 % CI −0.048 to 0.004)), respectively, and physician assessment of disease activity (β=−0.026 (95 % CI −0.042 to −0.010) and β=−0.0257 (95 % CI −0.0377 to −0.0137)) [18, 19].

Of the remaining four cross-sectional studies, two (1 in 145 patients with RA [20], the other in 104 patients with JIA [21, 22]) did not identify a significant correlation between RBC MTX_{Glu-n} concentration and either response to MTX or any measure of disease activity, although no quantitative estimate of the effect size was available. In 30 patients with JIA, Dolezalova et al. reported that the RBC $MTX_{Glu-total}$ concentration (mean±standard deviation) in non-responders and responders, respectively, was 227.6±161.9 and 188.8±245.8 nmol/L, $p=0.72$ [21, 22]. Finally, using Spearmans' rho coefficients, Stamp et al. (2010) reported that in 192 individuals with RA, those with higher RBC MTX_{Glu3}, RBC MTX_{Glu4}, RBC MTX_{Glu5}, RBC MTX_{Glu1-5} and RBC MTX_{Glu3-5} concentration had higher disease activity as measured by DAS28 (Table 3) [23].

Effect of changing RBC MTX_{Glu-n} concentration on disease activity

Five studies (three prospective cohort [13, 14] and two cross-sectional studies [18, 19]) included linear regression equations that allowed an approximate assessment of the projected impact of changes of RBC MTX_{Glu-n} concentration on disease activity, four in patients with RA and one in patients with JIA. The projected change in disease activity scores associated with RBC MTX_{Glu-n} concentrations is shown in Fig. 3. The maximum predicted reduction in DAS28 in the R-MTX study was 24 % (95 % CI 8 to 40 %) with doubling RBC MTX_{Glu1-5} concentration after 3 months, and in the treatment in Rotterdam study, it was 21 % (95 % CI 10 to 33 %) with RBC MTX_{Glu3} concentration after 6 months [13]. For example, data from the R-MTX study indicates that the typical patient had a DAS28 of 2.92 and RBC MTX_{Glu1-5} concentration of 117 nmol/L after 3 months of MTX therapy, if this concentration was 234 nmol/L, but it would be expected that their DAS28 would be 2.22 (95 % CI 1.75 to 2.69), representing a reduction of 24 % (95 % CI 8 to 40 %). In the one study in JIA, the largest predicted reduction in JADAS-27 was 31 % (95 % CI 0 to 42 %) with RBC MTX_{Glu1-5} concentration after

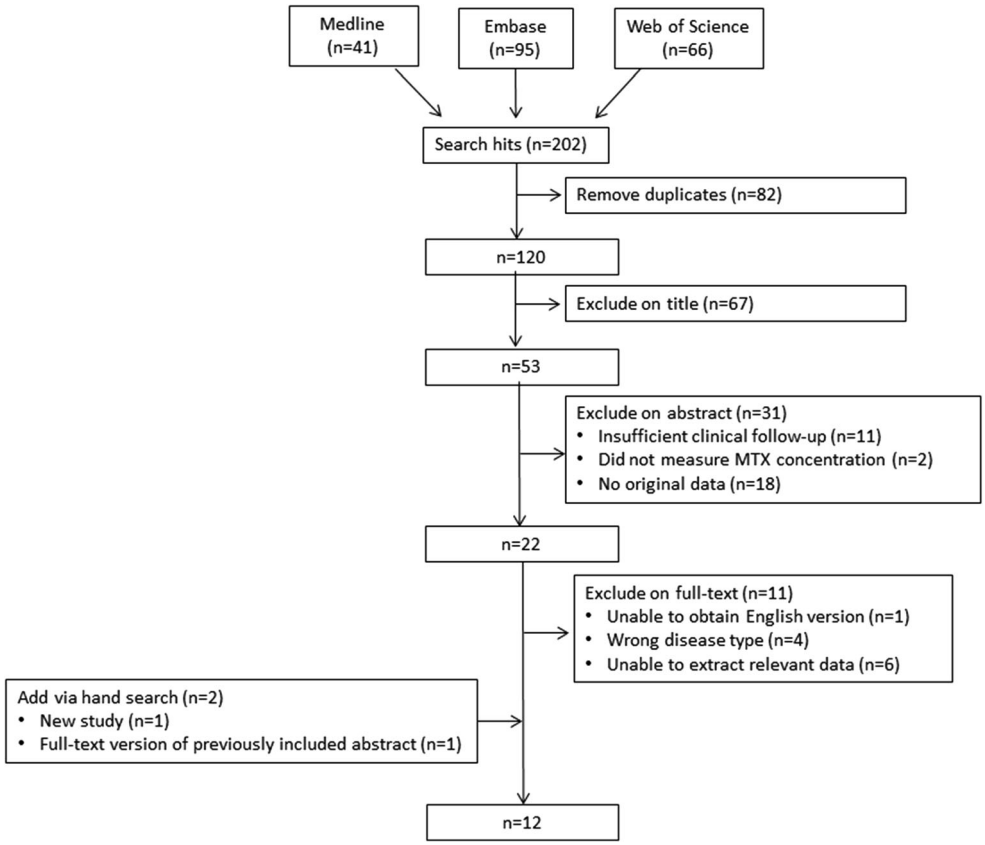


Fig. 1 PRISMA diagram of the identification of studies for the review

Table 1 Baseline patient characteristics of included studies for analysis

Author name, date	*N*	% Female	Age, mean (SD)	Other concurrent treatment (%)				Duration of disease at study entry (years), mean (SD)	Duration of MTX at study entry (years), mean (SD)
				DMARDs	CS	Folic acid	NSAIDs		
RCT and prospective cohort studies—RA patients									
Dervieux et al. 2006	48	–	55	29.2	58.3	96	62.5	1.0 (0.3–5.0)[a]	0
Stamp et al. 2011	30	77	51.8 (32–70)[b]	56.7	43.3	100	55.3	7.7 (0.75–21)[b]	3.3 (0.5–12)[b]
Hobl et al. 2012	19	68	56[c]	0	–	–	–	0	0
De Rotte et al. 2015 (MTX-R)	102	71	52 (16)	57	14	100	36	0	0
De Rotte et al. 2015 (treatment in Rotterdam)	285	70	54 (14)	62	94	100	14	0	0
Cross-sectional studies—RA patients									
Angelis-Stoforidis et al. 1999	61 (97)[d]	71	56.5 (11.3)	52.3	53.6	5.2	35.1	–	≥0.167[e]
Dervieux et al. 2004	108	70	65 (36–90)[a]	0	49	84	0	–	5.4 (0.25–22.2)[b]
Dervieux et al. 2005	225	73	66 (57–74)[a]	0	47	81	0	8.6 (4.2–17.9)[a]	4.25 (1.6–8.1)[a]
Stamp et al. 2010	192	73	60.5 (18–84)[b]	10.4	30.7	99.5	44.8	10.5 (0.25–53)[b]	3 (0.25–19)[b]
Ando et al. 2013	145	83	56.2 (31–78)[f]	–	64.1	73.1	–	–	≥0.25
Prospective cohort studies—JIA patients									
Bulatovic Calasan et al. 2015	113	68	12.1 (7.5–14.5)[a]	3.5	3.5	100	80.5	8.8 (3.8–12.3)[g]	0[h]
Cross-sectional studies—JIA patients									
Dolezalova et al. 2005	30	60	11.9 (4.1)	10.9	8.7	100	100	4.9 (3.4)	3.4 (2.3)
Becker et al. 2011	93	74	9.8 (4.7)	1	17.5	44.2	81.4	2.8 (1.1–5.3)[a]	1.5 (0.75–3.8)[a]

RCT randomised controlled trial, *RA* rheumatoid arthritis, *SD* standard deviation, *MTX* methotrexate, *CS* corticosteroids, *NSAIDs* non-steroidal anti-inflammatory drugs, *JIA* juvenile idiopathic arthritis, – not reported

[a] Median (IQR)

[b] Median (range)

[c] Median

[d] Indicates the number of assessable episodes from 61 patients where outcome data was available

[e] Participants were reported as having taken MTX for at least 2 months

[f] Mean (range)

[g] Age of onset of JIA (median (IQR))

[h] Patients who had previously ceased MTX but had ceased for more than 6 months but restarted due to relapse were included

3 months [14]. In the cross-sectional studies reported by Dervieux et al. (2004 and 2005), the estimated benefit with a doubling of RBC MTX_{Glu3} concentration in tender joint count (45 % (95 % CI 7 to 83 %) and 50 % (95 % CI 20 to 80 %), respectively), swollen joint count (48 % (95 % CI 2 to 94 %) and 18 % (95 % CI −3 to 38 %), respectively) and physician assessment of disease activity (30 % (95 % CI 11 to 48 %) and 29 % (95 % CI 16 to 43 %), respectively) were greater than with the prospective cohort studies [18, 19].

Toxicity

Four of the eight studies in which correlations between RBC MTX_{Glu-n} concentration and MTX toxicity were sought were prospective cohort studies (three in patients with RA and one in patients with JIA, Table 4) [13–15]. None of these studies reported a significant association between RBC MTX_{Glu-n} concentration and adverse effects. Specifically, the odds ratio in the R-MTX study describing the association between RBC $MTX_{Glu1–5}$ concentration and occurrence of toxicity in the first 3 months of treatment was 1.00 (95 % CI 0.99 to 1.01). They reported that the results were similar after 6 and 9 months of treatment and for all individual RBC MTX_{Glu-n}, and results in the treatment in Rotterdam cohort were also similar [13]. Stamp et al. (2011) reported the association between RBC MTX_{Glu-n} concentration and side effects as 'not significant', and in the trial including patients with JIA, Bulatovic Calasan et al. [14] reported that the odds ratio for the association between MTX intolerance and RBC $MTX_{Glu1–5}$ concentration was 0.99 (95 % CI 1.00 to 1.01, p=0.72).

Table 2 Risk of bias assessment of included studies

Study	Study Participation	Study Attrition	Prognostic Factor Measurement	Outcome Measurement	Study Confounding	Statistical Analysis and Reporting
Dervieux *et al* 2006						
Stamp *et al* 2011						
Hobl *et al* 2012						
de Rotte *et al* 2015 (MTX-R)						
de Rotte *et al* 2015 (Treatment in Rotterdam)						
Angelis-Stoforidis *et al* 1999		NA				
Dervieux *et al* 2004		NA				
Dervieux *et al* 2005		NA				
Stamp *et al* 2010		NA				
Ando *et al* 2013		NA				
Bulatovic Calasan *et al* 2015						
Dolezalova *et al* 2005		NA				
Becker *et al* 2011		NA				

Low	
Moderate	
High	
NA	Not applicable
NK	Unable to be determined

Two additional cross-sectional studies in RA patients did not identify a significant association with toxicity [10, 23]. One cross-sectional study in 30 JIA patients reported that the RBC $MTX_{Glu\text{-}total}$ concentration in those with and without gastrointestinal side effects was (mean±standard deviation) 340.7±329.7 versus 186.1±186.1 nmol/L (p=0.23) [22], whereas another study with 93 JIA patients found that those with gastrointestinal side effects (determined by symptoms at the time of taking the blood sample or by historical change in folic acid dose) had higher RBC $MTX_{Glu3–5}$ concentrations (mean±SD concentration in those with and without gastrointestinal intolerance was 159.2±134.4 vs 107±85.2 nmol/L, p=0.013) [21].

Seven studies analysed the association between hepatotoxicity and RBC $MTX_{Glu\text{-}n}$ concentration (four in patients with RA and three in patients with JIA) [10, 13–15, 21, 22]. The definition of hepatotoxicity was variable between studies. In patients with RA, two studies defined hepatotoxicity as alanine aminotransferase (ALT) three times the upper limit of normal [13], one as aspartate aminotransferase (AST) above the upper limit of normal [15] and another as liver function test abnormalities necessitating cessation or dosage change [10]. In studies that included patients with JIA, two studies defined hepatotoxicity as liver enzyme values above the normal range [21, 22], and the other as ALT and/or AST two times the upper limit of normal [14]. In the R-MTX study, the odds ratio describing the association with no hepatotoxicity was 0.92 (95 % CI 0.80 to 1.05), and results were said to be similar after 6 and 9 months, and the results in the treatment in Rotterdam study were described as comparable [13]. In only one of these studies, conducted in 93 JIA patients, the concentration of RBC $MTX_{Glu3–5}$ was higher in the 13 individuals with LFTs above the upper limit of normal (there were no elevations more than twice the upper limit of normal) compared to those with

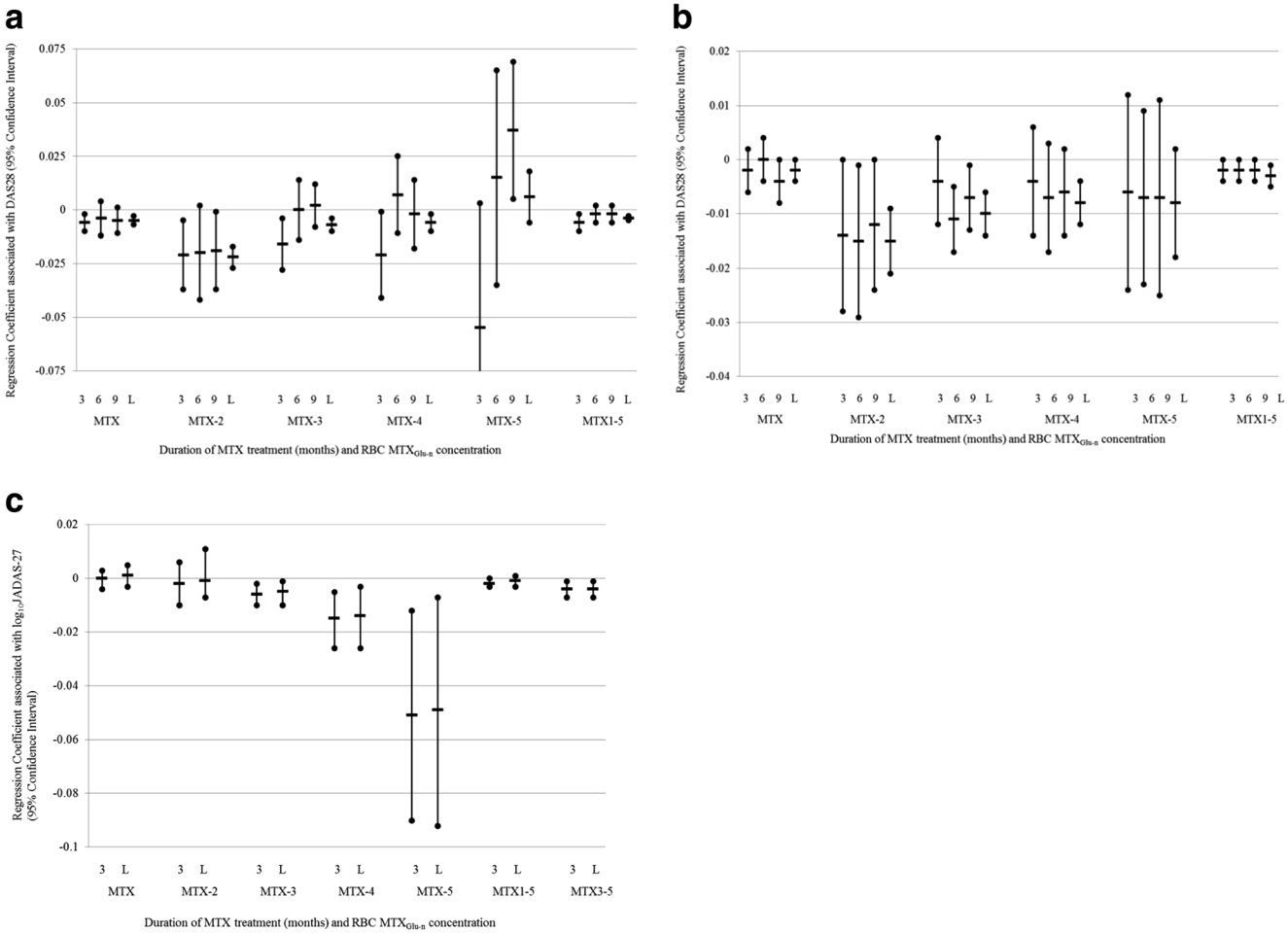


Fig. 2 Linear regression coefficients of the relationship between RBC $MTX_{Glu\text{-}n}$ concentrations and disease activity measures in identified prospective cohort studies. Results are presented as mean and 95 % confidence intervals (*L* longitudinal analysis). **a** Results from the R-MTX study. Regression coefficients are adjusted for age, gender, baseline DAS28, MTX dose, concurrent DMARDs (conventional and biological), NSAIDs and corticosteroids [13]. **b** The treatment in Rotterdam study. Regression coefficients are adjusted for age, gender, baseline DAS28, MTX dose, concurrent DMARDs (conventional and biological), NSAIDs and corticosteroids [13]. **c** The study by Bulatovic Calasan et al. in patients with JIA. Regression coefficients are adjusted for baseline JADAS-27, JIA sub-type and baseline NSAID use [14]

Table 3 Spearmans' rho coefficients associated with disease activity measures

Study	Time	RBC MTX_{Glu-n} concentration	Outcome measure	Spearmans' rho (*p* value)
Prospective cohort studies				
Hobl et al.	16 weeks	Maximum RBC MTX_{Glu2} concentration after 5 weeks of therapy	Change in DAS28	0.518 (0.023)
		Maximum RBC MTX_{Glu2} concentration after 10 weeks of therapy		0.475 (0.040)
Stamp (2011) et al.	24 weeks	RBC MTX_{Glu1}	DAS28	−0.06 (0.77)
		RBC MTX_{Glu2}		0.12 (0.55)
		RBC MTX_{Glu3}		−0.13 (0.52)
		RBC MTX_{Glu4}		−0.38 (0.056)
		RBC MTX_{Glu5}		−0.25 (0.035)
		RBC $MTX_{Glu1–5}$		−0.32 (0.11)
		RBC $MTX_{Glu3–5}$		−0.42 (0.032)
Cross-sectional studies				
Stamp (2010) et al.	NA	RBC MTX_{Glu1}	DAS28	0.01 (>0.05)
		RBC MTX_{Glu2}		0.09 (>0.05)
		RBC MTX_{Glu3}		0.15 (<0.05)
		RBC MTX_{Glu4}		0.17 (<0.05)
		RBC MTX_{Glu5}		0.21 (<0.01)
		RBC $MTX_{Glu1–5}$		0.19 (<0.01)
		RBC $MTX_{Glu3–5}$		0.19 (<0.01)

normal LFTs (mean±standard deviation 173±162.9 vs 111.8 ±85.5 nmol/L, p=0.03). Of note, Bulatovic Calasan et al. showed a positive trend for association between hepatotoxicity (ALT and/or AST two times the upper limit of normal) and RBC $MTX_{Glu-total}$ concentration (OR=1.02, 95 % CI 1.00 to 1.04, p=0.08) in 113 JIA patients [14]. The remaining two studies reported the relationship with hepatotoxicity as 'not statistically significant'—no quantitative estimate of the effect size was available [10, 15].

Discussion

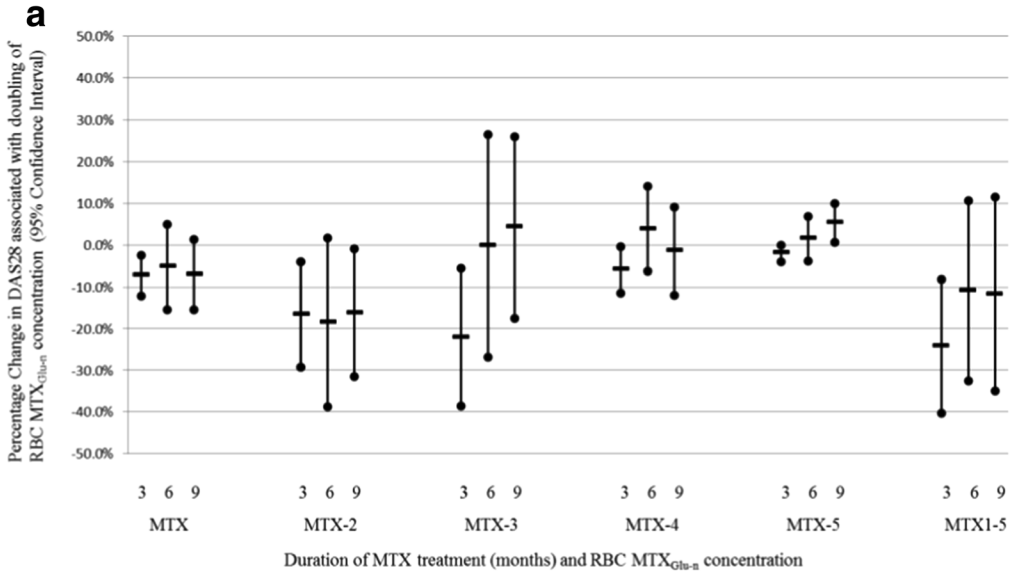
Identified studies that investigated the association between RBC MTX_{Glu-n} concentration and disease activity in inflammatory arthropathies were heterogeneous with respect to study design, concurrent treatments, duration of disease and MTX treatment at enrolment, the RBC MTX_{Glu-n} concentration measured (and reported), statistical methods and tools used to measure response/disease activity. Linear regression estimates suggested that doubling RBC MTX_{Glu-n} concentration may lead to potentially worthwhile reductions in disease activity, but since oral MTX bioavailability reduces with higher doses, doubling RBC MTX_{Glu-n} concentrations may require even greater increases in oral doses or a greater reliance on parenteral administration [24].

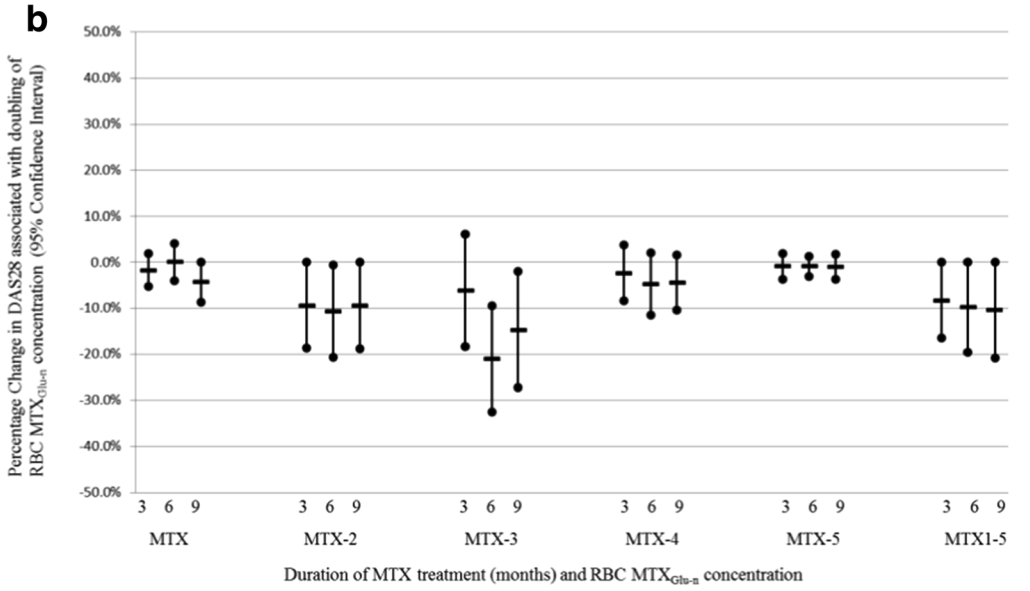
The strengths of this systematic review are the broad inclusion criteria and overall search strategy (including searches of clinical trial registries to minimise reporting bias) and the attempt to synthesise the magnitude of benefit that could be expected with concentration guided dosing. Regardless, reporting bias could not be avoided, and it is still possible that the search strategy did not detect all of the relevant reports.

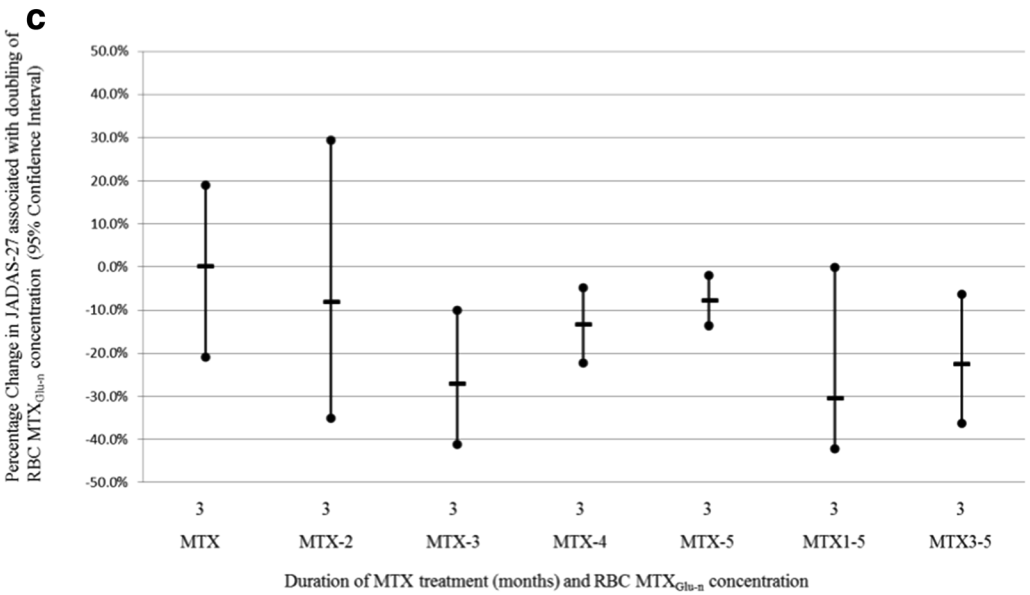
Weaknesses include the heterogeneity in the disease activity scales, methods of analysis and reporting of RBC MTX_{Glu-n} concentrations within the included studies, and as such, we were unable to provide simple and/or consistent assessments (such as regression coefficients with 95 % confidence intervals) of the outcome of each trial. We also chose to use the QuIPS tool to determine the risk of bias for the included studies, whereas others may prefer to use (either instead of or in addition to) a tool for therapeutic studies such as the GRADE criteria [25].

The better quality cohort studies tended to find positive relationships between RBC MTX_{Glu-n} concentration and disease activity, and the results from the cross-sectional studies were more variable. Robust historical data are necessary for

Fig. 3 Percentage change in disease activity scores predicted from a doubling of RBC MTX_{Glu-n} concentrations. Results are presented as mean and 95 % confidence intervals. **a** R-MTX study [13]. **b** The treatment in Rotterdam study [13]. **c** The study by Bulatovic Calasan et al. in patients with JIA [14]


a
Percentage Change in DAS28 associated with doubling of RBC MTX_{Glu-n} concentration (95% Confidence Interval)
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
-10.0%
-20.0%
-30.0%
-40.0%
-50.0%
3 6 9
MTX
MTX-2
MTX-3
MTX-4
MTX-5
MTX1-5
Duration of MTX treatment (months) and RBC MTX_{Glu-n} concentration


b
Percentage Change in DAS28 associated with doubling of RBC MTX_{Glu-n} concentration (95% Confidence Interval)
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
-10.0%
-20.0%
-30.0%
-40.0%
-50.0%
3 6 9
MTX
MTX-2
MTX-3
MTX-4
MTX-5
MTX1-5
Duration of MTX treatment (months) and RBC MTX_{Glu-n} concentration


c
Percentage Change in JADAS-27 associated with doubling of RBC MTX_{Glu-n} concentration (95% Confidence Interval)
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
-10.0%
-20.0%
-30.0%
-40.0%
-50.0%
3
MTX
MTX-2
MTX-3
MTX-4
MTX-5
MTX1-5
MTX3-5
Duration of MTX treatment (months) and RBC MTX_{Glu-n} concentration

these cross-sectional studies, as baseline disease activity is a significant predictor of future disease activity scores [26]. Another limitation of the cross-sectional design may be the practice of up-titrating MTX dose until either the desired response is achieved or until toxicity develops. This practice will result in individuals with more responsive disease receiving less drug (and hence having lower concentrations), and those with less responsive disease or inherent resistance to MTX may be taking higher doses (and have higher concentrations). The degree of dose titration prior to study entry may therefore impact significantly upon findings, such that a negative correlation between RBC MTX_{Glu-n} concentration and disease activity, as seen by Stamp et al. [23], may be a likely outcome. The negative findings in two cross-sectional studies should therefore be considered in this light, and not detract from the more positive results from the cohort studies.

To define the potential value of RBC MTX_{Glu-n} concentration, the most important and/or relevant individual or group of RBC MTX_{Glu-n} derivative(s) should be identified. In this review, there was substantial heterogeneity of the RBC MTX_{Glu-n} species that were associated with disease activity, and this represents a significant limitation in the published literature and represents a significant barrier to routine implementation of RBC MTX_{Glu-n} guided dosing. The potential to assess correlations of response and/or toxicity with a large number of single or groups of RBC MTX_{Glu-n} derivatives over multiple time points introduces the potential for error due to multiple hypothesis testing and reporting bias. The total and relative concentration of each RBC MTX_{Glu-n} derivative depends on MTX dose, route and duration of administration and genetic polymorphisms in enzymes involved in the polyglutamation process [4, 17, 18, 27, 28]. The RBC MTX_{Glu-n} derivative most associated with future disease activity may depend upon these variables and could even be dynamic over time, and these relationships need to be clarified via future research.

A number of other factors that can influence response and toxicity following MTX administration were either not captured or were variably reported in the included studies, including prior and concomitant DMARDs, use of corticosteroids, duration of disease and disease activity and presence of rheumatoid factor, anti-cyclic citrullinated peptide antibodies and/or shared epitope [26]. Standardisation and/or correction for these factors are essential for understanding the relationship between RBC MTX_{Glu-n} concentrations and response to MTX across the spectrum of uses in RA and JIA populations.

Table 4 Characteristics of studies assessing association with toxicity

Prospective cohort studies—RA patients					
Author name, date	*N*	Incidence of MTX toxicity (%)			
		GI	Hepatic	CNS	Total
Dervieux et al. 2006	48	30[b]	2[b]	28[b]	45[b]
Stamp et al. 2011	30	–	–	–	–
De Rotte et al. 2015 (MTX-R)	102	31[c]	4[c]	27[c]	85[c]
De Rotte et al. 2015 (treatment in Rotterdam)	285	[a]	[a]	[a]	[a]
Cross-sectional studies—RA patients					
Angelis-Stoforidis et al. 1999	61 (97)[d]	21	20	0	41
Stamp et al. 2010	192	42	–	61	73
Prospective cohort studies—JIA patients					
Bulatovic Calasan et al. 2015	113	–	5.3	–	50.6[e]
Cross-sectional studies—JIA patients					
Dolezalova et al. 2005	30	20	0	0	20
Becker et al. 2011	93	25	14	–	–

RA rheumatoid arthritis, *MTX* methotrexate, *GI* gastrointestinal, *CNS* central nervous system, *JIA* juvenile idiopathic arthritis, – not reported

CNS toxicity includes fatigue, loss of concentration, headache, dizziness, blurred vision, sleep disturbance or weepiness; GI toxicity includes nausea, vomiting, diarrhoea, mouth ulcers/stomatitis, dyspepsia or decreased appetite

[a] Not specifically stated, but said to be similar percentages to the MTX-R cohort

[b] Mean incidence per 4–6 weeks over a 6.9-month follow-up period

[c] Cumulative incidence after 9 months of treatment

[d] Indicates the number of assessable episodes from 61 patients where outcome data was available

[e] As determined by an MTX Intolerance Severity Score of ≥6

The overall evidence suggests that RBC MTX_{Glu-n} concentration is unlikely to be a useful predictor of MTX toxicity. The rate of serious toxicity (e.g. hepatotoxicity, haematological toxicity and pneumonitis) is relatively low, and permanent discontinuation occurs in about 10 % of individuals [29]; so, very large patient numbers would be required to identify concentration-toxicity relationships. Given that hepatic fibrosis and cirrhosis appear to be more common with higher cumulative MTX doses [30], it is reasonable to speculate that these serious hepatic toxicities would be related to long-term exposure of high RBC MTX_{Glu-n} concentrations. However, most of the studies included in this review did not include either adequate patient numbers or sufficiently long-term follow-up to assess this association.

Modest liver enzyme elevations (i.e. up to 3 times the upper limit of normal) secondary to MTX are relatively common in patients with RA, but their association with hepatic fibrosis/cirrhosis is unclear, and elevated liver enzymes necessitate cessation in <10 % of patients and generally resolve spontaneously without MTX dose adjustment [30]. Although data in patients with JIA are less abundant, liver function test abnormalities in these patients appear to be less common [31, 32]. There were little data identified to support an association between RBC MTX_{Glu-n} concentration and elevated liver enzymes in RA patients, but in those with JIA, we found one study with a significant association [21] and another with a non-significant trend [14]. This may be noteworthy, and further evaluation of this issue in JIA patients appears to be justified.

More common but less serious toxicities such as nausea and fatigue are subjective, and since cross-sectional studies will tend to select for 'tolerant' individuals, these should be assessed prospectively. It should also be recognised that RBC MTX_{Glu-n} concentration may not be a useful marker for some or all MTX-induced toxicities. For example, gastro-intestinal toxicity tends to occur in 24 h after MTX administration. This temporal association and the putative mechanism of nausea and vomiting being direct interaction of plasma MTX with the chemoreceptor trigger zone suggest that RBC MTX_{Glu-n} concentration is not likely to be a predictor of this adverse effect. Similarly, mechanisms of uptake (and efflux) into/from blood cells versus hepatocytes may differ, and there is no evidence to suggest that intracellular concentrations in different cell types are correlated. A relationship between RBC MTX_{Glu-n} concentration and various toxicities would therefore appear to be difficult to establish, and it appears unlikely that RBC MTX_{Glu-n} concentration will be more useful than traditional markers or predictors (e.g. MTX dose and liver function tests) of toxicity.

To demonstrate clinical applicability, it will be essential to demonstrate a clear benefit for the measurement of RBC MTX_{Glu-n} concentration as a biomarker over and above that achieved with 'conventional' predictors of efficacy. Furthermore, how this assay aligns with current treatment strategies, such as 'treat-to-target', will need to be defined since none of the studies identified compared MTX dosing to a targeted RBC MTX_{Glu-n} concentration with standard MTX dosing, and thus, no direct evidence was identified to support the clinical utility of such an approach.

Additional prospective studies in MTX naïve early RA or JIA patients are required to clarify RBC MTX_{Glu-n} concentration-response and concentration-toxicity relationships (including the best MTX_{Glu-n} derivative(s) to monitor). This systematic review has demonstrated that inconsistent and incomplete reporting has hampered overall interpretation of the data. In the future, results should be presented numerically as point estimates with associated uncertainty (either confidence intervals or standard errors), rather than p values and/or as non-significant. Furthermore, results should include univariate and multivariate analysis where the association is corrected for known confounders, and the relationship between each RBC MTX_{Glu-n} metabolite and efficacy and/or toxicity should be reported so that meta-analysis is facilitated. Response would ideally be measured by accepted composite measures of disease activity such as JADAS, DAS and/or ACR/EULAR response criteria [33]. Supplementary and on-line data repositories that are available for many journals should be used to present all of this data so that it can be evaluated comprehensively. Such data may form the basis for an RCT comparing the effect of pharmacokinetically informed and conventional dosing of MTX on short- and long-term outcomes in RA patients. Finally, current studies have assumed a linear relationship between RBC MTX_{Glu-n} and outcome, but drug concentration and drug toxicity more typically follow non-linear (sigmoidal) relationships. Investigation of these potentially informative non-linear relationships would be a useful advance in future studies.

In conclusion, there is some encouraging evidence of a relationship between the concentrations of various species of RBC MTX_{Glu-n} and response to MTX, but the derivative(s) that should be monitored are unclear and need to be established prior to routine implementation of RBC MTX_{Glu-n} concentration guided dosing. Relatively large increases in concentration appear to be required to achieve significant reductions in disease activity, and the place of RBC MTX_{Glu-n} concentration guided dosing in the era or treat to target therapy with combination DMARDs must be established.

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