



## The importance of inhibition of a catabolic pathway of methotrexate metabolism in its efficacy for rheumatoid arthritis

Sarah L. Morgan<sup>\*</sup>, Joseph E. Baggott

Division of Clinical Immunology and Rheumatology, Department of Medicine, School of Medicine, The University of Alabama at Birmingham, Birmingham, AL, United States



### ABSTRACT

Methotrexate (MTX), an antifolate, is the anchor drug for the treatment of rheumatoid arthritis (RA). It is inexpensive, effective, and generally safe. When clinical response is inadequate, biological therapies are commonly used in combination with MTX. However, biological agents have safety concerns (i.e. infections, malignancy) and the addition of a biologic agent is expensive, making strategies to improve MTX efficacy important.

Inhibition of pathways of folate metabolism involving purine metabolism by MTX, have been traditionally emphasized as important in MTX efficacy. However, inhibition MTX catabolism may also be important. MTX is irreversibly hydroxylated to form 7-hydroxy methotrexate (7-OH-MTX) by aldehyde oxidase (EC 1.2.3.1) (AOX). Catabolism of MTX to 7-OH-MTX is the first metabolic process imposed on an oral dose of MTX and will alter subsequent interactions of MTX with other enzymes. 7-OH-MTX is less potent than MTX in the treatment of rat adjuvant arthritis. RA patients with a low capacity to catabolize MTX to 7-OH-MTX do better clinically than individuals who are rapid formers of 7-OH-MTX. Therefore, altering the catabolism of MTX may be an innovative way to improve MTX efficacy.

Raloxifene is a FDA-approved therapy for postmenopausal osteoporosis and for the reduction of invasive breast cancers but has no known activity in RA. Raloxifene is a potent inhibitor of human liver AOX. Postmenopausal women with RA frequently have low bone mineral density and would be candidates for raloxifene and MTX combination therapy. The effect of raloxifene on MTX metabolism has never been studied.

Our hypothesis is that in postmenopausal women with RA and osteoporosis treated with MTX and raloxifene, the inhibition of AOX with resultant decreased formation of 7-OH MTX; will increase MTX levels and improve MTX efficacy. This hypothesis could be studied in an open-label, proof of concept clinical study in individuals before and after the addition of raloxifene. Red blood cell MTX and 7-OH-MTX levels and RA disease activity (DAS28) would be measured. In possible future studies, there are dietary substances, as supplements, (e.g. epigallocatechin gallate in green tea and resveratrol) which inhibit human liver AOX which could be evaluated.

### Introduction/background

Rheumatoid arthritis (RA) is a chronic inflammatory arthritis that affects ~1% of the US population [1]. Because of the significant morbidity and mortality of RA, effective treatment regimens are needed [2,3]. Methotrexate (MTX) has become the most commonly prescribed disease-modifying antirheumatic drug (DMARD) therapy worldwide for RA and the gold standard to which other DMARD therapies are compared [4–6]. MTX is inexpensive, has long-term effectiveness as a sole therapy, and is useful in early, poor-prognosis RA [7]. When clinical response is inadequate, MTX is sometimes combined with other DMARDs [8–10]. Biological therapies, such as anti-tumor necrosis factor  $\alpha$  therapies, are commonly used in combination with MTX [11–16]. However, biological agents have safety issues (e.g. infections and malignancy) [17–20] and the addition of a biologic agent will greatly increase treatment costs [21]. If MTX could be made more efficacious, that could obviate the need for more expensive therapies.

Inhibition of pathways of folate metabolism involving purine metabolism, have been traditionally emphasized as important in MTX efficacy [22–26]. However, inhibition of MTX catabolism may also be important in MTX efficacy. Aldehyde oxidase (E.C. 1.2.3.1) (AOX) irreversibly converts MTX to 7-hydroxymethotrexate (7-OH-MTX) in the catabolism of MTX (Fig. 1). AOX is an iron, molybdopterin and riboflavin-dependent enzyme, therefore iron and riboflavin status can affect its activity [27]. Once hydroxylated, MTX is metabolically trapped as 7-OH-MTX and it is the major catabolite of MTX in tissues, accounting for 30–100% of all MTX equivalents [28].

7-OH-MTX has been shown to be one-eighth as efficacious as MTX in the Lewis rat adjuvant arthritis model [29]. Patients with RA who excrete less 7-OH-MTX after a MTX dose have a better clinical response [26]. It has also been demonstrated that larger areas under the curve for plasma MTX are associated with better efficacy [30], suggesting that the increased catabolism of MTX to 7-OH MTX, which would decrease the area under the curve for MTX, may be an important determinant of

<sup>\*</sup> Corresponding author at: Division of Clinical Immunology and Rheumatology, School of Medicine, UAB, 830 Faculty Office Tower, 510 20th Street South, Birmingham, AL 35294-3408, United States.

E-mail address: [slmorgan@uab.edu](mailto:slmorgan@uab.edu) (S.L. Morgan).

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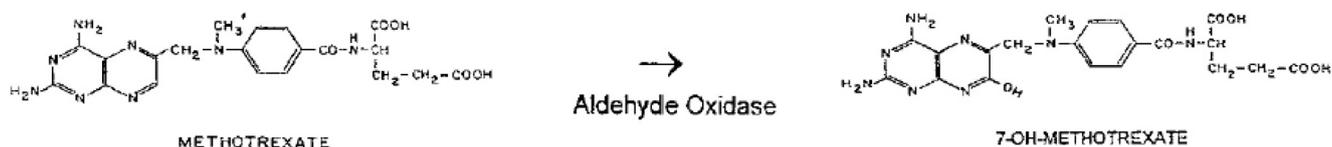


Fig. 1. Metabolism of MTX to 7-OH MTX by aldehyde oxidase (E.C. 1.2.3.1).

lowered efficacy in RA therapy. There is a positive dose response with increasing MTX dose in RA [31,32], so that increasing MTX levels in the plasma (by lowering 7-OH MTX formation) could improve efficacy.

The liver and intestinal mucosa are involved in the catabolism of MTX to 7-OH-MTX [33]. The bioavailability of an oral dose of MTX is approximately 15–20% lower than intramuscular (IM) or intravenous (IV) administration of MTX [34–36], which will affect efficacy [37,38]. The improved efficacy of parenteral vs. oral MTX [39–43], where the first pass through the liver is eliminated is also suggestive that MTX catabolism is important in clinical inefficacy in RA.

It may be possible to combine drugs that inhibit AOX with MTX, thereby potentiating the efficacy of MTX. Raloxifene, which is FDA-approved for osteoporosis treatment, is a potent AOX inhibitor and could presumably be used in combination with MTX therapy to potentiate MTX efficacy [27,44]. Osteoporosis is the third most common comorbidity in RA [45] and that bone mineral density is often low in RA [46], the addition of raloxifene to the treatment regimen in a postmenopausal woman taking MTX, who has low bone mineral density, is reasonable and may be beneficial and the combination is likely already used clinically. Raloxifene alone has no efficacy in RA [47,48]. In humans oral raloxifene will be extensively glucuronidated by intestinal and liver enzymes with  $K_m$ 's of  $10^{-6}$  to  $10^{-5}$  M [49], such that systemic blood concentrations of the parent drug will be only 1.4 to  $6.5 \times 10^{-9}$  M one to 2-h after a 60 mg dose [50,51]. However, the  $K_i$  of raloxifene for AOX is 0.9 to  $2.3 \times 10^{-9}$  M, indicating that it will be preferentially bound to liver and intestinal AOX and will inhibit the enzyme [52]. In addition, liver glucuronidases reform raloxifene from its glucuronide metabolites [50]. Both raloxifene and MTX undergo extensive enterohepatic cycling, repeatedly exposing them to AOX [50].

The combinations of MTX with leflunomide, cyclosporine A (CSA), or hydroxychloroquine have been shown to be more effective than either drug alone in the treatment of RA [7,53–59]. It is assumed that the combination of MTX and other drugs act synergistically; but it may be that these drug combinations also block the conversion of MTX to 7-OH-MTX, thereby increasing the plasma concentration of MTX. For example, it is known that azathioprine and 6-mercaptopurine (a metabolite of azathioprine) are substrates for rabbit liver AOX [60]. When azathioprine and 6-mercaptopurine are acted upon by the oxidase, these drugs would exclude MTX from the active site and spare it from oxidation to 7-OH-MTX. A pharmacokinetic study combining MTX and CSA indicated that plasma MTX levels were moderately elevated and 7-OH-MTX levels substantially diminished compared to MTX alone [61]. This finding suggests that one of the effects of CSA or its metabolites, when combined with MTX, may be to inhibit the activity of AOX.

It may be possible to combine other drugs or nutritional supplements that are inhibitors of AOX with MTX, thereby potentiating the clinical efficacy of MTX. There are dietary substances (available as supplements) that are inhibitors of AOX, such as epigallocatechin gallate (found in green tea) and resveratrol (found in grape skin, wine, grape juice, peanuts, cocoa and blueberries and cranberries) (Inhibition constants [ $K_i$ ] 0.34 to  $4.8 \times 10^{-6}$  M) that also could ultimately be considered in combination with MTX to improve efficacy [62]. These  $K_i$ 's are similar to blood levels of these compounds and they are available as supplements [62].

### Hypothesis/theory

Our hypothesis is that the combination of MTX and an AOX

inhibitor, such as raloxifene may be beneficial in the treatment of RA, compared to MTX alone because of less formation of 7-OH-MTX which would allow more MTX to flow through other pathways of MTX metabolism. This hypothesis is different from current thinking which has postulated only the importance of adenosine trapping and inhibition of enzymes involved in purine biosynthesis as important [22–26]. This hypothesis evolved through data collected in our trials related to folic acid supplementation in lowering low dose MTX toxicity in RA. This is an important hypothesis because it presents an alternative biochemical target for increasing MTX efficacy in RA.

### Evaluation of the hypothesis/idea and empirical data

We have evaluated our hypothesis based upon preliminary data in rats with adjuvant arthritis and humans with RA.

#### Efficacy of 7-OH MTX in Lewis rat adjuvant arthritis

The dose-dependent effects of subcutaneously administered MTX and 7-OH-MTX were studied in the Lewis rat adjuvant arthritis model [29]. Using differences in mean erythema and edema scores in the model, MTX was found to be 8-fold more efficacious than 7-OH-MTX in preventing paw disease activity. This would suggest that prevention of formation of 7-OH-MTX may be important in arthritis therapy.

#### Pharmacokinetic study of MTX and CSA therapy

A study evaluating the effect of CSA on MTX pharmacokinetics was completed. CSA, when given in combination with MTX, blocks 80% of the metabolism of MTX to 7-OH-MTX [61]. Figs. 2 and 3 display the mean plasma MTX and 7-OH-MTX concentrations after a dose of MTX

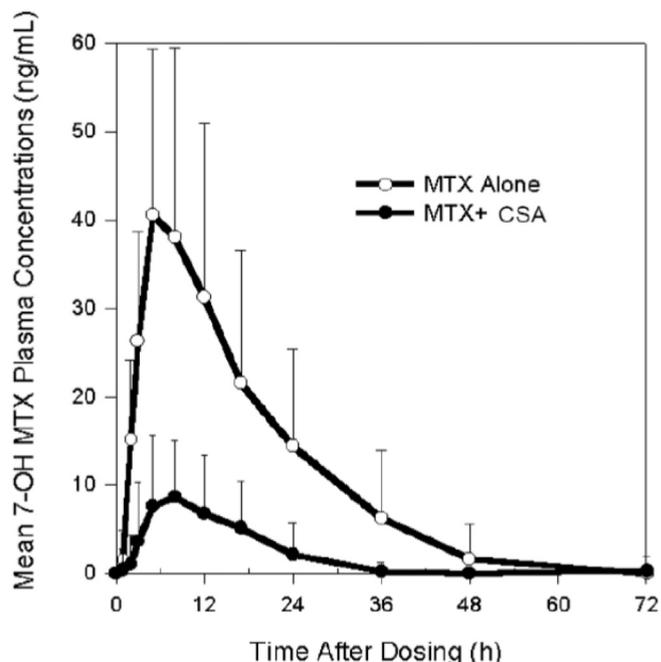


Fig. 2. Plasma 7-OH-MTX and MTX concentrations after an oral dose of MTX alone or MTX plus CSA.

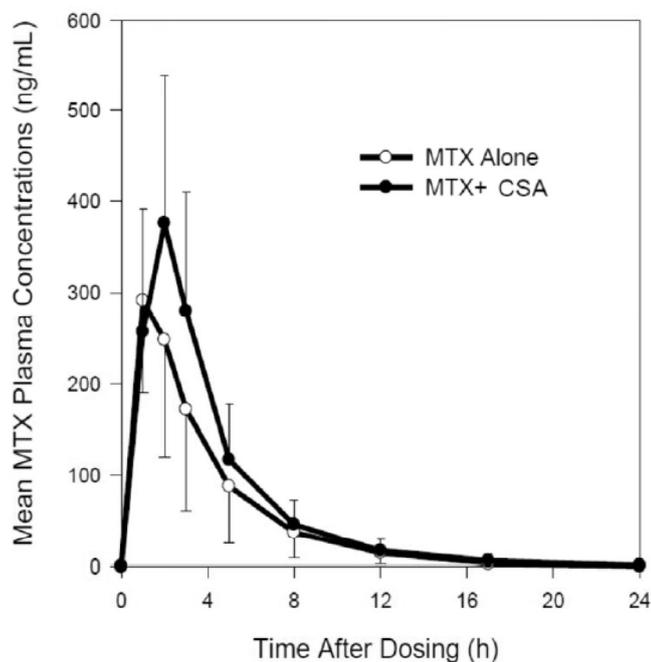


Fig. 3. Plasma 7-OH-MTX and MTX concentrations after an oral dose of MTX alone or MTX plus CSA.

or MTX plus CSA. CSA, or its metabolites, when given with MTX, blocks the metabolism of MTX to 7-OH-MTX; raising the possibility that adding CSA to MTX therapy lowers 7-OH formation and potentiates MTX efficacy.

#### Urinary pharmacokinetics data of MTX plus CSA

A subset within the study above of 13 patients was selected who took 10 mg of MTX with or without CSA. The amount of MTX or 7-OH-MTX in a 72-h urine is shown in Table 1 [61]. There was no statistically significant difference in the excretion of MTX when CSA was added. In contrast, the excretion of 7-OH-MTX decreased to 13% of controls when CSA was included. Therefore, CSA lowers the catabolism of MTX to 7-OH-MTX.

#### Variability in formation of 7-OH-MTX in RA patients on MTX

An analysis of 29 patients with RA on MTX, in which 72-h urinary MTX and 7-OH-MTX concentrations were measured, suggested that there are two phenotypes in the catabolism of MTX to 7-OH-MTX [63]. Fig. 4 shows the distribution of the percent of the oral dose of MTX excreted in urine as 7-OH-MTX in 72 h. The distribution is bimodal with high metabolizers of MTX excreting  $\geq 9\%$  of the dose and low metabolizers excreting  $\leq 5\%$  of the dose. Mean doses were not statistically different (11 to 13 mg) in the high and low metabolizers, respectively [63]. This data suggests that there is variability in the ability to form 7-OH-MTX in RA patients, raising the possibility that this variability is important in explaining the response to MTX.

Table 1

Total amount of MTX and 7-OH MTX in a 72-h urine in 13 RA Patients on MTX (10 mg/week).

Study Arm	Mean MTX excretion (mg) $\pm$ SEM	Mean 7-OH-MTX excretion (mg) $\pm$ SEM
MTX alone	8.37 <sup>1</sup> $\pm$ 0.71	0.69 <sup>2</sup> $\pm$ 0.13
MTX + CSA	7.89 <sup>1</sup> $\pm$ 0.48	0.09 <sup>2</sup> $\pm$ 0.03

<sup>1</sup>  $p > 0.4$ ; paired  $t$ -test, two-tailed, <sup>2</sup>  $p < 0.001$ ; paired  $t$ -test, two-tailed.

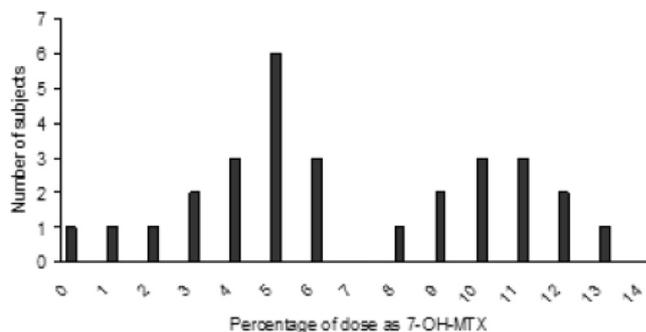


Fig. 4. Distribution of the percentage of the MTX dose excreted in a 72-h urine as 7-OH-MTX in 29 patients with RA.

#### Effect of folic acid and folinic acid on MTX and 7-OH-MTX levels at 6 weeks

Another protocol evaluated urine for aminoimidazole-carboxamide and adenosine levels as markers for MTX interference with purine metabolism [26]. Forty subjects with RA who had not received MTX were admitted to the General Clinical Research Center for patient and physician assessment of pain, the modified Health Assessment Questionnaire, joint counts and scores for pain and tenderness and physician and patient visual analog scales for pain and assessment of disease activity. Weekly MTX therapy was started. On the day that the patient was to take their 6th dose of MTX (median dose 10 mg/week), the patient was readmitted and a 24-h urine was begun at the time of taking the dose of MTX. For the 6th week, the patient was randomized to either 5 mg of folic acid or 5 mg of folinic acid per day.

The percentage of the dose excreted as 7-OH-MTX in a 24-h urine varied from 1% to 7.33% in 39 subjects after 6 weeks of MTX therapy [26]. The fact that urinary excretion was only up to 7% does not mean that 7-hydroxylation is a minor pathway. In patients treated with low dose MTX, 30–100% of the MTX equivalents in bone marrow are in the 7-OH-MTX form and 7-OH-MTX, like MTX, is polyglutamylated [28]. Bone marrow is the target tissue for immunosuppressive action by MTX. Thus, urinary 7-OH-MTX is a marker for tissue levels, which will be higher because they include polyglutamates of 7-OH-MTX. The distribution of the data is shown in Fig. 5. There was no statistical difference in the mean dose of the high capacity catabolizers ( $> 3.6\%$ , mean dose  $9.3 \pm 2.2$  mg,  $n = 11$ ) and the low capacity catabolizers ( $< 1.8\%$ , mean dose  $8.8 \pm 2.2$  mg,  $n = 12$ ). These results are similar to those reported before in 29 patients with RA where a 72-h urine was collected, except that the data was shifted to lower percentages in the 24 h urines, because not all of the 7-OH-MTX to be excreted was produced in 24 h. The data were expressed as mg 7-OH-MTX/g creatinine to correct for body (organ) mass. A similar distribution of data is shown in Fig. 6 where 7-OH-MTX excretion is expressed as mg 7-OH-MTX/g creatinine. Therefore, body (organ) mass does not determine the catabolism of MTX to 7-OH-MTX. Race and gender were also equally distributed among the high and low capacity catabolizers. However, as shown in Table 2, those patients who excreted less 7-OH-MTX tended to have a better response to MTX, providing important preliminary data for our first hypothesis.

In summary, we have data which evaluates the importance of MTX catabolism in MTX efficacy and that 7-OH MTX is less efficacious than MTX in RA therapy.

In conclusion, we hypothesize the inhibition of a catabolism of MTX metabolism, the formation of 7-OH-MTX from MTX, is important in modulating the efficacy of MTX therapy in RA.

#### Consequences of the hypothesis and discussion

Since hydroxylation reactions function to detoxify xenobiotic compounds, AOX serves an important metabolic and homeostatic role.

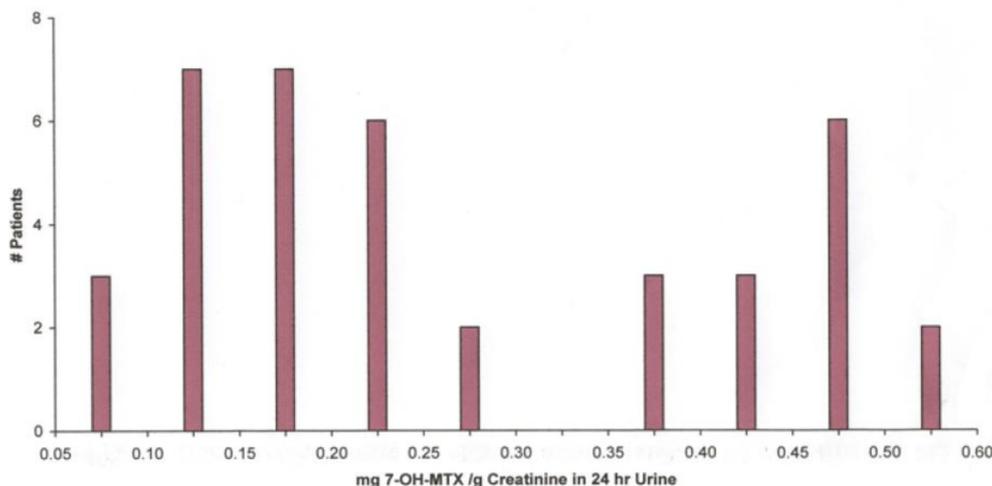


Fig. 5. Distribution of the % of the Dose Excreted as 7-OH-MTX in 24-h Urine Samples at Visit 2 (6-weeks) in Patients with RA taking MTX (N = 39).

Therefore substantially blocking this enzyme may increase the toxicity of all levels of MTX dosing. Because of the very high levels of AOX in the liver, the rabbit is remarkably resistant to MTX toxicity [60]. Thus, the extent to which AOX is inhibited may parallel increases in MTX toxicity and this should be carefully clinically evaluated.

There may be side effects of the combination which would also need to be evaluated in the protocol. By increasing intracellular MTX concentrations, it is possible that pulmonary, hepatic, and hematological toxicity may increase (which may or may not be affected by folic acid supplementation). In addition, when raloxifene was prescribed to postmenopausal women with coronary heart disease or multiple risk factors for coronary heart disease, there was no difference in death from any cause or total stroke between the raloxifene and placebo groups, but there was an increased risk of fatal stroke and venous thromboembolism between the raloxifene and placebo groups [64]. A further subgroup analysis of this trial confirmed the pattern of increased fatal stroke and venous thromboembolic events and confirmed that the risk of stroke differed by smoking status [65]. Since individuals with RA have a higher risk of cardiovascular disease [66], it will be prudent to avoid patients who smoke and who have a history of stroke or venous thromboembolism and carefully monitor for toxic side effects, including

Table 2

Joint Improvements in Patients is Dependent Upon Mean % of MTX Dose Excreted as 7-OH-MTX.<sup>1,2</sup>

Category of Joint Improvement	N	Mean % of MTX Dose Excreted as 7-OH-MTX (S.D.)
Marked Improvement	9	1.95 (1.48) <sup>1</sup>
Moderate Improvement	18	3.15 (1.30)
No Change	8	3.29 (1.34)

<sup>1</sup> Significantly different from other categories by ANOVA, F = 5.4, p ≅ 0.01, data log transformed before analysis, followed by Scheffe’s test (P < 0.05)

<sup>2</sup> Marked improvement: greater than 50% decrease in swelling (SW) and pain/tenderness (PT) indices at both visit 2 (6 weeks) and visit 3 (7 weeks); no change: less than 30% change in SW and PT indices at both visit 2 and 3; Moderate improvement: all other patients. No patients were in the category of worsening.

cardiovascular disease, between placebo and raloxifene groups. Until the biochemical effects of raloxifene on methotrexate metabolism can be confirmed, it is not possible to speculate the length of such combination therapy, however, most osteoporosis practitioners stop raloxifene in women over 70 because of a rising incidence of venous

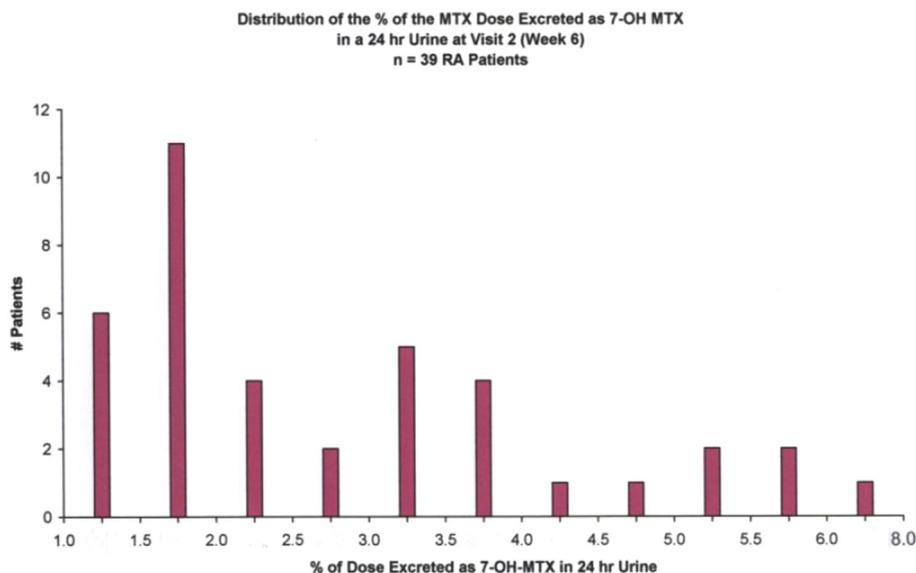


Fig. 6. Distribution of mg 7-OH-MTX/g creatinine in a 24-h urine at Visit 2 (6 weeks) (N = 39).

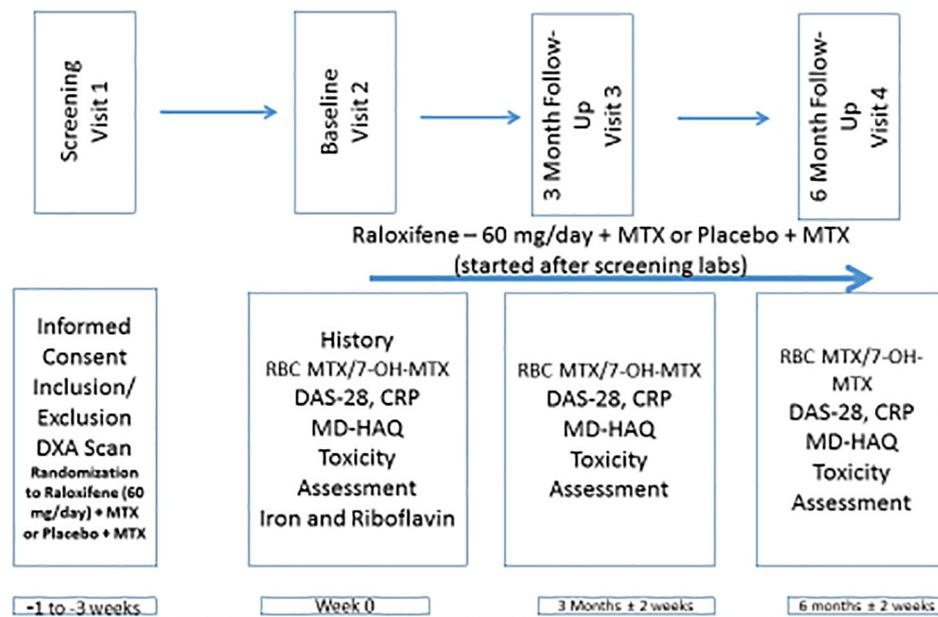


Fig. 7. Synopsis of a Possible Clinical Study DAS-28 is a disease activity score which measures disease activity in RA. MD-HAQ is a multidimensional health assessment questionnaire for RA.

thromboembolic events after the age of 70 [67].

We believe that the combination of raloxifene and MTX may be more efficacious than MTX alone in the treatment of RA. If so, this would be the first data documenting the importance of MTX catabolism which could affect the efficacy of low-dose MTX in RA. The importance of this hypothesis, if true, is that it would improve the efficacy of a well-tolerated, inexpensive drug for RA. If this hypothesis is true, it is likely that other AOX inhibitors, such as epigallocatechin gallate in green tea and resveratrol might also be used in combination with MTX.

We believe that this hypothesis could be tested in a double-blind, placebo-controlled study. A possible study design is outline in Fig. 7. In development of the study design it will be important to also evaluate if increasing MTX levels affect toxicity. In addition, the development of a clinical assay to evaluate capacity to form 7-OH-MTX would be a useful clinical addition.

In summary altering the catabolism of MTX may be an innovative way to improve MTX efficacy.

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## Conflict of interest

Neither of the authors has any conflicts of interest to report

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