ORIGINAL ARTICLE

Genetic variants associated with methotrexate efficacy and toxicity in early rheumatoid arthritis: results from the treatment of early aggressive rheumatoid arthritis trial

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Methotrexate (MTX) has emerged as first-line therapy for early moderate-to-severe rheumatoid arthritis (RA), but individual variation in treatment response remains unexplained. We tested the associations between 863 known pharmacogenetic variants and MTX response in 471 Treatment of Early Aggressive Rheumatoid Arthritis Trial participants with early RA. Efficacy and toxicity were modeled using multiple regression, adjusted for demographic and clinical covariates. Penalized regression models were used to test joint associations of markers and/or covariates with the outcomes. The strongest genetic associations with efficacy were in CHST11 (five markers with \( P < 0.003 \), encoding carbohydrate (chondroitin 4) sulfotransferase 11. Top markers associated with MTX toxicity were in the cytochrome p450 genes CYP20A1 and CYP39A1, solute carrier genes SLC22A2 and SLC7A7, and the mitochondrial aldehyde dehydrogenase gene ALDH2. The selected markers explained a consistently higher proportion of variation in toxicity than efficacy. These findings could inform future development of personalized therapeutic approaches.

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INTRODUCTION

Methotrexate (MTX) is a disease-modifying anti-rheumatic drug commonly used as a first-line agent in the treatment of rheumatoid arthritis (RA).1,2 There is considerable inter-individual heterogeneity in response to MTX, both in treatment efficacy and associated toxicity. Although smoking, female sex, younger age, longer disease duration and the presence of serum rheumatoid factor (RF) are associated with poor response to MTX therapy, they explain a rather low proportion of response variability, suggesting a role for pharmacogenetic factors.3,4 Consistent with that hypothesis, several reports identified polymorphisms in the MTX metabolic pathway to be significantly associated with treatment outcomes.5,6 However, much of the currently available genetic evidence remains conflicting and limited to a small number of candidate genes involved in the mechanism of MTX action, that is, the major histocompatibility locus including HLA–DRB1 and LTA–TNF as well as purine, folate and adenosine pathways.7–10

Genetic determinants of MTX response can be evaluated early in the course of RA, when pharmacologic intervention confers maximum benefits. Thus, identification and validation of new markers has important clinical implications, including development of personalized therapeutic approaches that result in greater efficacy and lower toxicity. To that end, this study used the Affymetrix drug metabolism, excretion and transport (DMET) plus platform to comprehensively examine the role of genetic variation in key pharmacokinetic pathways in MTX efficacy and toxicity among participants of the Treatment of Early Aggressive Rheumatoid Arthritis Trial (TEAR). The early RA phenotype of the TEAR cohort is uniquely suited for exploring pharmacogenetic associations as improvements associated with disease-modifying anti-rheumatic drug therapy are on average greater for participants with early rather than advanced disease, potentially increasing statistical power and informing future clinical interventions.11

PATIENTS AND METHODS

Study population

The TEAR Trial \( (n = 755) \), described in previous publications,12–14 is a 2-year, double-blind, active control, multicenter phase IV clinical trial in patients with early (<3 years disease duration) RA characterized by an ‘aggressive’ clinical phenotype, defined as autoantibody positivity or the presence of erosions on radiographs of hands and feet. Using a \( 2 \times 2 \) factorial design, the TEAR Trial compared two treatment strategies (early intensive therapy vs step-up therapy) and two combinations of medications (etanercept plus MTX vs hydroxychloroquine plus sulfasalazine plus MTX). After 24 weeks of treatment, participants randomized to MTX monotherapy were ‘stepped up’ to either oral triple therapy or MTX + etanercept if their Disease Activity Score on 28 joints (DAS28) exceeded 3.2. All participants received a daily 1 mg supplement of folic acid. If participants developed toxicity to MTX or sulfasalazine, the drug was discontinued or the dosage was decreased at the discretion of the treating physician. If the treatment changes resolved the toxicity in 2 weeks, the drug was continued at that dose.

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Eligibility criteria were reported in detail in prior publications. Briefly, entry criteria included age of 18 years or older; duration of disease ≤3 years; RA diagnosis by American College of Rheumatology (ACR) criteria; have active disease at the time of screening, defined as at least four swollen and four tender joints because of RA (using the 28 joint count) and either the presence of erosions or positive RF; if taking corticosteroids, receiving stable doses (<10 mg day⁻¹ of prednisone) at least 2 weeks before screening; if taking non-steroidal anti-inflammatory drugs, receiving stable doses for at least 1 week before screening. Participants were excluded if they were pregnant or lactating; had contraindications to study medications; received corticosteroid injections during the 4 weeks before screening; had a diagnosis of serious infection; or used biologic therapy. Mean disease duration ranged from 2.9 to 4.5 months across study arms and RF seropositivity approached 90%. Prior use of leflunomide, hydroxychloroquine and sulfasalazine was allowable if for no >4 months, as was a total dose of 40 mg of MTX. The TEAR Trial was registered with clinicaltrials.gov (NCT00259610) and approved by the appropriate institutional review board committees.

The primary outcome of the TEAR Trial was defined as the mean DAS28 from weeks 48 to 102, with secondary end points defined by ACR criteria for improvement (ACR20, ACR50 and ACR70) as assessed by a questionnaire and radiographic evidence of joint damage. DAS28 has been previously validated as an outcome measure for RA disease activity. Participants were evaluated during clinic visits at 6-week intervals for the first 48 weeks and at 12-week intervals thereafter. Drug toxicity was assessed at 6-week intervals via laboratory measures and a self-report of adverse events at each scheduled visit. As participants were given a folic acid supplement, the risk of adverse events was reduced, limiting our statistical power to consider different classes of toxicity outcomes. Thus, we have grouped together all instances of adverse events, including infections (that is, upper respiratory, urinary tract or general infection), as well as gastrointestinal, oral and skin conditions.

Of the 755 participants, 630 consented to genotyping and provided DNA. Of those, 471 had complete information on adverse events and covariates, and were included in the analysis.

Genotyping
Genomic DNA was extracted from a whole blood sample using the PureGene system (Gentra Systems, Minneapolis, MN, USA). The DMET platform (Affymetrix, Santa Clara, CA, USA) enables multiplexed genotyping of 1936 markers in 225 genes previously determined to have functional significance in phase I and phase II drug metabolism enzymes and drug transporters. Genotyping was carried out using methods as described by the manufacturer. Genotype calls were made using the DMET Command Console. Samples were considered passed or in bounds if they had genotyping calls of ≥95%. Samples that were out of bounds or had genotyping calls of <95% were re-run. Of the original 1936 markers, 1931 were successfully genotyped; of those, 1068 were found to be monomorphic in the study population and removed, leaving 863 markers in 224 genes in the analysis.

Statistical analysis
We tested the hypothesis that genes represented by DMET chip single-nucleotide polymorphisms are associated with MTX efficacy and toxicity using two different approaches. The first approach was a conventional multiple regression model of the association between genetic markers from the DMET chip and DAS28 at 24 weeks, adjusted for the baseline DAS28, treatment arm, race (European American, African American or other), sex, age (as linear and quadratic terms) and smoking status. The
genetic marker effects were assumed to be co-dominant with the
genotypes coded as (0, 1, 2) according to the number of minor alleles.
In addition, we conducted sensitivity analyses modeling genotype effects
using recessive and dominant modes of inheritance, as well as restricting
the analysis to Caucasian participants (data not shown). The 863 markers
were tested one at a time. The freely available software for genetic data
analysis, PLINK v1.07, was used to fit each of the models. We were
primarily interested in the $P$-value from the test of the additional variation
explained by the marker vs a null model, which included all covariates
except the marker effect. The same variables were used to test the
association of the individual markers on the toxicity outcome except that
except the marker effect. The same variables were used to test the
explanatory variables to consider far exceeds the number of genotyped
participants, we used a form of penalized regression that shrinks small
marker genotypes coded as (0, 1, 2) according to the number of minor alleles.

The issue of adjusting the type I error rate to account for multiple
testing is not as simple as performing a Bonferroni correction because of
the linkage disequilibrium between markers on the DMET chip. Assuming
complete pairwise linkage disequilibrium between markers within a gene,
we implemented a gene-wise correction for multiple testing, estimating
the significance level at $\alpha = 0.05/224$ genes $= 2.2 \times 10^{-4}$.

The second approach to testing the hypothesis that DMET markers were
associated with variation in efficacy and toxicity was to regard the gene
discovery analysis as a model selection problem. As the number of
explanatory variables to consider far exceeds the number of genotyped
participants, we used a form of penalized regression that shrinks small
marker associations while allowing for greater probability of detecting
markers with larger associations. In this framework, we tested the
hypothesis that the covariates, genes and markers were jointly associated
with toxicity or the change in DAS28. To test this hypothesis, we used a
regression model that penalizes both individual markers as well as groups
of markers, for example, all markers within a specific gene. This
approach is a natural application to the DMET chip data because each
marker can be grouped into a specific gene. The approach differs from
standard penalized regression approaches (for example, LASSO, ridge),
which penalize only the individual markers. The tuning constants, $\lambda_1$ and
$\lambda_2$, both control the penalty on the marker effects, but $\lambda_1$ (lasso penalty)
applies to individual markers, and the $\lambda_2$ (Euclidian or ridge penalty)
applies to groups of markers, that is, reflecting the combined influence of
markers on the outcome across an entire gene.

The key component of the penalized regression model fitting process is
choosing the total tuning constant (penalty), $\lambda = \lambda_1 + \lambda_2$. The model
optimization procedure developed by Wu et al. and Zhou et al. is
controlled by the number of explanatory variables, that is, as the dimension
of the model grows the penalty constant is relaxed. Given a preset
number of explanatory variables and ratio of individual and group
penalties $\lambda_1/\lambda_2$, the algorithm uses a ‘bracketing and bisection’
strategy that finds a solution for the total tuning penalty. In order to
observe the model optimization as the penalty varied, we extracted
shrinkage estimates of regression parameters as the number of variables
changed from 1 to 20. The order of entry of markers/covariates into the
model provides a rubric for selecting an appropriately sized model, which a
priori is expected to contain markers from a small proportion of the 224
analyzed genes from the DMET array. The models were fit using the
software Mendel (University of California at Los Angeles, Los Angeles, CA,
USA), following the methods outlined by Zhou et al.

The purpose of the penalties $\lambda_1$, $\lambda_2$ is to limit the actual number of
variants, because clearly a small minority of the 863 markers is expected
to be important. Here we do not consider models with $>20$ variables,
including markers and covariates. As simulation studies suggest
that penalizing both is the optimal choice, we examined the

Figure 2. Genetic variants selected by group lasso penalized regression for entrance into the additive model of methotrexate-associated adverse events in Treatment of Early Aggressive Rheumatoid Arthritis Trial (TEAR) participants. Panels show the following proportions of lasso to total penalty: $\lambda_1/\lambda = 0$ (pure group penalty), $\lambda_1/\lambda = 0.25$, $\lambda_1/\lambda = 0.5$ and $\lambda_1/\lambda = 1$ (pure lasso penalty).
robustness of our findings by fitting models with the ratio of individual penalty to total penalty $\lambda_2/(\lambda_1 + \lambda_2)$ of 1 (pure lasso penalty), 0.5, 0.25 and 0 (pure group penalty; Figures 1 and 2). The models were fit using the software Mendel.24

Finally, multiple regression was used to compare the proportion of variance in efficacy and toxicity explained by the markers selected from the penalized regression analysis with five allowed variables and penalties of $\lambda_2/(\lambda_1 + \lambda_2) = 1$ or 0.5. These models were fit using R software.24

RESULTS

Demographic and clinical characteristics of the study population are listed in Table 1. In this analysis, the proportion of patients in each treatment arm did not differ from that in the parent study.12 The majority of participants were female and of self-reported European ancestry, with a mean age of 50 years and body mass index of 30 kg m$^{-2}$ (obese). The average decrease in DAS28 from baseline to week 24 across treatment arms was 1.9 units.

In single-marker analyses of MTX efficacy, the DAS28 score at 24 weeks was associated with 42 out of 863, or 4.8%, of the markers at a $P<0.05$. However, no marker was statistically significant after Bonferroni correction at the marker or gene level. Five of the top six associated with DAS28 were in CHST11, which encodes carbohydrate (chondroitin 4) sulfotransferase 11. For all these markers, the minor allele was associated with greater MTX efficacy, as indicated by the change in DAS28 from baseline to 24 weeks. Modeling the genotype as dominant vs additive or restricting the data set to Caucasian participants only did not appreciably change the estimates of association (data not shown).

Table 2 shows the marker groups and genes selected by the penalized regression model listed by order of entrance into the model under varying proportions of lasso to overall penalty. In all four penalized regression models, the baseline DAS28 score was selected first, followed by sex in all models except with the pure lasso (that is, marker only, $\lambda_1/\lambda = 1$) penalty. The percentage of overall variance in efficacy explained by DMET chip markers when five variables were allowed in the model was 2% and 1% with $\lambda_2/\lambda = 0.5$ and 1.0, respectively (Table 3).

In single-marker analyses adjusted for multiple testing, toxicity was associated with 43 out of 863, or 5.0%, of the markers with $P<0.05$. No single marker was associated after the conservative multiple testing correction. Similarly to the efficacy analyses, the top hits did not vary depending on the assumed mode of inheritance or by racial group (Caucasians vs the total study population). In contrast to efficacy, non-genetic covariates such as age, race and treatment were not among the top variables included in the penalized regression model of MTX toxicity. Notably, one of the top hits for toxicity, rs670 in the ALDH2 gene, was significantly out of Hardy–Weinberg equilibrium in our population ($P$-value $= 8 \times 10^{-37}$), whereas other single-nucleotide polymorphisms in the same gene were not. The percentage of overall variance in toxicity explained by DMET chip markers when five variables were allowed in the model was more than threefold higher than for efficacy when $\lambda_2/\lambda = 0.5$ (5% vs 2%) and twice as high under the pure lasso penalty (9% vs 1%), suggesting that functional variants such as those included on the DMET array may have a larger role in explaining the heterogeneity in MTX treatment toxicity rather than efficacy.

DISCUSSION

Using the DMET genotyping array, we have evaluated associations between a set of known pharmacogenetic variants and MTX response in patients with early RA enrolled in the TEAR Trial. Of these, the ALDH2 finding is of particular interest because evidence suggests that in vitro human aldehyde dehydrogenase activity is highly variable, and that genetic variation in enzymes that catalyze the conversion of MTX to 7-OH-MTX is associated with two phenotypes of MTX metabolism among RA patients.25,26

In single-marker analysis, the presence of one copy of the minor allele at the rs886205 locus was associated with a 42% reduction in the relative odds of adverse events. One possible explanation is that the variant genotype is associated with higher enzymatic activity in the formation of 7-OH-MTX from MTX, reduced amount of cellular MTX, and therefore reduced MTX toxicity. Under that hypothesis, a positive relation would also be expected between the dosage of the minor allele at the rs886205 locus and MTX efficacy; however, the association with the DAS28 at 24 weeks observed in our study population was negative and did not reach statistical significance in the single-marker analysis.

Associations with solute carrier genes were observed for both efficacy and toxicity outcomes. These results are notable as SLC19A1, which encodes solute carrier family 19 member A1 and belongs to the same family of genes as SLC4A1, SLC22A2 and SLC28A2, has a crucial role in the pharmacokinetics of both MTX and 7-OH-MTX by transporting them into the cell.27 Accordingly,
Table 3. Top groups of DMET platform SNPs selected under varying ratios of lasso to total penalties and an additive model of methotrexate toxicity in TEAR participants

| λ/λ₀ | First                        | Second                      | Third                       | Fourth                      |
|------|------------------------------|-----------------------------|-----------------------------|-----------------------------|
| 1 (Pure lasso penalty) | SLC22A2<sup>a</sup> rs624249 | CYP20A1 rs2043449           | ALDH2 rs886205              | CYP39A1 rs7761731           |
| 0.5  | SLC22A2 rs316019 ALDH2 rs886205 | CYP39A1 rs9369629           | CYP20A1 rs938048013         |                              |
| 0.25 | SLC22A2 rs316019 ALDH2 rs886205 | CYP39A1 rs9369629           | SLC28A2 rs10519020         |                              |
| 0 (Pure group penalty) | SLC22A2 CYP39A1 ALDH2 SLC28A2 |                              |                              |                              |

Abbreviations: DMET, Drug Metabolism, Excretion, and Transport; TEAR, Treatment of Early Aggressive Rheumatoid Arthritis Trial; SNP, single-nucleotide polymorphism.

<sup>a</sup>Genes listed in the table encode the following proteins: SLC22A2, solute carrier family 22 member 2; CYP20A1, cytochrome p450 family 20 subfamily A polypeptide 1; ALDH2, aldehyde dehydrogenase 2; CYP39A1, cytochrome p450 family 39 subfamily A polypeptide 1; SLC28A2, solute carrier family 28 member 2.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES

1. Saag KG, Teng GG, Patkar NM, Anuntiyo J, Finney C, Curtis JR et al. American College of Rheumatology 2008 recommendations for the use of nonbiologic and biologic disease-modifying antirheumatic drugs in rheumatoid arthritis. Arthritis Rheum 2008; 59: 762–784.
2. Smolen JS, Landewe R, Breedveld FC, Dougados M, Emery P, Gaujoux-Viala C et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biologic disease-modifying antirheumatic drugs. Ann Rheum Dis 2010; 69: 964–975.
3. Drouin J, Harouei B. 3e Initiative Group. Predictors of clinical response and radiographic progression in patients with rheumatoid arthritis treated with methotrexate monotherapy. J Rheumatol 2010; 37: 1405–1410.
4. Saevarsdottir S, Wallin H, Seddighzadeh M, Ernestam S, Gelborek P, Petersson IF et al. Predictors of response to methotrexate in early DMARD naive rheumatoid arthritis: results from the initial open-label phase of the SWEFOT trial. Ann Rheum Dis 2011; 70: 469–475.
5. Kremer JM. Methotrexate pharmacogenomics. Ann Rheum Dis 2006; 65: 1121–1123.
6. Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dose escalation in rheumatoid arthritis. Nat Clin Pract Rheumatol 2007; 3: 256–257.
7. Criswell LA, Lum RF, Turner KN, Woelch B, Zhu Y, Wang J et al. The influence of genetic variation in the HLA–DRB1 and LTA–TNF regions on the response to treatment of early rheumatoid arthritis with methotrexate or etanercept. Arthritis Rheum 2004; 50: 2750–2756.
8. Sharma S, Das M, Kumar A, Manwaha V, Shankar S, Singh P et al. Purine biosynthetic pathway genes and methotrexate response in rheumatoid arthritis patients among north Indians. Pharmacogenet Genomics 2009; 19: 823–828.
9. 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: 1087–1095.
22 Zhou H, Kooloo WM, De Jonge R, De Vries-Bouwstra JK, Allaart CF, Linssen A et al. Relationship between genetic variants in the adenosine pathway and outcome of methotrexate treatment in patients with recent-onset rheumatoid arthritis. *Arthritis Rheum* 2006; **54**: 2830–2839.

21 Wu TT, Chen YF, Hastie T, Sobel E, Lange K. Genome-wide association analysis by penalized regression. *Methods Mol Biol* 2010; **632**: 99–124.

20 Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; **31**: 315–324.

19 Draper N, Smith H. *Applied Regression Analysis*, 3rd edn. (Wiley: New York, NY, USA, 1998).

18 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D et al. PLINK: a toolset for whole-genome association and population-based linkage analysis. *Am J Hum Genet* 2007; **81**: 559–575.

17 Breedveld FC, Weisman MH, Kavanaugh AF, Cohen SB, Pavelka K, van Vollenhoven R et al. The PREMIER study: a multicenter, randomized, double-blind clinical trial of combination therapy with adalimumab plus methotrexate versus methotrexate alone or adalimumab alone in patients with early, aggressive rheumatoid arthritis who had not had previous methotrexate treatment. *Arthritis Rheum* 2006; **54**: 26–37.

16 Prevoo ML, van ’t Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* 1995; **38**: 44–48.

15 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D et al. PLINK: a toolset for whole-genome association and population-based linkage analysis. *Am J Hum Genet* 2007; **81**: 559–575.

14 Moreland LW, O’Dell JR, Paulus HE, Curtis JR, Bathon JM, St Clair EW et al. Relationship between genetic variants in the adenosine pathway and outcome of methotrexate treatment in patients with recent-onset rheumatoid arthritis. *Arthritis Rheum* 2006; **54**: 2830–2839.

13 Moreland LW, O’Dell JR, Paulus HE, Curtis JR, Bathon JM, St Clair EW et al. Two-year radiographic results from the TEAR trial. *Arthritis Rheum* 2010; **62**: S1368.

12 Moreland LW, O’Dell JR, Paulus HE, Curtis JR, Bathon JM, St Clair EW et al. Folate supplementation during methotrexate therapy for rheumatoid arthritis alters drug efficacy and retention and is reduced by folic acid supplementation. *Arthritis Rheum* 2009; **60**: 2257–2261.

11 Breedveld FC, Weisman MH, Kavanaugh AF, Cohen SB, Pavelka K, van Vollenhoven R et al. The PREMIER study: a multicenter, randomized, double-blind clinical trial of combination therapy with adalimumab plus methotrexate versus methotrexate alone or adalimumab alone in patients with early, aggressive rheumatoid arthritis who had not had previous methotrexate treatment. *Arthritis Rheum* 2006; **54**: 26–37.