Personalized medicine in rheumatoid arthritis

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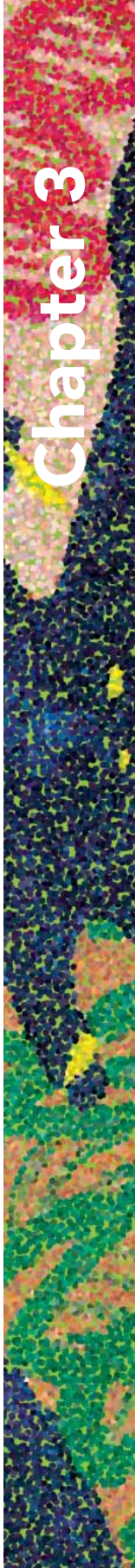

SLC04A1, *SLC22A2* and *SLC28A2* variants not related to methotrexate efficacy or toxicity in rheumatoid arthritis patients

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Abstract

Aim: A third of rheumatoid arthritis patients discontinue methotrexate treatment due to inefficacy or toxic side effects. Recently, an association between *SLC04A1* rs2236553, *SLC22A2* rs624249 and rs316019, and *SLC28A2* rs10519020 and rs1060896 with the efficacy and toxicity of methotrexate was reported. This study aims to replicate these findings in an independent cohort (n=324).

Methods: Regression analyses tested the associations between genotype and methotrexate response or toxicity.

Results: In the discovery study, there was a significant association between toxicity and rs624249, and rs1060896. These associations were not replicated in the independent cohort. Neither study observed an association between methotrexate efficacy and *SLC04A1*, *SLC22A2* or *SLC28A2* variants.

Conclusion: Current evidence does not support associations between variants in *SLC04A1*, *SLC22A2* and *SLC28A2* with methotrexate efficacy or toxicity.

INTRODUCTION

Methotrexate (MTX) is the first line disease-modifying antirheumatic drug (DMARD) in the treatment of patients with rheumatoid arthritis (RA). However, a third of the patient fails to achieve clinical remission or are unable to tolerate the drug due to side effects, often necessitating a switch to another DMARD or biological drug. Several nongenetic factors are known to influence the efficacy and toxicity of MTX, including gender, disease activity, disease duration, ethnicity and smoking.[1,2] The predictive value of these factors, however, remains limited for MTX response or toxicity. In contrast, genetic variation is found to play a substantial role, and several studies have reported a predictive role of variants in candidate genes related to MTX pharmacology.[3–7]

The precise mechanism of action of MTX in the treatment of RA is unknown, but MTX as a folate antimetabolite may exert its immunological function (after polyglutamation) via pathways involving adenosine, ubiquitin, methionine, folate, *de novo* pyrimidine and *de novo* purine synthesis.[8] Variants in genes encoding proteins in these pathways could play a role in predicting efficacy or toxicity of low-dose MTX, such as demonstrated in the meta-analysis by Chen *et al.*, which reported the *AMPD1* 34C (rs17602729) and *ATIC* T675C (rs4673993) mutations to be associated with MTX efficacy and linked *TYMS* 1494 del6 (rs34489327), *FPGS* (rs10106) and *MTHFR C677T* (rs1801133) to the risk of adverse events.[4]

Solute carriers (SLC) are constitutively expressed folate transporters that mediate the influx of MTX in the cell. Hence, genetic variation in genes encoding these transporters was previously examined for their association with MTX response. The most investigated SNP, *SLC19A1* 80G>A (rs1051266, also called *RFC-1*), was examined in two meta-analyses by Kung *et al.*[6] and Qiu *et al.*[9]. Kung *et al.* found an association with MTX efficacy, but not with toxicity. Qiu *et al.* showed an association with MTX toxicity in Europeans (OR: 1.36; p=0.041). Other SNPs found to be associated with MTX toxicity were: *SLC19A1* G carriers (rs7499; OR: 3.72; p=0.017), *SLC46A1* GG (rs2239907; OR: 2.32; p=0.030) and *SLC01B1* T carriers (rs4149056; OR: 2.78; p=0.040) and TT (OR: 2.82; p=0.019; Lima *et al.*).[10] Also, *SLC22A11* T>A rs11231809 T-allele carriership (OR: 0.19; p=0.031) was associated with MTX response (as measured by the changes in the disease activity score [DAS] at the 6-month time interval).

Recently, a study by Aslibekyan *et al.*[11] reported that the SNPs in *SLC04A1* (rs2236553), *SLC22A2* (rs624249 and rs316019) and *SLC28A2* (rs10519020 and rs1060896) are associated with MTX toxicity or efficacy in RA patients in the Treatment of Early Rheumatoid Arthritis trial (TEAR). Those variants were relatively common with a minor allele frequency greater than 5% in the global population. The present study aims to investigate if the initial findings can

be replicated in the BeSt (Dutch acronym for 'behandelstrategieën', treatment strategies) cohort, thus informing future precision medicine efforts in RA.

MATERIALS & METHODS

Patient characteristics

DNA samples and clinical response data from 352 RA patients receiving MTX therapy were available on 508 patients participating in the BeSt study.[12] The BeSt study is a multicenter randomized clinical trial that recruited early RA patients and compared the clinical and radiographic outcomes of four different treatment strategies as detailed below. The patient eligibility criteria were: age of ≥18 years, disease duration of ≤2 years, active disease on onset defined as ≥6 of 66 swollen joints, ≥6 of 68 tender joints and either an erythrocyte sedimentation rate of ≥28 mm/h or a global health score of ≥20 mm on a 0–100 mm visual analog scale, where 0 reflects the best and 100 the worst. Figure 3-1 illustrates the different treatment groups until the first evaluation point after 3 months of treatment.

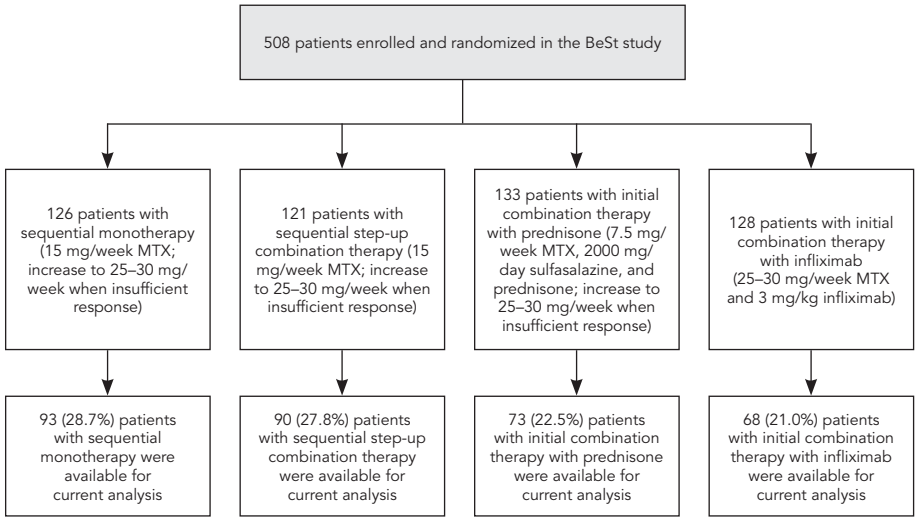

Figure 3-1. Flowchart of the enrolled BeSt patients.
Abbreviations: MXT: Methotrexate.

Written informed consent was obtained from all patients, and the local ethics committees of all participating hospitals approved the study (CME LUMC; registration number P258/99). Information about gender, the age of disease onset, age at treatment onset, smoking, MTX dosage, concomitant drugs (DMARDs, corticosteroids and nonsteroidal anti-inflammatory

drugs), rheumatoid factor and anticitrullinated protein antibody seropositivity and the DAS28 score before and after 6 months of treatment were provided. In the BeSt study, individual ethnicity data were not available, but most patients (>95%) were self-reported Caucasians.

Genotyping

The five SLC SNPs that emerged from the LASSO regression in the previous explorative study from Aslibekyan *et al*.[11] were genotyped in the BeSt cohort. Thermocycler (SensoQuest GmbH, Gottingen, Germany) was used for PCR to amplify the preferred SNPs and Q-solution was added to facilitate the SNP amplification. For all PCRs, 45 cycles were performed. SLC28A2rs10519020 was genotyped by pyrosequencing (PyroMark Q96 ID system, Qiagen, Hilden, Germany), while *SLC22A2* rs624249, *SLC22A2* rs316019, *SLC28A2* rs1060896 and SLC04A1 rs2236553 were genotyped by high-resolution melting (Lightscanner, Idaho Technology Inc., UT, USA). The obtained genotypes were confirmed by Sanger sequencing (Applied Biosystems, MA, USA).

End points

Similar end points were used as in the discovery study by Aslibekyan *et al*.[11] as discussed below. Efficacy was defined as DAS28 at 24 weeks, and the MTX-related toxicity was defined as any adverse or severe adverse event within the first 2 years of treatment. The research nurse determined toxicity during hospital visits in the BeSt study.

Treatment of early rheumatoid arthritis trial data

The design of the TEAR trial was previously described in detail elsewhere.[13] Briefly, TEAR is a 2-year, double-blind clinical trial of two treatment strategies (early intensive vs step-up therapy) and two medication combinations (MTX + etanercept and MTX + hydroxychloroquine + sulfasalazine) in early RA (<3 years since disease onset). Efficacy was ascertained at the 24-week time point using the change in DAS28 score, while toxicity was ascertained by self-report during clinic visits over the 2 years of follow-up; because of low rates of adverse events, all types of toxicity were combined. In the present analysis, smoking was ascertained using a validated biomarker (cotinine) to ensure accuracy. Genotyping was performed using the DMET array as previously described[13] as well as the Affymetrix 6.0 chip during a subsequent effort. TEAR data were reanalyzed for the present study to ensure methodological consistency with BeSt; specifically, we analyzed the data using standard regression rather than using the LASSO as described in the original paper (see below).

Statistical analysis

Statistical analyses were performed using SPSS 23.0 (SPSS, Inc., IL, USA) and Plink (version 1.9, http://pngu.mgh.harvard.edu/purcell/plink/).[14] Each SNP was checked for Hardy–Weinberg equilibrium. The associations of SNPs with efficacy or toxicity were analyzed using multiple regression models, adjusted for DAS28 at baseline, age, gender, smoking and treatment arm.

We tested associations of SNPs with efficacy in both complete study populations as well as in the subgroup of patients initially treated with MTX monotherapy. Due to the timing of both studies, a subgroup analysis on MTX monotherapy was not possible for toxicity. We applied a Bonferroni correction for the five *SLC* SNPs to adjust for multiplicity, which results in a significance threshold of 0.01 (0.05/5 SLC SNPs).

RESULTS

Study population

DNA from 351 patients from the BeSt cohort was available for the current analysis, but for 25 patients, data on either DAS28 (n=2), MTX dose (n=2), or both (n=21) were missing at 24 weeks. An additional two patients had stopped MTX before 24 weeks, yielding a total of 324 patients for the association analysis.

Table 3-1. General characteristics of both study populations

Variables	BeSt (n=324)	TEAR (n=480)
Age, years†	54.3±13.4	49.5±12.6
Female, n (%)	220 (67.9)	349 (72.7)
Methotrexate dosage at 6 months, mg/wk†	19.9±6.9	13.9±3.5
RF-positive, n (%)†	211 (65.1)	430 (89.6)
Smoking, n (%)	116 (35.8)	150 (39.0)
DAS28 at baseline, points	5.7±0.90	5.8±1.1
DAS28 at 24 weeks, points†	3.6±1.23	3.9±1.4
Experience adverse events within 2 years of treatment, n (%)	114 (35.2)	174 (36.3)

† Significant difference between BeSt and TEAR study ($p<0.05$).
Abbreviations: DAS28: Disease activity score measured in 28 joints, RF: Rheumatoid factor, BeSt: Behandelstrategieën (Dutch acronym for treatment strategies), TEAR: Treatment of early rheumatoid arthritis trial.

Table 3-1 summarizes the demographic and clinical characteristics of the patients enrolled in the TEAR and BeSt cohorts. The mean age of the BeSt patients was 54 years and the mean baseline DAS28 was 5.7; 68% were female. Of the 324 patients, 116 (36%) patients were current smokers, and 211 (65%) were rheumatoid factor positive. After 6 months, the mean

dose of MTX was 19.8 mg/week, ranging from 7.5 to 30.0 mg/week, and all patients used concomitant folic acid supplementation (5 mg/week). Within 6 months of enrolment, 188 patients (58%) received MTX monotherapy, and 136 patients received MTX combination therapy, either with infliximab (n=66, 20%) or with sulfasalazine and prednisolone (n=70, 22%).

Genetic association results

In the BeSt cohort, all five genotyping assays had a call rate of >95%, and all five genotypes did not violate Hardy–Weinberg equilibrium (p>0.05). The minor allele frequencies (MAF) of the investigated SNPs were >5%, except the SNP in *SLC28A2* (rs10519020, MAF in BeSt=0.95%) which occurred more frequently in the general population: MAF=6.55% (1000 genome project, Caucasians: 1.91%).[15] The associations between the investigated genetic variants and efficacy are shown in Table 3-2, and the associations with toxicity are summarized in Table 3-3.

Table 3-2. Associations between DAS28 at 24 weeks and SLC SNPs

			BeSt study		TEAR study	
Gene	SNP	Allele	B (SE)	p-value†	B (SE)	p-value†
SLCO4A1	rs223655	C	-0.11 (-1.20)	0.23	0.11 (0.14)	0.45
SLC22A2	rs624249	A	-0.10 (-1.06)	0.29	-0.13 (0.13)	0.34
SLC22A2	rs316019	T	0.12 (0.86)	0.39	0.18 (0.21)	0.39
SLC28A2	rs1060896	C	-0.01 (-0.08)	0.78	0.17 (0.13)	0.18
SLC28A2	rs10519020	C	-0.26 (-0.56)	0.73	0.25 (0.30)	0.40

† The p-values were adjusted for gender, DAS28 at baseline, randomization groups, age and smoking status.
Abbreviations: B: Regression coefficient, DAS: Disease activity score, SE: Standard error, BeSt: Behandelstrategieën (Dutch acronym for treatment strategies), TEAR: Treatment of early rheumatoid arthritis trial.

Table 3-3. Associations between toxicity (within 2 years of therapy) and SLC SNPs

			BeSt study		TEAR study	
Gene	SNP	Allele	OR (95% CI)	p-value†	B (SE)	p-value†
SLCO4A1	rs223655	C	1.31 (0.92–1.85)	0.13	0.85 (0.71–1.03)	0.10
SLC22A2	rs624249	A	0.87 (0.62–1.23)	0.44	1.55 (1.16–2.07)	0.003
SLC22A2	rs316019	T	1.03 (0.62–1.71)	0.92	1.28 (0.82–1.99)	0.28
SLC28A2	rs1060896	C	1.27 (0.92–1.74)	0.15	0.72 (0.54–0.95)	**0.02**
SLC28A2	rs10519020	C	4.03 (0.71–22.87)	0.12	0.87 (0.72–1.06)	0.17

† The p-values were adjusted for gender, DAS28 at baseline, randomization groups, age and smoking status. Nominally significant (p<0.05) associations are marked in bold, and significant association after Bonferroni correction (p<0.01) are underlined.
Abbreviations: DAS: Disease activity score, OR: Odds ratio, BeSt: Behandelstrategieën (Dutch acronym for treatment strategies), TEAR: Treatment of early rheumatoid arthritis trial.

Both studies showed null associations between MTX efficacy and SLC SNPs. Further tests carried out with the MTX monotherapy groups consistently showed no significant associations. Although in the TEAR study two nominally significant SLC-variants were associated with MTX toxicity, these associations in the BeSt cohort were not statistically significant.

DISCUSSION

In this study, we aimed to replicate previously reported associations of selected variants in *SLC04A1*, *SLC22A2*, and *SLC28A2* with the efficacy and toxicity of MTX in RA patients participating in the TEAR study. Before pharmacogenetics biomarkers can be used in clinical practice, it is essential that potential biomarkers from explorative studies are replicated in independent cohorts; such replication was not achieved in our study, redirecting future pharmacogenetics investigations of MTX to other genomic regions. The SLC superfamily comprises 55 gene families with at least 362 putatively functional protein-coding genes. SLC04, SLC22A and SLC28 function, respectively as bicarbonate transporter, organic cation/anion/zwitterion transporter and Na-coupled nucleoside transporter. Although no further studies published on relationships of the investigated variants with either MTX efficacy or toxicity, associations have been described with *SLC22A2* rs316019 and either cisplatin,[16] metformin,[17] smoking cessation,[18] diabetic nephropathy and hypertension[19] and *SLC28A2* rs1060896 with ribavirin.[20]

There are several potential explanations why the reported pharmacogenetics markers could not be replicated. The most likely reason is that the biomarkers found in the TEAR study were false positive findings. Alternatively, contradictory findings could be explained by patient differences between the two study cohorts. For instance, we observed significant differences in age, rheumatoid factor positivity, DAS28 at 24 weeks, and MTX dosage at 24 weeks. By adjusting for differences in age, DAS28 at baseline, and group assignment in the regression models, baseline differences were taken into account, but we could not correct for differences that occurred during treatment, although to a large extend the drug treatment regimens between the studies were comparable. In both studies, one group was treated with MTX, and a biological (TEAR etanercept and BeSt infliximab), one group with MTX and sulfasalazine (and in case of BeSt also with prednisolone), and one group was treated with MTX monotherapy. Moreover, subgroup analysis in both TEAR and BeSt patients receiving MTX monotherapy showed no significant associations between efficacy and the genetic SLC variants. A potential limitation is that our study has a limited number of patients involved to detect the previously reported associations. However, post hoc analysis showed that the positively associated SNPs (rs624249 and rs1060896) have more than 90%

power to detect an effect. Another limitation could be the effect introduced by different ethnicities in the two cohorts. Yet, in both cohorts most patients were from Caucasian origin (TEAR ~80%, BeSt >95%), and the investigated significant SNPs showed no different allele frequencies according to the 1000 genome data.

Penalized regression, as used in the discovery study,[11] offers an attractive way to select relevant SNPs in the application of pharmacogenomics, especially when the considered number of SNPs exceeds the number of individuals in the study. The two most widely used techniques are Ridge and LASSO regression,[21] and various combinations thereof, such as elastic net[22] and group LASSO.[23] These methods are widely accepted for explorative studies but were developed for prediction problems, in other words, false positives among selected SNPs are acceptable as long as outcome prediction performs well. No associations are established by penalized regression. Replication in independent cohorts is a prerequisite for clinical application, and the presence of false positives in the SNPs selected by penalized regressions tends to hamper such efforts, as evidenced by our study. An improved strategy seems to be that penalized regression should only be considered a screening step, followed by a step that demonstrates associations.

In conclusion, our study provides no evidence that genetic variants in *SLCO4A1*, *SLC22A2* and *SLC28A2* are associated with either efficacy or toxicity in early RA patients treated with MTX. To better understand the role of SLC, future research should focus whether other SLC variants are associated with the effectiveness or toxicity of MTX in RA patients.

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