Potential role of pharmacogenetics for optimalization of drug therapy in rheumatoid arthritis

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Chapter 4

Functional polymorphisms and methotrexate treatment outcome in recent-onset rheumatoid arthritis

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Abstract

Objective

Clinical response to methotrexate (MTX) treatment differs among rheumatoid arthritis (RA) patients. Genetic variation, can at least partly, account for this phenomenon. In this study, functional polymorphisms in genes related to the mechanism of action of MTX or immunopathogenesis of RA were studied for association with treatment outcome in a Dutch cohort of patients with early RA.

Methods

Seven polymorphisms in seven genes were analyzed in 205 genotyped patients with active rheumatoid arthritis. All received standardized MTX treatment (up to 25 mg per week orally), combined with folic acid. MTX treatment outcome was evaluated by disease activity score criteria (DAS) and adverse drug events (ADE). The following genetic variants were analyzed and correlated: *DHFR* 829C/T, *ABCB1* 3435C/T, *ITPA* IVS2 +21A/C, *HLA-G* (-/+14bp), *IMPDH2* +787C/T, *TGFB1* +869T/C and *TLR4* +896A/G. In case of significant differences, regression analyses were applied.

Results

No significant associations of these genetic variants with MTX efficacy were demonstrated. Regarding toxicity, patients carrying the *ABCB1* 3435 T-allele and *TLR4* G-allele were 2.5 times more likely to develop ADEs at 6 months (OR 2.6; 95%C.I. 1.1-6.2 and OR 2.5; 95%C.I. 1.1-6.1, respectively). Additionally, the chance of experiencing ADEs at 6 months increased almost 4 times in patients with the two unfavourable genotypes (OR 3.9; 95%C.I. 1.5-10.3). However, none of these associations remained significant after correction for multiple testing ($p<0.004$).

Conclusion

In conclusion, it is demonstrated that in RA patients treated with MTX toxicity was potentially associated with *ABCB1* 3435C/T and *TLR4* +896A/G. However, after correction, none of these associations remained significant. Further, no significant associations of these seven functional variants with efficacy were found.

Introduction

The optimal strategy in treatment of early rheumatoid arthritis (RA) is to use effective disease modifying antirheumatic drugs (DMARDs) in an early stage of disease in order to reduce disease activity and to prevent destructive arthropathy. In clinical studies, this treatment goal is frequently achieved by methotrexate (MTX) (1;2). Accordingly, this drug developed as a mainstay in effective drug therapy of RA (3;4). Still, a substantial part of patients (40-60%) , fail to achieve an efficacious clinical result and/or experience adverse drug events (15-30%) with MTX monotherapy (5;6).
Interestingly, these differences in clinical response to MTX can at least partially be explained by pharmacogenetics (7). The ultimate aim of pharmacogenetics in RA is to use genetic profiles of RA patients for selecting the optimal antirheumatic drug treatment for the individual patient in an early phase of the disease. So far, most studied genetic variants were chosen based upon their hypothetical relation to the mechanism of MTX or inflammatory process in RA, such as genetic variants in the folate and adenosine pathway (8;9). Ideally, functional genetic variants are selected because the alteration in protein function is thought to influence drug action and thus may explain interindividual differences in drug response (10;11).
To date no genetic factors are proven and validated markers for predicting MTX therapy outcome. Specifically, several genetic markers such as *MTHFR* 677 C>T and *RFC* 80G>A have been correlated with treatment outcome in RA patients over the last years (12;13) but have been found hard to replicate in large and independent cohorts of patients with valid clinical endpoints. Moreover, only a minority of the genetic variants have proven functional consequences, e.g. to impact gene expression and subsequent enzyme activity. Particularly, this lack of validation and replication limits the application of pharmacogenetics of MTX in clinical decision making (11;14;15).
Therefore, in this study we selected a series of functional genetic polymorphisms for association with MTX treatment outcome in early RA patients. The genetic polymorphisms included are *DHFR* 829C>T, *ABCB1* 3435C>T, *ITPA* IVS2 +21A>C, *HLA-G* (->+14bp), *IMPDH2* +787C>T, *TGFB1* +869T>C and *TLR4* +896A>G. These variants are in genes, which are thought to be related with the mechanism of action of MTX and/or immunopathogenesis of RA (Table 1).

Patients and methods

Patients.
The 205 patients enrolled in this study comprised a subcohort of the 508 patients who participated in the multicenter BeSt study. Inclusion criteria were a diagnosis of early RA as defined by the American College of Rheumatology (ACR) 1987 criteria for RA, age ≥18 years, and a symptom duration of <2 years. Patients were also required to have active RA, with ≥6 of 66 swollen joints and ≥6 of 68 tender joints and either an erythrocyte sedimentation rate (ESR) ≥28 mm/hour or a global health score of ≥20 mm on 0 – 100 mm visual analogue scale (VAS) where 0= best and 100 = worst. Main exclusion criteria were previous treatment with DMARDs other than antimalarials and concomitant treatment with an experimental drug. Further details have been published elsewhere (16). The local ethics committee at each participating hospital approved the study protocol. All patients gave informed consent before enrolment into the study.
Regarding ethnicity, our population consisted of 93.2% Caucasian (n= 191), 2.4% Asian (n= 5), 1.0% African (n= 2), 3.4% other (n= 3 Hindustani, n=3 Surinamese, n= 1 Israeli).

Study design and treatment.
Patients who were allocated to initial monotherapy with MTX (n=247) and for whom DNA samples were available (n= 205) were included in the current analysis. The primary goal, regarding efficacy of therapy in the BeSt study (16) groups was a clinical response as defined by the Disease Activity Score (DAS) of ≤2.4 at 6 months (17;18). The DAS is a validated composite outcome measure consisting of the Ritchie articular index (RAI) (19), the swollen joint count (SJC), general well-being as indicated by the patient on a VAS and the ESR. A research nurse who was blinded to the allocated treatment group assessed the DAS.
All patients included in this analysis began treatment with a regimen of oral MTX 7.5 mg weekly, with the dosage increasing to 15 mg weekly after 4 weeks, in combination with folic acid (1 mg daily). In the event of insufficient clinical response (DAS >2.4) at the 3-month follow up visit, the MTX dosage was increased stepwise from 5 mg every 2 weeks to 25 mg weekly. In case of intolerance, MTX could also be given by parenteral route of administration. In case of adverse drug events, MTX was continued at the highest tolerated dose. If MTX was not tolerated at all, the patient was treated with the next DMARD according to the BeSt protocol. Concomitant therapies with nonsteroidal anti-inflammatory drugs as well as intra-articular injections with corticosteroids were allowed for all treatment groups.

Evaluation of clinical efficacy and toxicity
Responders were defined as patients who were receiving MTX and had a DAS of ≤2.4 at 6 months (good clinical response). Nonresponders were defined as patients who were receiving MTX and had a DAS of >2.4. A total of 186 patients were available for the analysis of associations between selected SNPs and response to MTX at 6 months (16). Patients who experienced adverse events but continued to be treated with MTX at 6 months were included in the efficacy analysis. Baseline variables possibly influencing the patient's disease state and MTX response were previously selected on the basis of literature (20).
Toxicity was evaluated by counting each reported adverse drug event (ADE) and its consequences for the patient and treatment. Adverse drug events were spontaneously reported by the patients, or were reported as a result of non-specific questioning on patients' wellbeing by the investigator, by physical examination or laboratory measurements during follow up. In case of adverse drug events, MTX was continued at the lowest tolerated dose, or if MTX was not tolerated at all, the rheumatologists adjusted DMARD therapy according to the protocol. Of all reported ADEs, the following non-infectious adverse drug events were evaluated explicitly: gastrointestinal adverse drug events defined as patients' general wellbeing, nausea, vomiting, diarrhoea, and constipation; liver adverse drug events defined as all cases of elevated functional liverenzymes resulting in MTX dose adjustment or discontinuation; pneumonitis; skin and mucosal disorders. Moreover, patients were evaluated for leucopenia (<4.10^9/L), ALAT 3 times upper limit of normal (>135 U/L) and for alkaline phosphatase (AF) 3 times the upper limit of normal (>360 U/L). The analyses of laboratory measurements were performed for completers only.

Selection of SNPs
Seven nucleotide polymorphisms (SNPs) with proven functional consequences in seven candidate genes related to the mechanism of action of MTX and/or immunopathogenesis of RA were selected. Based on literature, the following criteria were taken into consideration: validated SNP, proven functional consequences of the variant (21-26) and indications for clinical relevance, as defined by previous associations with treatment outcome in RA (tables 1 and 3) (25;27-32).
The genetic polymorphisms included were in genes coding for dihydrofolate reductase (*DHFR*), multi-drug resistance-1 (*ABCB1*), inosine triphosphate pyrophosphatase (*ITPA*), human leukocyte

antigen-G (*HLA-G*), transforming growth factor β1 (*TGFB1*), toll-like receptor 4 (*TLR4*), inosine 5'-monophosphate dehydrogenase (*IMPDH2*).
The following SNPs were analyzed: *DHFR* –829C>T (rs34764978), *ABCB1* 3435C>T (rs60023214), *ITPA* IVS2 +21A>C (rs7270101), *HLA-G* ->+ATTTGTTCATGCCT (->+14bp) (rs16375), *TGFB1* +869T>C (rs1982073), *TLR4* +896A>G (rs4986790), *IMPDH2* +787C>T.

Genotyping
DNA was isolated from peripheral white blood cells by the standard manual salting-out method. As a quality control, positive controls (Control DNA CEPH 347-02; Applied Biosystems) and negative controls (water) were used. In addition, 5-10% of samples were genotyped in duplicate and no inconsistencies were observed. Real-time polymerase chain reaction (PCR) using Taqman technique, according to the protocol provided by the manufacturer (Applied Biosystems), was performed for genotyping SNPs in the genes *ABCB1, ITPA, TLR4, TGFB1*. Regarding HLA-G, the 14 bp deletion was determined with fragment length analysis on the ABI PRISM 3730xl Analyzer, according to standard procedures.
Briefly, DNA was PCR-amplified using primers 5'-AAGGAATGCAGTTCAGCATG-3' and 5'-CTCACGGCTTGTAAATGTGAC-3' of which the forward primer was FAM-labeled. PCR product was 20 times diluted and 1 μl was mixed with 9 μl formamide containing ROX dye. The fragments were analysed with peakscanner software (Applied Biosystems).*IMPDH2* +787C/T was analysed by lightscanner (Idaho) using PCR primers 5'-CTGCTGTGTGGGGCAGCC-3' and 5'-TAGCAGCTCACCAAAACCAC3' and as probe 5'-TGGACTTGCTCGCCCAGG-3'. *DHFR 829C>T* was detected using a multiplex pyrosequencing method. The sequence of *DHFR* 829C>T (rs34764978) appeared to be highly homologous with a sequence on chromosome 18. This specific gene has a T at position 829. To discriminate 829T of the DHFR gene with that on chromosome 18, allele-specific PCR primers were chosen in such a way that chromosome 18 will not be amplified. In addition, pyrosequencing was performed as a multiplex assay in which the second SNP discriminates another mismatch between both genes. PCR primers are 5'-CTTCTCCAAGACCCCAACTG-3' and biotinylated revrese primer 5'- CTTCCAGGTTGTTTTCAATTTTT-3'. Sequence primers are 5'-AGTCCCCAGCACCTGCTA-3' (sequence to analyze: C/TAGTGAGCTGCC) and 5'- AGTGGAAA-TACCAAAA-3' (sequence to analyze GC/ATTCCTACGT). Dispensation order was ACTCAG-CAGTCTG.
Success rates for these assays were as follows: for *ABCB1* 3435 C>T, 94.6%; for *ITPA* IV2 +21 A>C, 98.5%; for *HLA-G* ->+14bp, 89%; for both *TGFB1* +869T>C and *TLR4* +896A>G, 95.6%; for *DHFR* 829C>T 96.0% and for *IMPDH2* +787 C>T, 97.2%.
Genotype distributions in percentages were for DHFR 829C>T, 100% CC, 0% CT, 0% TT; for *ABCB1* 3435C>T, 22% CC, 45% CT, 33% TT; for *ITPA* IVS2 +21A>C, 80% AA, 17% AC, 3% CC; for *HLA-G* ->+14bp, 31% -14bp/-14bp, 48% -14bp/+14bp, 21% +14bp/+14bp; for *TGFB1* +869T>C, 37% TT, 49% TC, 14% CC; for *TLR4* +896A>G, 87% AA, 12% AG, 1% GG; for IMPDH2 +787C>T, 100% CC, 0% CT, 0% TT. Notably, the variants within the *DHFR* gene (genotypes CT and TT) and *IMPDH2* gene (genotypes CT and TT) were not observed during PCR sequence analysis in our patient cohort.
Genotype frequencies for all genetic polymorphisms were in Hardy-Weinberg equilibrium, except for *ITPA* IVS2 +21 A>C (p=0.025). However, the genotype distribution of this SNP in our population was not significantly different from allele frequencies presented in the NCBI SNP database (33).

Gene	Role in relation with MTX and/or RA	Genetic variant	Hypothetical effect
ABCB1	Efflux transporter; (hypothetically) efflux of MTX	3435C>T	Decrease of mRNA stability and enzyme expression; May increase intracellular MTX levels (36-38)
DHFR	Conversion DHF to THF; target of MTX	-829C>T	Increase of enzyme expression leading to increased enzyme production and MTX resistance (21;22)
HLA-G	Downregulation of inflammatory process; involved in immune tolerance	->+14 bp	Decrease of HLA-G mRNA stability; Decrease of sHLA-G protein production (25;48)
IMPDH2	Catalyzation of conversion IMP to XMP; involved in the purine metabolism	787C>T	Decrease of enzyme activity (24)
ITPA	Conversion ITP to IMP; involved in the purine metabolism; indirect target MTX	IVS2 +21A>C	Alteration of enzyme structure and Decrease of enzyme activity (26)
TGFB1	Involved in immunosuppression and inflammatory conditions	869T>C	May decrease or increase enzyme production (59-62)
TLR4	Mediation of secretion of inflammatory cytokines;	896A>G	Decrease of inflammatory cytokine; May associate with susceptibility to RA (53;55)

Table 1. Characteristics of functional genetic polymorphisms

Abbreviation(s): bp= basepair; *DHFR*= dihydrofolate reductase , *ABCB1*= multi-drug resistance-1; *ITPA*= inosine triphosphate pyrophosphatase; *HLA-G*= human leukocyte antigen-G; *TGFB1*= transforming growth factor β1; *TLR4*= toll-like receptor 4, *IMPDH2*= inosine 5'-monophosphate dehydrogenase; MTX= methotrexate; N.A.= not available; RA= rheumatoid arthritis; mRNA= messenger ribonucleic acid; IMP= inosine monophosphate; XMP= xanthosine monophosphate; ITP= inosine triphosphate

Statistical analysis.
Genotype distributions were related to efficacy and toxicity using 3 by 2 cross tabs for each genotype, and 2 by 2 cross tabs for carriers versus non-carriers analysis with the two-sided Chi-square test. Generally, if genotype distributions differed, defined as p-value of ≤0.1 between responders and nonresponders (or ADE versus non-ADEs) as tested in the Chi-square univariate analysis, the SNP was selected for the multiple regression analysis. Hereby, other factors related to MTX treatment outcome were used as covariates in all regression analyses, as previously reported (9;20). The p-values that were considered significant were $p<0.05$ and corrected for multiple testing: 0.05 divided by 7 (SNPs) times two (efficacy and toxicity) = 0.004. All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Power calculation
With the appliance of a power calculation it was demonstrated that for our study population of 205 patients, minimal allele frequencies ranging from 7% (*TLR4* 896A>G) to 45% (*ABCB1* 3435C>T), an exposure of 40% and a chosen type 1 error probability $\alpha=0.004$ (because of multiple testing), a power of >80% could be achieved to detect an odds ratio of 1.8 (*ABCB1* 3435C>T) to 4.3 (*TLR4* 869A>G).

Results

Patient population
Baseline demographic and disease characteristics between responders and nonresponders of the 205 genotyped patients were prevalent as earlier reported (34). After 6 months of treatment with MTX, 87 patients (47%) responded (DAS ≤2.4) to MTX monotherapy. Among these patients, 43% received MTX at a dosage of 15 mg weekly and 57% received MTX at a dosage of 25 mg weekly. All results remained similar when performed with and without inclusion of non-Caucasian patients (data not shown).

Functional polymorphisms in relation with MTX efficacy
Table 2 displays the comparison of wildtype and/or mutant allele carriers for successfully genotyped variants between responders and nonresponders. Generally, no significant differences in genotype frequency distribution between responders and nonresponders were seen in the univariate analysis for *ABCB1* 3435C>T, *ITPA* IVS2 +21 A>C, *HLA-G* ->14bp, *TGFB1* +869T>C and *TLR4* +896A>G ($p>0.05$). Notably, the SNPs *DHFR* 829 C>T and *IMPDH2* +787C>T could not be used for correlation analysis due to the presence of 100% homozygous wildtype genotype in the cohort of patients.
Regarding subsequent analysis, only *ABCB1* 3435C>T was a candidate to include in the multivariate regression analysis ($p=0.082$). Patients with the *ABCB1* 3435TT genotype were more frequent in the group of nonresponders, as defined by to DAS >2.4, than in the group of responders (36% vs. 24%, respectively). However, in the multivariate regression analysis including the co-variates gender, rheumatoid factor status, smoking status, DAS at baseline the SNP in the *ABCB1* gene was no longer significant ($P>0.05$; OR= 1.93 and 95%C.I. 0.88-4.26).

Genetic polymorphism	Response in % (DAS ≤2.4)		Nonresponse in % (DAS >2.4)		P-value
***ABCB1* 3435C>T**					
CC vs. T-allele carriers	25	75	23	77	0.769
C-allele carriers vs. TT	76	24	64	36	0.082
***ITPA* +21A>C**					
AA vs. C-allele carriers	79	21	81	19	0.687
A-allele carriers vs. CC	97	3	98	2	0.555
***HLA-G* ->+14 bp**					
-14bp/-14bp vs. +14bp carriers	25	75	33	67	0.306
-14bp carrier vs. +14bp/+14bp	78	22	80	20	0.781
***TGFB1* +869T>C**					
TT vs. C-allele carriers	39	61	38	62	0.709
T-allele carriers vs. CC	88	12	85	15	0.541
***TLR4* +896A>G**					
AA vs. G-allele carriers	88	12	88	12	0.967
A-allele carriers vs. GG	99	1	100	0	0.289

Table 2. Comparison of genetic polymorphisms between responders and nonresponders to MTX monotherapy at 6 months[a,b]

a. Abbreviation(s): bp= basepair; *ABCB1*= multi-drug resistance-1; *ITPA*= inosine triphosphate pyrophosphatase; *HLA-G*= human leukocyte antigen-G; *TGFB1*= transforming growth factor β1; *TLR4*= toll-like receptor 4.
b. The SNPs *DHFR* -829 C>T and *IMPDH2* +787C>T could not be used for statistical analysis due to the presence of 100% homozygous wildtype genotype

Functional polymorphisms in relation with toxicity

MTX toxicity data at 6 months were present for 200 RA patients. Thirty percent of these patients (n= 60) experienced at least 1 ADE during 6 months of treatment (34). ADEs at 6 months were more frequent in patients carrying the mutant alleles of *ABCB1* 3435C>T and in those with *TLR4* 896A>G in comparison with patients carrying homozygous wildtypes (34% vs. 16% and 50% vs. 28%, respectively). For *ITPA* IVS2 21A>G, patients with the homozygous mutant genotype experienced more frequently ADEs in comparion with A-allele carriers (67% vs. 29%, respectively).
In multivariate regression analyses, *ABCB1* T-allele- and *TLR4* G-allele carriers were approximately 2.5 times more likely to experience toxicity at 6 months (OR 2.6; 95%C.I. 1.1-6.2 p=0.037 and OR 2.5; 95%C.I. 1.1-6.1; p=0.037, respectively). Moreover, patients carrying both the *ABCB1* T-allele and *TLR4* G-allele (N=21) had an almost four times risk on a ADE (OR 3.9; 95%C.I. 1.5-10.3; p=0.005) (Figure 1). Additional multivariate analysis for the SNP in the *ITPA* gene revealed no significant association with developing toxicity at 6 months. However, none of these associations remained significant after correction for multiple testing (p<0.004).

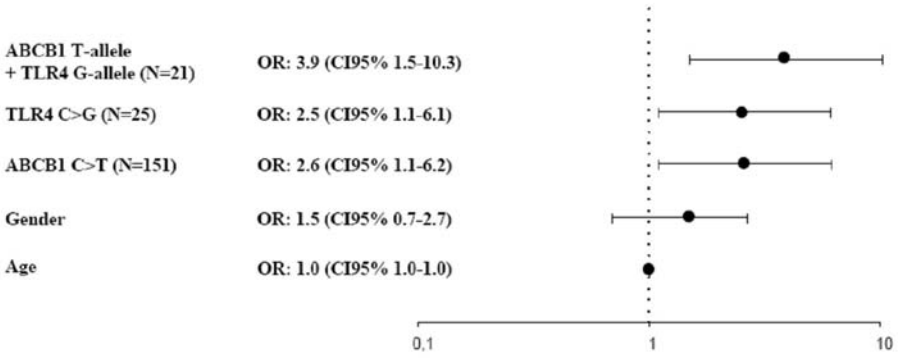

Figure 1. Genetic variants and occurrence of adverse drug events during 6 months of MTX therapy[a,b]

a. Abbreviation(s): *ABCB1*= multi-drug resistance-1; *TLR4*= toll-like receptor 4; OR= Odds ratio; CI95%= confidence interval of 95%
b. None of these genetic associations remained significant after correction for multiple testing (p<0.004)

Finally, in order to assess whether the observed relationship of toxicity and *ABCB1* T-allele and *TLR4* G-allele carriership was attributable to achieving a response, toxicity regression analyses were performed at 6 months for the group of responders and nonresponders. No significance with obtaining response or nonresponse in developing ADEs was seen in patients genotyped for *ABCB1* T-allele (N=74). For carriers of both the *TLR4* G-allele and *ABCB1* T-allele and for carriers of the *TLR4* G-allele alone in patients, which achieved a DAS of >2.4 at 6 months (N=9 and N=11, respectively), a significantly higher percentage of toxicity and increased odds ratio was demonstrated (OR=8.2; 95%C.I. 1.6-43.5; p=0.013 and OR= 4,0; 95%C.I. 1.0-15.4; p=0.043, respectively). Regarding obtaining response at 6 months in patients carrying both the *TLR4* G-allele and *ABCB1* T-allele and the *TLR4* G-allele alone no significance was found.

Discussion

In this study on the association between functional genetic variants and MTX treatment outcome in RA patients, it is shown that toxicity was potentially associated with *ABCB1* 3435C/T and *TLR4* +896A/G. However, none of these associations remained significant after correction for multiple testing. No significant associations of *DHFR* 829C/T, *ABCB1* 3435C/T, *ITPA* IVS2 +21A/C, *HLA-G* (-/+14bp), *IMPDH2* +787C/T, *TGFB1* +869T/C and *TLR4* +896A/G with MTX efficacy were found.

The genetic polymorphisms included in this study were selected based upon literature findings on their proven functional consequences of the variant.

The *ABCB1* (*MDR-1*) gene product P-glycoprotein (P-gp) is a member of the ATP-binding cassette (ABC) superfamily of membrane proteins which functions as an exporter of xenobiotics from cells. Despite the fact that it is yet unclear whether MTX is a substrate for P-gp, it was demonstrated in in-vitro studies that the *ABCB1 3435C>T* variant alters the drug specificity of P-gp in general (35). Further, it was shown that this genetic variant decreases the mRNA stability, expression and hence the activity of the efflux-transporter P-gp (36-38). In this way, hypothetically *ABCB1* 3435C>T could influence MTX treatment by a decreased MTX cellular efflux. However, studies have demonstrated conflicting results regarding a relation between P-gp expression and resistance of MTX therapy (39;40). Future research on this topic has to be performed.

Interestingly, in one in-vitro study (23), an other functional change of *ABCB1* 3435C>T has been demonstrated: this SNP is (along with *ABCB1* 2677 G>T) of influence on the release of several inflammatory cytokines, from mononuclear cells treated with MTX and dexamethasone. Specifically, the release of IFN-γ, IL-2, IL-4 and TNF-alpha at cellular level in subjects with the haplotype pair 3435TT-2677TT was significantly decreased in comparison with subjects with other haplotypes. It was hypothesized that this may lead to a reduced incidence and activity of RA and higher probability of remission of disease symptoms after treatment with MTX in patients with the 3435TT-2677TT haplotype. This would mean that patients with the *ABCB1* 3435TT before treatment of MTX, have a lower disease activity compared to patients genotyped for *ABCB1* CC and *ABCB1* CT. However, in our cohort no significant differences in baseline DAS between ABCB1 genotypes were seen.

Previously, in clinical studies conflicting results have been found regarding the relationship of *ABCB1* 3435 C>T and MTX efficacy in RA patients (table 3). In our data, only a trend towards significance was seen in the analysis of *ABCB1* 3435C>T and our primary efficacy parameter (DAS of ≤2.4 at 6 months) (table 2). This result strengthens the insignificant results seen in the study of Bohanec Grabar et al (27). However, an opposite result was reported by Drozdzik et al (28), which found that *ABCB1* TT was related with an increased likelihood of response according to ACR20. However, in our study a higher dosage, 7.5-25mg MTX (compared with 7.5-15 mg in the study of Drozdzik et al (28)), was given. Hypothetically, intergenotype differences within the SNP *ABCB1* 3435C>T will become apparent if lower dosage is prescribed. Specifically, in our cohort 77% of patients genotyped for *ABCB1* TT (N=52) had a dosage of more than 15 mg MTX. In this way, dosage could interfere with a possible effect of the mutation on treatment outcome.

	Author	N of patients (ethnicity/ country)	Dose regimen	Clinical endpoint	Reported results	Replication results in BeSt cohort
***ABCB1* 3435C>T**	Bohanec Grabar et al (27)	213 (Caucasian/ Slovenia)	10-14.4 mg/ weekly	EULAR criteria based on DAS28 (good, moderate and poor response and remission)	- No association with (in)efficacy MTX - TT associated with toxicity	- EULAR good response: NS - EULAR moderate response: NS - EULAR poor response: NS - EULAR remission: NS
	Drozdzik et al (28)	174 (Caucasian/ Poland)	7.5-15 mg/ weekly	ACR20	TT associated with efficacy MTX	- ACR20 at 3 and 6 months: NS
	Takatori et al (32)	124 (Asian/ Japan)	N.D.	Decrease in dose (≤6mg/week) during 3 months	TT associated with lower MTX maintenance dosage compared with CC/CT	N.A.
***HLA-G* ->14 bp**	Rizzo et al (25)	156 (Caucasian/ Italy	10 -15 mg/ weekly	EULAR criteria based on ΔDAS_{44} >0.6 or ≤0.6	-14bp/-14bp associated with efficacy MTX	- EULAR >0.6 and ≤0.6: NS
	Stamp et al (31)	130 (N.D./ New Zealand)	N.D.	EULAR criteria based on absolute DAS28 ≤3.2 or >3.2 (low disease activity)	No association with (in)efficacy MTX	- EULAR low disease activity: NS
***TGFB1* +869T>C**	Mattey et al (30)	208 (Caucasian/ United Kingdom)	N.D.	HAQ score (disease outcome)	CT associated with higher HAQ-score compared with CC[c]	- HAQ score at 3 and 6 months: NS
***TLR4* +896A>G**	Kuuliala et al (29)	169 (Caucasian/ Finland)	7.5-15 mg/ weekly	- EULAR criteria based on DAS28 <2.6 (remission) at 6 months - Change in absolute DAS28 at 3 and 6 months	No effect on efficacy with MTX as combination therapy[d]	-EULAR remission: NS - Change in absolute DAS28 at 3 and 6 months: NS

Table 3. Replication of results earlier reported in pharmacogenetic studies concerning MTX in RA patients[a,b,c,d]

a. Abbreviation(s): bp= basepair; *ABCB1*= multi-drug resistance-1; *HLA-G*= human leukocyte antigen-G; *TGFB1*= transforming growth factor β1; *TLR4*= toll-like receptor 4; N.A.= not available; N.D.= not defined; HAQ= Health Assessment Questionnaire; ACR= American College of Rheumatology; EULAR criteria= European League Against Rheumatism-criteria; DAS= Disease activity score
b. The SNPs *DHFR* -829 C>T and *IMPDH2* +787C>T could not be used for analysis due to the presence of 100% homozygous wildtype genotype
c. DMARDs: hydroxychloroquine, sulfasalazine, gold, or MTX
d. In combination with sulfasalazine, methotrexate, hydroxychloroquine and prednisolone

With regard to toxicity, experiencing ADEs at 6 months was more frequent in patients carrying the mutant allele of *ABCB1* 3435C>T in comparison with patients carrying homozygous wildtype. Previously, three studies concerning *ABCB1* 3435C>T and MTX toxicity have been performed

(27;32;41). Only, in the study of Bohanec Grabar et al (27), a comparable significant result was seen: patients with *ABCB1* TT genotype experienced more frequently ADEs than C-allele carriers.
HLA-G, represents the nonclassical major histocompatibility complex (MHC) class Ib molecules, and have demonstrated to play an important role as anti-inflammatory particles in autoimmune diseases (42). Since, it was reported that increased anti-inflammatory IL10 production was related with an improved clinical result to MTX (43;44) and that *HLA-G* and IL-10 are functionally correlated (e.g. IL-10 modulates *HLA-G* expression) (45-47), a pharmacogenetic role of the SNP *HLA-G* -/14bp in MTX treatment could be present. Indeed, it was demonstrated that a homozygous deletion of this SNP (-14bp/-14bp) within this gene was associated with *HLA-G* stability and regulation of sHLA-G production, induced by MTX (25;48).
Two studies explored the relationship between *HLA-G* genotype and MTX efficacy in RA patients. In the study of Rizzo et al (25), *HLA-G* -14bp/-14bp was associated with clinical response according to ΔDAS >0.6. In contrast, in a smaller cohort of patients (N=133), Stamp et al (31) found no significant relationship with response based upon obtaining low disease activity (absolute DAS28 ≤3.2).
The intracellular conversion of MTX into its polyglutamated form (MTXPGs) is thought be essential for the inhibition of folate enzymes, like DHFR, which converts dihydrofolate to tetrahydrofolate for purine synthesis and, consequently, for cellular proliferation of inflammatory cytokines. Despite it is unclear whether MTXPGs is of influence in the response to MTX, hypothetically, a genetic polymorphism within the DHFR gene can account for a defect in polyglutamation of MTX leading to a decreased response. Previously, the genetic variant *DHFR* 829C>T was associated with an increased expression of *DHFR* (21) leading to enzyme overproduction and hypothetically MTX resistance (22). Besides our report, so far no pharmacogenetic studies have been performed on *DHFR* 829C>T and MTX treatment outcome in RA patients.
Like DHFR, also both the enzymes IMPDH2 and ITPA are involved in the purine metabolism. *IMPDH2*, an isoform type 2 of *IMPDH2*, is involved in transformation of IMP (inosine 5' monophosphate) to XMP (xanthosine 5' monophosphate) .Expression of this type, compared to type 1, is significantly up regulated in rapidly proliferating cells involved in the inflammatory process. In contrast, *ITPA* catalyzes the conversion of ITP (inosine triphosphate) to IMP, whereas ITP is formed by phosphorylation of IMP. Deficiency of *ITPA* interrupts this cycle and possibly influences its balance with AMP and the release of adenosine, a potential anti-inflammatory agent (49). *IMPDH2* 787 C>T and *ITPA* IVS2 +21A>C, have demonstrated a negative effect on their respective gene expression resulting in diminished enzyme activity. Besides our study, so far no studies have related *IMPDH2* 787 C>T and *ITPA* IVS2 +21A>C with MTX treatment outcome in RA patients.
As a first line defence mechanism against endogenous ligands, *TLR*, like *TLR4,* are potentially involved in inflammatory conditions of RA (50). Specifically, *TLR* are highly expressed in RA synovium and higher inflammatory products upon TLR activation were demonstrated in RA patients(51;52). Polymorphisms within genes coding for TLR4, like *TLR4* +896 A>G, have been related to several other diseases (53). *TLR4* +896 A>G has been associated with the potency of mediating the magnitude of inflammatory response (53;54) and controversially of susceptibility to RA (55;56). Still, a relation with expression of TLR4 was not seen (54). Regarding an association of this SNP with MTX treatment outcome, only a pharmacogenetic effect of this SNP on sulphasalazine, not MTX, monotherapy outcome was seen (29).
Like TLR4, the cytokine TGFB1 has been reported to be of influence in many inflammatory diseases (57;58). In RA, TGFB1 is thought to down regulate the inflammatory response. Conflicting result have been reported regarding the functionality of the SNP *TGFB1* 869T>C in decreasing or increasing enzyme production (59-62). Regarding efficacy, overexpression of the *TGFB1* gene has been related to *TGFB1* 869 C>T and decreased disease activity possibly resulting in enhanced MTX response (30). Notably, a separate analysis of the association of this SNP with MTX monotherapy was

not reported. So far no studies have been performed, besides our report, which have analyzed a relation of TLR4 896A>G with toxicity in patients on MTX monotherapy.
In an earlier study, performed in the same cohort of patients, we developed a clinical pharmacogenetic model, including four genetic polymorphisms, in order to predict the response to MTX (20). This predictive algorithm showed a positive predictive value of 95% and a negative predictive value of 86%. Potentially, the seven SNPs included in the current study could be useful to refine and improve the model. However, since no significant results for association were found this study does not add novel parameters for the predicting MTX efficacy. Interestingly, the current study revealed two genetic polymorphisms potentially related with MTX toxicity and may therefore be useful in combination with our predictive model for MTX efficacy.
In this study, Bonferroni correction for multiple testing is applied. By using this conservative method, the chance of missing a genuine effect (type 2 error) is increased. However, if this correction was not applied, the chance of finding false positive results (type 1 error) would have been substantially large.
The relatively large cohort used in this pharmacogenetic study comprises well characterized patients with recent-onset RA treated with MTX monotherapy without prior DMARD usage. A robust and widely accepted measure for efficacy (DAS of ≤2.4 at 6 months) and registration of ADEs was applied (16). Four of the seven included genetic polymorphisms (*ABCB1* 3435C/T, *HLA-G* (-/+14bp), *TGFB1* +869T/C and *TLR4* +896A/G) were studied before in relation to MTX efficacy in RA patients but with inconclusive results. Therefore, the current study can partly be considered as a replication study. Importantly, the original studies applied different clinical endpoints for efficacy such as American College of Rheumatology (ACR) (63) and Health Assessment Questionnaire (HAQ) (64) (Table 3). Since from our clinical study database all these efficacy measures could be retrieved, we were able to replicate the studies using the previously described clinical endpoints for efficacy. With these analyses in our cohort, no significant results were detected for efficacy.
This analysis is important, as many replication studies use different endpoints as compared to the original studies which make them incomparable and leads to inconclusive results. However, beside endpoints, alternative factors such as population differences, dosage of MTX, co-medication (e.g. other DMARDs) and treatment- and disease duration may hamper the replication of explored associations in pharmacogenetic studies. Concerning ethnic differences, our DHFR and IMPDH2 genotyping results demonstrate that remarkable differences are seen between populations, since no mutant alleles were detected in our cohort. Generally, differences in study design could be an explanation for the findings in our cohort of patients.
We are convinced that the publication of replication studies is important. The publication of negative replication studies is even more important as to prevent publication-bias (65). Indeed, association studies for MTX treatment outcome have increasingly presented new genetic variants in the last decade, but replication of these variants could not be retained. Fortunately, growing evidence exists in acknowledging negative associations and replications (65). In this way, along with the performance of genome wide association studies, false positive findings could be reduced and evidence of a genetic effect could be declared.
Furthermore, analyses in our cohort revealed a lower effect size in experiencing ADEs in the *ABCB1* TT group compared with patients genotyped for *ABCB1* TT in the study of Bohanec Grabar et al (27). In general, it is demonstrated that the initial reported effect size is overestimated of the true effect size in genetic association studies (66). Consequently, this could account for the different degree of effect sizes in experiencing overall toxicity demonstrated within our replication cohort of patients and the cohort of Bohanec Grabar et al (27). Ideally, if multiple studies are performed on one specific genetic variant, a meta-analysis should be performed in order to calculate an effect size, which is close to a genuine effect size. Alternatively, but a more extensive approach is a pooled data

analysis, which overcomes heterogeneity between patient cohorts in order to clarify the role of the variant in drug response.

In conclusion, in this study it is demonstrated that in RA patients treated with MTX toxicity was potentially associated with *ABCB1* 3435C/T and *TLR4* +896A/G. However, none of these associations remained significant after correction for multiple testing. Further, no significant associations of *DHFR* 829C/T, *ABCB1* 3435C/T, *ITPA* IVS2 +21A/C, *HLA-G* (-/+14bp), *IMPDH2* +787C/T, *TGFB1* +869T/C and *TLR4* +896A/G with efficacy were found.

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