Personalized medicine in rheumatoid arthritis

Eektimmerman, F.

Personalized medicine in rheumatoid arthritis

Frank Eektimmerman

The research presented in this thesis was performed at the department of Rheumatology and the department of Clinical Pharmacy and Toxicology of Leiden University Medical Center, Leiden, The Netherlands.

Financial support for publication of this thesis was provided by departmental funds from the department of Clinical Pharmacy & Toxicology of the Leiden University Medical Center, Leiden, the Netherlands.

Cover	Paulien Frederiek Lipman
Layout	Renate Siebes | Proefschrift.nu
Printed by	Proefschriftmaken.nl | De Bilt
ISBN	978-94-90791-89-6

Personalized medicine in rheumatoid arthritis

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op woensdag 11 mei 2022
klokke 16.15 uur

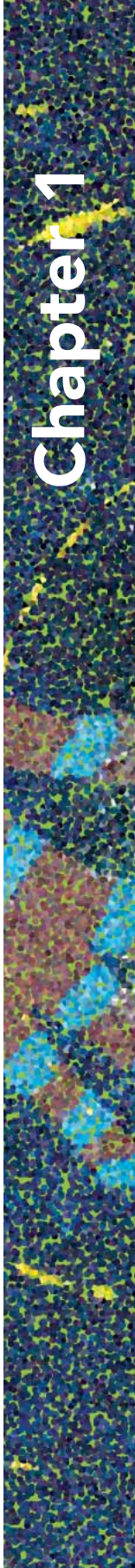
door

Frank Eektimmerman

geboren te Almelo
in 1987

Promotor	Prof.dr. H.-J. Guchelaar
Copromotores	Dr. J.J. Swen Dr. C.F. Allaart
Promotiecommissie	Prof.dr. T. van Gelder Prof.dr. A.H.M. van der Helm - van Mil Prof.dr. E.N. van Roon (University of Groningen, Groningen) Prof.dr. J.G.W. Kosterink (University of Groningen, Groningen) Dr. M. Teichert

Contents



General introduction

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune disease characterized by pain, multiple joint swelling, and stiffness.[1,2] Untreated RA results in severe joint destruction, dysfunctionality, and eventually bone erosion.[3,4] Furthermore, RA leads to an increased prevalence of comorbidities such as cardiovascular diseases, depression, and pulmonary diseases and a threefold elevated risk of cardiovascular and respiratory mortality.[5–8] RA is an incurable disease, whereby the goals of drug treatment are to relieve pain, reduce inflammation and to reach a low-disease activity ("remission"), and prevent joint damage. Therefore, it is crucial for delaying the development of the disease by prompt diagnosis with early (aggressive) treatment.

The classification criteria of RA were first developed in 1987 (ACR 1987 criteria)[9] and updated in 2010 (ACR/EULAR 2010 criteria),[10] see Figure 1-1. Particularly, the 2010 criteria identified RA patients that present the first signs and symptoms of the disease. Consequently, RA patients with recent-onset inflammatory arthritis will be identified earlier and receive treatment at an earlier stage. The ACR/EULAR 2010 classification criteria consist of joint involvement, serology testing, acute-phase reactants, and symptom duration. The classification criteria of RA are well accepted by rheumatologists and are used as inclusion criteria in clinical trials.

MEASUREMENTS OF THE DISEASE ACTIVITY

Several standardized measurements for the assessment of the disease activity are available. The variety of disease subsets results in the use of multiple measurements to evaluate the status and activity of RA. A commonly used index that measures RA activity is the Disease Activity Score (DAS) or the DAS in 28 joints (DAS28). This measurement assesses the number of tender and swollen joints, general health status, and the erythrocyte sedimentation rate (ESR).

The DAS indicates the activity of RA by providing a score, in which a higher score indicates higher disease activity.[11,12] The DAS measures 44 joints, while the DAS28 measures 28 joints. The DAS28 is a validated simplified measurement tool, which makes it in practice more feasible (Formula 1-1). However, a combination of both measurements is used to support clinical decision-making.

$$DAS28 = 0.56 \times \sqrt{(TJC28)} + 0.28 \times \sqrt{(SJC28)} + 0.70 \times \ln(ESR) + 0.014 \times VASGH$$

Formula 1-1. DAS28 formula.
Abbreviations: tender joint count (TJC28), swollen joint count (SJC28), erythrocyte sedimentation rate (ESR) and the patient's assessment of global general health (VASGH).

ACR 1987 criteria	ACR/EULAR 2010 criteria	
1. Morning stiffness Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement	**A. Join involvement (tender/swollen)** 1 large joint 2–10 large joints 1–3 small joints (with or without large joints) 4–10 small joints (with or without large joints) >10 joints (at least one small joint)	 0 1 2 3 5
2. Arthritis of ≥3 joint areas At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints	**B. Serology** Negative RF and ACPA Low-positive RF or low-positive ACPA High-positive RF or high-positive ACPA	 0 2 3
3. Arthritis of hand joints At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint	**C. Acute phase reactants** Normal CRP and ESR Abnormal CRP or abnormal ESR	 0 1
4. Symmetric arthritis Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)	**D. Duration of symptoms** <6 weeks ≥6 weeks	 0 1
5. Rheumatoid nodules Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician		
6. Serum RF Demonstration of abnormal amounts of serum RF by any method for which the result has been positive in <5% of normal control subjects		
7. Radiographic changes Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)		
For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 or these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded. Designation as classic, definite, or probable rheumatoid arthritis is not to be made.	The criteria are meant to be applied in patients with at least one swollen joint, after the exclusion of the other causes of synovitis. Patients with a score ≥6 are classified as having RA. Also subjects with typical bone erosions can be classified as RA regardless of the score.	

Figure 1-1. Classification criteria for RA.
Abbreviations: ACPA: anti-cyclic citrullinated peptide antibodies, ACR: American College of Rheumatology, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, EULAR: European League against Rheumatism, MCP: metacarpophalangeal, MTP: metatarsophalangeal, PIP: proximal interphalangeal, RF: rheumatoid factor.

Disease activity is interpreted as low (DAS≤2.4), moderate (DAS2.4 to ≤3.7), or high (DAS>3.7).[12] Based on the DAS, the EULAR response criteria have been developed. The EULAR response criteria combined the change and the level of the DAS. Responders are classified when they have a significant change in the DAS (of at least 1.2 DAS points) and have a low disease activity (DAS≤2.4). Remission, with the absence of disease activity and with the possibility of the recurrence of the disease activity predicts the best clinical, functional, and structural outcomes and is therefore frequently used in both clinical trials and clinical practice. The American Rheumatism Association (ACR) has a strict definition of remission: patients must have at least five or more fulfilled criteria for at least two consecutive months of either; morning stiffness not exceeding 15 minutes; no fatigue; no joint pain (by history); no joint tenderness or pain on motion; no soft tissue swelling in joints or tendon sheaths; erythrocyte sedimentation rate <30 mm/h (female) or <20 mm/h (male).[13] Due to the strict definition, attainment is very rare in RA trials, and therefore, the DAS or DAS28 are used, whereas the DAS28<2.6 or DAS<1.6 represents remission.[12]

Improvement in RA can also be determined by the ACR response criteria. Here, improvement is 20, 50 or 70% or more, referred as ACR20, ACR 50, or ACR70 respectively. The ACR20 improvement criteria are standard for DMARD trials, that consisted of ≥20% of improvement in the tender joint count and swollen joint count plus ≥20% improvement in three of the following: patient pain, patient global disease activity, physician global disease activity, physical function, e.g., HAQ-DI and acute-phase reactants, ESR, or C-reactive protein (CRP).[14] Different endpoints can be used, but using the ACR or EULAR response criteria are almost equal with a discrepancy of less than 5%.

PREDICTIVE BIOMARKERS IN RA

Biomarkers in RA are molecules of the immune system that can be used to measure the level of inflammation, the presence or progress of the disease, or the responsiveness of a patient to drug treatment. The first biomarker found in RA was rheumatoid factor (RF). RF is detectable in the blood of circa 80% of adults with RA and is mainly an IgM antibody directed against the FC portion of IgG. Higher RF titers suggest more severe disease manifested as radiographic joint damage, poorer functional status (DAS), and developing extra-articular manifestations, such as rheumatoid nodules or rheumatoid lung disease. There are, however, exceptions, particularly among patients with chronic inflammatory disorders, such as systemic lupus erythematosus, Sjögren syndrome, and bacterial diseases like bacterial endocarditis, leprosy, tuberculosis, syphilis, and malaria.[15]

Another predictive biomarker for RA is the immune regulator C-reactive protein (CRP). CRP is an acute-phase protein, that will rise in the circulation in the presence of an infection, an injury, or a chronic disease. CRP measurements are used in the management and prognosis of RA because patients with persistently high levels of CRP are at high risk of joint degradation and require more intensive drug treatment. Acceptable levels of CRP offer rheumatologists an indication of the therapeutic efficacy of the medication. Although CRP is commonly used as a diagnostic and predictive biomarker, it has the limitation that circa 40% of RA patients have normal levels of CRP, and elevated levels have been found in other conditions than RA such as infections and inflammatory bowel disease.[16–18]

The most commonly available biomarker in RA is the anti-cyclic citrullinated peptide (anti-CCP). Anti-CCPs, also known as anti-citrullinated protein antibodies (ACPA), are autoantibodies that are directed against peptides and proteins that are citrullinated (e.g. proteins with a conversion of the amino acid arginine into the amino acid citrulline). The advantage of anti-CCP over RF is that the specificity of anti-CCP is higher than RF (95% vs. 70%) because other inflammation or infections can raise the RF titers, while both have the same sensitivity (~50%). Also, both biomarkers may be present before symptoms of RA, but anti-CCP can be measured at an earlier stage of RA. Notable, 35% of patients with a negative RF may test positive for the anti-CCP antibody. Additionally, elevated ESR or CRP in early RA can be used as a predictor of greater radiographic joint damage and poorer functional status.[19]

TREATMENT OF RA

The disease-modifying anti-rheumatic drugs (DMARDs) suppress the inflammation and reduce joint swelling and erosion. DMARDs are divided into conventional (cDMARDs), targeted synthetic (tsDMARDs) and biological (bDMARDs), whereas the latter can be separated into original and biosimilar (boDMARDs and bsDMARDs, respectively).[20] In general, the precise mechanisms of action of the cDMARDs are only partially understood. Unlike bDMARDs and tsDMARD that selectively inhibit a pro-inflammatory cytokine or block its receptor, cDMARDs interfere with combinations of pathways in the inflammatory cascade. Among the cDMARDs, several are cytotoxic causing either cell death or impaired proliferation, such as azathioprine, cyclophosphamide, and methotrexate (MTX).

Treatment guidelines are based on the therapeutic benefits, the drug costs, and experience with the drug. The choice of drug treatment depends on the severity of the disease, the response to prior treatment, and contraindications. Because of the cost issues involved in the use of biological therapies and JAK inhibitors, rheumatologists initiate the treatment with

a csDMARD, mostly MTX. MTX is the most prescribed drug to treat RA, due to the efficacy, low costs, and multiple decades of experience. However, MTX and other csDMARDs are slow acting and take several weeks to exert their full effect. Therefore, anti-inflammatory agents, such as non-steroidal anti-inflammatory drugs and glucocorticosteroids, are added as concomitant therapy to rapidly suppress inflammation. When RA patients have an insufficient response to MTX, another DMARD will be used or will be combined with MTX.[21,22]

In recent years, tsDMARDS (JAK inhibitors) are introduced and applied in clinical trials and clinical practice. Compared to bDMARDs, tsDMARDs have the advantage that they are small molecules and thereby can be orally administered, while bDMARDs are large molecules and can only be given by injection (subcutaneously or intravenously). Moreover, not only the availability of a wide range of new drugs, such as TNF or JAK inhibitors leads to a better prognosis, but also the treat-to-target principle with the assessment of the disease activity. The disadvantage of the tsDMARDs and bDMARDs are the high cost and the increased susceptibility of infections or reactivation of tuberculosis or hepatitis B, and therefore, it is a necessity to screen for tuberculosis, hepatitis B and C, and HIV before starting drug therapy.[23]

PHARMACOGENETICS

Over the last decades, considerable progress has been made in addressing the role of genes in drug response or drug-related toxicity in RA. The results of pharmacogenetic testing may be used to predict therapeutic failure or (severe) adverse drug reactions, and therefore could be used to optimize the drug dose or choice, avoid adverse effects and decrease medical costs. MTX for instance is a good candidate for pre-treatment pharmacogenetic testing, because it is a highly toxic drug and the treatment effect can only be assessed after a substantial treatment time. Unfortunately, so far, pharmacogenetic tests in RA led to limited success with still unpredictable effectiveness or drug-related side effects.

The candidate gene approach has been widely applied to identify risk alleles and their association with the clinical response of drugs. This approach focuses on associations between pre-specified genes of interest, e.g. based upon the mechanism of action of the drug, and the disease state or a typical phenotype. In contrast to the hypothesis-free driven methods of the genome-wide association study (GWAS) and whole genome sequencing, that investigate the genome for common variants (Minor Allele Frequency [MAF] >5%) or the entire genome respectively. Still, there is a considerable amount of pharmacogenetic results that were not validated, due to lack of replication, or were not associated with a functional phenotype.

SCOPE OF THIS THESIS

This thesis aims at identifying and validating genetic variants related to the efficacy or toxicity of MTX or biological DMARDs in the treatment of RA. Ultimately, those findings benefit the patient, whereas adequate drug treatment without unnecessary toxicity could be achieved faster.

Chapter 2 gives an overview of known genetic variants associated with MTX efficacy and results are divided into eight different pathways related to the mechanism of action of MTX. All previously associated significance results (p-values) are adjusted for multiple testing by Bonferroni correction. The goal of this systematic review is to explore which SNPs related to MTX pharmacology are associated with efficacy in RA by selecting only studies with the validated endpoints DAS(28), EULAR, or ACR response criteria.

The transporter solute carrier (SLC) is responsible for transporting MTX from the blood into the cell, where MTX exerts its function after polyglutamation. The focus in **Chapter 3** is on four genetic variants in the SLC gene, that might be associated with the efficacy or toxicity of MTX.

In **Chapter 4** an earlier developed pharmacogenetic model, including four genes in the folate pathway in combination with the variables gender, baseline DAS, smoking status, and RF positivity is validated in patients treated with combination therapies. While the initial model is applied in an MTX monotherapy cohort, nowadays, patients are increasingly treated with combination therapies. Therefore, this study aimed to test the performance of the model in patients treated according to daily clinical practice (MTX combination therapies).

In **Chapter 5** a genome-wide association study (GWAS) with over 600,000 common genetic variants is performed to identify genetic loci associated with MTX-induced hepatotoxicity injury (ALT level of ≥3X upper level of normal). For this study, cases and controls were collected from seven international research groups.

In **Chapter 6**, 223 genetic variants are tested with the efficacy (EULAR good response and EULAR remission) of adalimumab. This pathway selection method contained 223 genetic variants in 124 genes related to the mechanism of action of adalimumab.

A general discussion about genetic testing in RA and future perspectives is presented in **Chapter 7**. Finally, an English and Dutch summary of this thesis are described in **Chapter 8**.

REFERENCES

1. Choy E. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. Rheumatology (Oxford) 51 Suppl 5, v3-11 (2012).
2. Gibofsky A. Overview of epidemiology, pathophysiology, and diagnosis of rheumatoid arthritis. Am. J. Manag. Care 18, S295–S302 (2012).
3. Agarwal SK. Biologic agents in rheumatoid arthritis: an update for managed care professionals. J. Manag. Care Pharm. 17, S14–S18 (2011).
4. Sen D, Paul JR, Ranganathan P. Pharmacogenetics in rheumatoid arthritis. Methods Mol. Biol. 1175, 625–660 (2014).
5. Dougados M, Soubrier M, Antunez A *et al*. Prevalence of comorbidities in rheumatoid arthritis and evaluation of their monitoring: results of an international, cross-sectional study (COMORA). Ann. Rheum. Dis. 73, 62–68 (2013).
6. Sparks JA Chang SC, Liao KP *et al*. Rheumatoid Arthritis and Mortality Among Women During 36 Years of Prospective Follow-Up: Results From the Nurses' Health Study. Arthritis Care Res. (Hoboken). 68, 753–762 (2016).
7. Aviña-Zubieta JA, Choi HK, Sadatsafavi M *et al*. Risk of cardiovascular mortality in patients with rheumatoid arthritis: a meta-analysis of observational studies. Arthritis Rheum. 59, 1690–1697 (2008).
8. Gibofsky A. Epidemiology, pathophysiology, and diagnosis of rheumatoid arthritis: A Synopsis. Am. J. Manag. Care 20, S128–S135 (2014).
9. Arnett FC, Edworthy SM, Bloch DA *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 31, 315–324 (1988).
10. Felson DT, Anderson JJ, Boers M *et al*. American College of Rheumatology. Preliminary definition of improvement in rheumatoid arthritis. Arthritis Rheum. 38, 727–735 (1995).
11. van Riel PLCM, Renskers L. The Disease Activity Score (DAS) and the Disease Activity Score using 28 joint counts (DAS28) in the management of rheumatoid arthritis. Clin. Exp. Rheumatol. 34, S40–S44 (2016).
12. Fransen J, Stucki G, van Riel PLCM. Rheumatoid arthritis measures: Disease Activity Score (DAS), Disease Activity Score-28 (DAS28), Rapid Assessment of Disease Activity in Rheumatology (RADAR), and Rheumatoid Arthritis Disease Activity Index (RADAI). Arthritis Rheum. 49, S214–S224 (2003).
13. Pinals RS, Masi AT, Larsen RA. Article preliminary criteria for clinical remission in rheumatoid arthritis. Arthritis Rheum. 24, 1308–1315 (1981).
14. Aletaha D, Neogi T, Silman AJ *et al*. 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/ European League Against Rheumatism collaborative initiative. Arthritis Rheum. 62, 2569–2581 (2010).
15. Ingegnoli F, Castelli R, Gualtierotti R. Rheumatoid factors: Clinical applications. Disease Markers 35, 727–734 (2013).
16. Pope JE, Choy EH. C-reactive protein and implications in rheumatoid arthritis and associated comorbidities. Semin. Arthritis Rheum. 51, 219–229 (2021).
17. Shervington L, Darekar A, Shaikh M, Mathews R, Shervington A. Identifying Reliable Diagnostic/Predictive Biomarkers for Rheumatoid Arthritis. Biomark Insights 13, 11772719 18801005 (2018).
18. Maksymowych WP. Biomarkers for diagnosis of axial spondyloarthritis, disease activity, prognosis, and prediction of response to therapy. Front. Immunol. 10, 305 (2019).

19. Samanci N, Ozdem S, Akbas H *et al*. Diagnostic value and clinical significance of anti-CCP in patients with advanced rheumatoid arthritis. J. Natl. Med. Assoc. 97, 1120–1126 (2005).

20. Smolen JS, van der Heijde D, Machold KP, Aletaha D, Landewé R. Proposal for a new nomenclature of disease-modifying antirheumatic drugs. Ann. Rheum. Dis. 73, 3–5 (2014).

21. Smolen JS, Landewé RBM, Bijlsma JWJ *et al*. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2019 update. Ann. Rheum. Dis. 79, 685–699 (2020).

22. Rein P, Mueller RB. Treatment with Biologicals in Rheumatoid Arthritis: An Overview. Rheumatol. Ther. 4, 247–261 (2017).

23. Harrington R, Al Nokhatha SA, Conway R. Jak inhibitors in rheumatoid arthritis: An evidence-based review on the emerging clinical data. J. Inflamm. Res. 13, 519–531 (2020).

Predictive genetic biomarkers for the efficacy of methotrexate in rheumatoid arthritis: a systematic review

Frank Eektimmerman
Jesse J. Swen
Moenira B. Madhar
Cornelia F. Allaart
Henk-Jan Guchelaar

Pharmacogenomics J. 20, 159–168 (2020)

Abstract

Multiple pharmacogenetic studies investigated the effectiveness of methotrexate. However, due to the use of non-validated outcomes, lack of validation or conflicting results it remains unclear if genetic markers can help to predict response to MTX treatment. Therefore, a systematic review was performed. PubMed was searched for articles reporting potential pharmacogenetic biomarkers associated ($p<0.05$) with MTX efficacy using the validated endpoints DAS(28), EULAR, or ACR response criteria. The PICO method was used for study selection, and PRISMA guidelines to prepare the report. Thirty-five studies met the inclusion criteria, providing 39 potential genetic biomarkers in 19 genes. After Bonferroni correction, six genetic biomarkers were associated with the efficacy of MTX: *ATIC* rs7563206; *SLC19A1* rs1051266; *DHFR* rs836788; *TYMS* rs2244500, rs2847153, and rs3786362 in at least one study. Only *SLC19A1* rs1051266 was replicated in an independent cohort and promising for predicting methotrexate efficacy.

INTRODUCTION

Low-dose methotrexate (MTX) is considered the "anchor drug" for the treatment of rheumatoid arthritis (RA). The precise mechanism of action of MTX remains to be elucidated, but it is known that MTX is transported over the membrane by multiple solute carriers (SLC) and that intracellular MTX has to be bound to polyglutames molecules by folylpolyglutamate synthase (FPGS) to exert its function. As illustrated in Figure 2-1, the polyglutamated MTX affects multiple cellular pathways, e.g., adenosine, *de novo* purine synthesis, folate, methionine, and *de novo* pyrimidine synthesis.

In particular, an essential function of the folate pathway is to provide cofactors for key enzymes, such as dihydrofolate reductase (DHFR) that converts dihydrofolate into the folic acid derivative tetrahydrofolate (THF). THF and other derivatives are required for the purine and pyrimidine synthesis, which are important for cell proliferation and cell growth.[1] The methionine pathway is responsible for the synthesis of adenosine, which is an anti-inflammatory agent, alterated by methionine synthase and methionine synthase reductase (MTRR). Further, methionine is a precursor for S-adenosyl-methionine, which is a methyl donor that serves a variety of cellular functions, including DNA methylation.[2] The ubiquitin pathway is not directly related to the other pathways, but has an essential function in homeostasis and recognition of MHC class 1 for the cytotoxic T cells.[3]

Approximately one-third of RA patients experience insufficient clinical response to MTX. Pharmacogenetics studies the impact of genetic variation to drug response and genetic variants in the MTX pathways described above may affect the potential effects of methotrexate on inflammation in RA. Indeed, multiple studies reported associations between single nucleotide polymorphisms (SNPs) and the efficacy of MTX. However, to date, none of the proposed markers are applied in clinical practice due to lack of validation or conflicting results. In addition, previous systematic reviews[4–10] described the effect of SNPs on the efficacy of MTX, but some included studies with MTX in different diseases such as juvenile idiopathic arthritis[10] or leukemia[5] or applied non-validated endpoints, such as red blood cell MTX polyglutamate concentrations[5,11] or physicians' assessment of patient's response.[9]

The goal of this review is to systematically explore which SNPs related to MTX pharmacology are associated with efficacy in RA by selecting only studies with the validated endpoints DAS(28), European League Against Rheumatism (EULAR), or American College of Rheumatology (ACR) response criteria.[12,13]

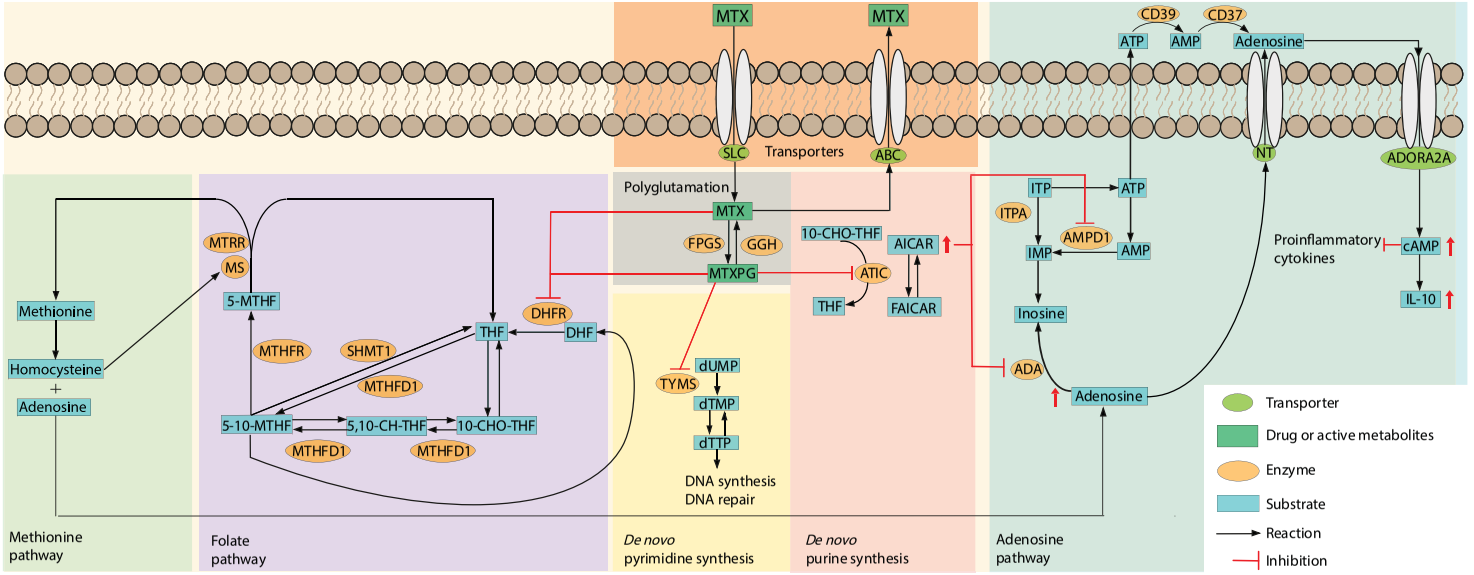


Figure 2-1. Intracellular MTX mechanism pathway, divided into the methionine, folate, *de novo* pyrimidine synthesis, *de novo* purine synthesis, and adenosine pathway.

Abbreviations: 10-CHO-THF: 10-formyltetrahydrofolate, 5,10-CH-THF: 5,10-methylenetetrahydrofolate, 5-MTHF: 5-methyltetrahydrofolate, ABC: ATP-binding cassette transporter, ADA: adenosine deaminase, ADORA2A: adenosine A2A receptor, AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide, AMP: adenosine monophosphate, AMPD1: adenosine monophosphate deaminase 1 ATIC 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, CD37: transmembrane protein, CD39: transmembrane protein, DHF: dihydrofolate, DHFR: dihydrofolate reductase, dTMP: deoxythymidine monophosphate, dTTP: deoxythymidine triphosphate, dUMP: deoxyuridine monophosphate FAICAR 5-formamidoimidazole-4-carboxamide ribotide, FPGS: folylpolyglutamate synthase, GGH: γ-glutamyl hydrolase, IL-10: interleukin-10, IMP: inosine monophosphate, IPTA: inosine triphosphatase, MS: methionine synthase, MTHFD1: methylenetetrahydrofolate dehydrogenase 1, MTHFR: methylene tetrahydrofolate reductase, MTRR: methionine synthase reductase, MTX: methotrexate, MTXPG: methotrexate polyglutamate, NT: nucleoside transporter, SHMT-1: serine hydroxymethyltransferase 1, SLC: solute carrier, THF: tetrahydrofolate, TYMS: thymidylate synthase.

METHODS

Data extraction and identification of eligible studies Identification and selection of studies were performed according to the PICO method.[14] Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were used to prepare the report.[15] PubMed was used to identify and extract all relevant articles published between April 2002 and March 2017. Search terms consisted of rheumatoid arthritis, methotrexate, pharmacogenetics, and SNP. The full search string is provided in Supplementary File S2-1. Also, we manually checked reference lists from reviews to identify relevant cross-references.

Records were screened on title and abstract. Comments, editorials, narrative reviews, letters (without original data), abstracts, and publications in languages other than English were excluded. Only studies utilizing the DAS(28), the response criteria of the ACR or the EULAR were eligible for inclusion. Included SNPs were analyzed under the additive, allelic, genotypic or haploid genetic model, and had at least one association with either DAS(28), ACR or EULAR response ($p<0.05$, uncorrected for multiple testing). SNPs were divided into MTX-related pathways: adenosine, *de novo* purine synthesis, transporters, polyglutamation, folate, methionine, *de novo* pyrimidine synthesis, and ubiquitin. Results from included studies were summarized, and reported odds ratio (OR) with 95% confidence interval (CI), p-value, type of association and SNP ID were collected. Finally, SNPs were checked on linkage disequilibrium by SNP Annotation and Proxy Search (SNAP, Broad Institute),[16] with the LD threshold of $R^2>0.8$.

To control the risk of false positive findings, Bonferroni correction was applied when no correction for multiple testing was performed in the original study by calculating a significant cutoff p-value at α/n ($p=0.05$ divided by the number of tested SNPs within each study). SNPs were significantly associated if the p-value was <0.05 after Bonferroni correction. Ultimately meta-analyses were used to support our findings of potential significant SNPs.

RESULTS

Study selection

Figure 2-2 shows the results of the study selection. Initially, 115 publications were identified. We excluded 30 comments, editorials, letters, narrative reviews, and seven non-English written publications. Of the remaining 78 studies, 41 were excluded because none of our defined endpoints was reported and one because the report of the study could not be obtained. By cross-references, three more studies were included. In total, 35 original studies were available for analysis in this systematic review and seven meta-analyses were used to support our findings.

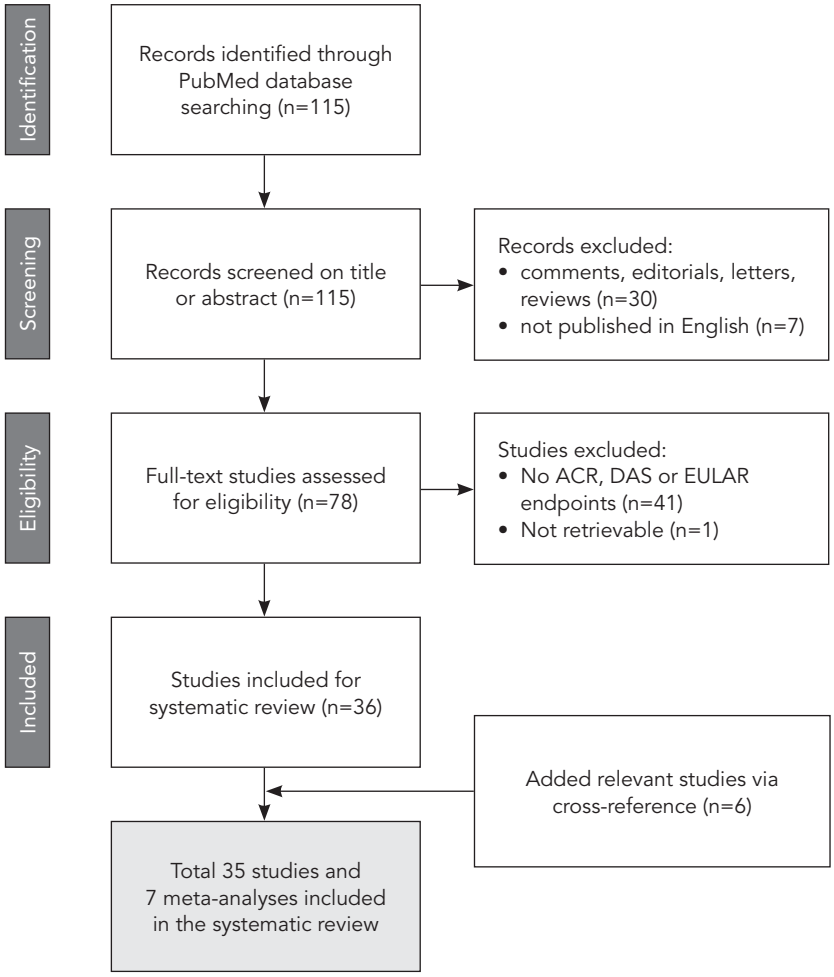


Figure 2-2. Study flow diagram of the systematic review inclusion.[15]
Abbreviations: MTX: methotrexate, MAF: minimum allele frequency, ACR: American College of Rheumatology, DAS: Disease Activity Score, EULAR: European League Against Rheumatism.

Study characteristics

Most studies (34 out of 35) were candidate gene studies investigating 1–35 polymorphisms. There was one genome-wide association study (GWAS) investigating 559,007 polymorphisms.[17] The mean study population of the studies was 197 patients (ranging from 48 to 422 patients). Most studies used the EULAR good response criteria (32%), tested <10 SNPs (76%), were conducted in Europe with RA patients of (self-)reported Caucasian origin. The average rate of good EULAR response to MTX monotherapy at t=6 months was 55%, ranging from 23[18] to 85%.[19]

The included studies reported 39 SNPs in 20 genes associated with either DAS(28), EULAR, or ACR response with a p<0.05. After Bonferroni correction, 16 SNPs in 10 genes remained significantly associated with MTX efficacy.

Adenosine pathway – *ADA*, *ADORA2A*, *AMPD1*, and *ITPA*

AMPD1 rs17602729 (allelic T) showed a significant association with DAS28≤3.2 (OR: 6.73, 95% CI: 1.74–26.01) between t=3 and 6 months.[20] However, this was not confirmed with the genotypic CC model at t=6 months.[21] None of the other SNPs in the adenosine pathways – *ADA* (rs244076), *ADORA2A* (rs5751876), and *ITPA* (rs1127354) – were significantly associated with the MTX response at t=6 months using allelic or genotypic genetic models.

De novo* purine synthesis – *ATIC

Four SNPs in *ATIC* (rs2372536,[22] rs4673993,[23] rs7563206,[1] and rs12995526[1]) had at least one study reporting a significant association with MTX efficacy. *ATIC* rs7563206 (allelic T-carrier) was tested in one study, and showed an association with MTX non-response with the endpoint DAS28≤3.2 at t=6 months (OR: 0.20, 95% CI: 0.09–0.46).[1] At t=6 months, *ATIC* rs4673993 (genotypic TT) showed a significant association with a better response (DAS28≤3.2, OR: 3.86 95% CI:1.50–9.91), while rs12995526 (allelic T-carriers) showed a significant association with a worse response (DAS28≤3.2, OR: 0.23, 95% CI: 0.10–0.53) to MTX.[23]

ATIC (rs2372536, genotype CC) was significantly associated with DAS≤2.4 at t=6 months, with an OR of 2.5 (95% CI: 1.3–4.8).[22] Three other studies – using *ATIC* rs2372536 genotypic CC at t=6 months – reported no significant association, of which one study reported that the CC genotype was related to MTX non-response with a OR below 1.0 (OR: 0.27, 95% CI: 0.08–0.92).[1,20,24]

Transporters – *ABCB1C1*, *ABCC1*, *SLC19A1* (*RFC1*), and *SLC22A11*

None of the SNPs in *ABCB1* (rs1045642), *ABCC1* (rs246240 and rs3784864), and *SLC22A11* (rs11231809) were significantly associated with DAS28≤3.2 or EULAR good response at t=6 months. The most studied genetic *SLC19A1* SNP was rs1051266, which was investigated in 11 studies. Three studies reported a significant association with MTX efficacy at t=6 months using ACR20 or DAS28 and different genetic models (either allelic A-carriers, genotypic GG or genotypic AA). Other studies did not investigate the same genetic models, using the same efficacy endpoints with the same time evaluation point for *SLC19A1* rs1051266.

Polyglutamation – *FPGS* and *GGH*

FPGS rs4451422 (allelic C-carriers) was associated with MTX efficacy using EULAR good response at t=6 months, with an OR of 0.73 (0.54–0.98).[17] *FPGS* SNPs (rs1544105, rs10106, and rs10987742) and *GGH* SNPs (rs2305558 and rs1800909) were not significantly associated with MTX efficacy.

Folate pathway – *DHFR*, *MTHFR*, and *SHMT*

Both *MTHFR* rs1801131 (A1298C) and rs1801133 (C677T) have frequently been studied (>10 studies). One study showed a significant association with *MTHFR* rs1801133 CC genotype with DAS28 ≤.2 at t=6 months, with an OR of 3.4.[25] Three other studies investigated the association of *MTHFR* genotypic CC at t=6 months, and did not find an association using other endpoints (EULAR GR, ΔDAS44 <0.6, and ACR20).[26–28] For two other SNPs in *MTHFR* (rs17421511 and rs1476413) there was no significant association with MTX response. Also, no association was found between *MTHFD1* rs17850560 or *SHMT-1* rs1979277 with MTX response using DAS28 (≤3.2) or EULAR GR. *DHFR* rs836788 was associated in one study with EULAR response at t=6 months, with an OR of 1.44 (95% CI: 1.09–1.93) and 1.47 (95% CI: 1.09–1.96), respectively for the allelic A-carriers and the genotypic AA.[17]

Methionine pathway – *MTR* and *MTRR*

Six studies investigated the role of the *MTR* A2756G (rs1805087), of which one study reported a significant association.[19] Here, *MTR* rs1805087 was associated with MTX efficacy at t=12 month, and the use of the endpoint EULAR good response with the genotypic AA (OR was not available). Other studies could not confirm the association with rs1805087, using the DAS28 with genotypic AA on t=4 months,[29] EULAR GR with the allelic G-carriers on t=4 months,[30] or with the DAS28≤3.2 allelic G-carriers on t=6 months.[31] No significant association was reported with *MTRR* rs162040 and rs1801394.

De novo* pyrimidine pathway – *TYMS

TYMS rs2244500, rs2847153, and rs3786362 were all significantly associated with EULAR good response at t=6 months and had OR of resp. 1.48, 0.68, and 0.51.[17] No other studies investigated the effect of *TYMS* with MTX response.

Ubiquitin pathway – *CUL1*

Negi *et al*. investigated the association of *CUL1* haplotypes with MTX efficacy using the DAS28≤3.2 at t=6 months.[32] Here, *CUL1* rs122571 haplotype A-T-T (OR: 2.83, 95% CI: 1.33–6.04) and rs243480 haplotype G-T-T (OR: 0.16, 95% CI 0.04–0.67) were significant.

***KIR* – gene**

One study tested multiple length variants of the KIR gene and showed that the full-length *KIR2DS4* gene was significantly associated with DAS28≤2.5 (OR: 0.4344, 95% CI: 0.215, 0.987) at t=6 months.[33] Here, possessing the KIRSDS4 gene had a lower chance of responding to MTX treatment.

Most promising genetic variants related to MTX efficacy

Table 2-1 lists the most promising SNPs that were significantly associated with MTX efficacy after Bonferroni correction without having conflicting results from other studies. For instance, it is ATIC rs467393 genotypic TT with better response, while allelic T-carriers results in worse response or lacks validation.

The most promising SNPs were derived from the pathways *de novo* purine (*ATIC*), *de novo* pyrimidine (TYMS), and transporters (SLC19A1). The SNPs have a minor allele frequency >0.2, except TYMS rs3786362 (MAF<0.2 for all races). ATIC rs7563206 and TYMS rs2244500 were found significantly associated with an OR below 1.0, while the other eight SNPs had an OR between 1.42 and 2.83. The used genetic models were with either allelic, genotypic or haplotype. No linkage disequilibrium (R^2>0.8) was observed for any of the SNPs in Table 2-2. SLC19A1 rs1051266 was tested in multiple studies and positively associated in three studies.

Of the six promising SNPs, ATIC rs7563206, TYMS rs2847153, and rs3786362 were associated with non-response to MTX, while SLC19A1 rs1051266, DHFR rs836788, and TYMS rs2244500 were associated with response to MTX. ORs range from 0.2 to 0.68 for MTX non-response and 1.42–2.76 for MTX response. The six SNPs had a MAF of >0.2 in all races except for TYMS rs3786362 which is sparse and even does not occurred in the European population.

Despite the findings of one significant association of ATIC rs473993 and rs12995526, AMPD1 rs17602729, MTHFR rs1801133, and MTR rs180508, and FPGS rs4451422, we did not mark those as promising genetic variants due to conflicting results. Also, we did not include the full-length KIR2DS4 gene as a promising genetic marker for the response to MTX, due to the complexity of the determination of the whole KIR2DS4 gene (with 15,894 bases) and the fact that it is not one SNP. This was also the case of CUL1 that was significantly associated with MTX response for two haplotypes; A-T-T (rs122571) and G-T-T (rs243480).

Table 2-1. Genetic biomarkers related to MTX efficacy

Gene	SNPs	Genetic model	Endpoint	Time of response evaluation (months)	N	Reported P-value	OR (95%CI)	Study
Adenosine pathway								
ADA	rs244076	Allelic A carriers	EULAR GR	6	281	**0.02**	1.66 (1.01–2.75)	Sharma (2009)[21]
		Genotypic AA	EULAR GR	6	281	0.17	-	Sharma (2009)[21]
ADORA2A	rs5751876	Allelic C carriers	EULAR GR	6	281	**0.04**	1.55 (1.01–2.37)	Sharma (2009)[21]
		Genotypic TT	EULAR GR	6	281	0.12	-	Sharma (2009)[21]
AMPD1	rs17602729	Allelic T carriers	DAS≤2.4	6	204	**<0.05**	2.1 (1.0–4.5)	Wessels (2006-2)[22]
	(C34T)	Allelic T carriers	DAS28≤3.2	3–6	205	**0.006***	6.73 (1.74–26.01)	Grabar (2010)[20]
		Allelic C carriers	EULAR GR	6	281	0.39	-	Sharma (2009)[21]
		Genotypic CC	EULAR GR	6	281	0.38	-	Sharma (2009)[21]
ITPA	rs1127354	Genotypic CC	DAS≤2.4	6	204	**<0.05**	2.7 (1.1–8.1)	Wessels 2006-2[22]
	(C94A)	Allelic A carriers	EULAR GR	4	255	**0.006**	2.95 (1.36–6.38)	Dervieux (2009)[30]

De novo purine synthesis pathway								
ATIC	rs2372536 (C347G)	Allelic C carriers	DAS28≤3.2	6	233	0.57	0.83 (0.43–1.69)	Lima (2016)[1]
		Allelic C carriers	EULAR GR	6	281	0.96	-	Sharma (2009)[21]
		Allelic C carriers	EULAR GR	12	98	0.56	-	James (2008)[19]
		Allelic C carriers	EULAR GR	6	319	0.94	0.98 (0.67–1.43)	Muralidharan (2016-1)[36]
		Allelic C carriers	ACR 20 & 50	12	217	NS	-	Ghodke-Puranik (2015)[18]
		Allelic C carriers	DAS28≤2.4	6	422	0.23	1.29 (0.87–1.91)	Kurzawski (2016)[24]
		Allelic G carriers	EULAR GR	4	255	0.71	1.09 (0.66–1.80)	Dervieux (2009)[30]
		Genotypic GG	DAS28	6	170	NS	-	Hayashi (2013)[37]
		Genotypic GG	DAS28≤2.4	6	422	**0.005**	2.40 (1.30–4.42)	Kurzawski (2016)[24]
		Genotypic CC	EULAR GR	6	281	0.17	-	Sharma (2009)[21]
		Genotypic CC	EULAR GR	12	98	0.85	-	James (2008)[19]
		Genotypic CC	DAS28≤3.2	6	233	**0.036**	0.27 (0.08–0.92)	Lima (2016)[1]
		Genotypic CC	DAS28≤3.2	3–6	208	NS	-	Grabar (2010)[20]
		Genotypic CC	DAS≤2.4	6	205	**0.007***	2.5 (1.3–4.8)	Wessels 2006-2[22]
		Genotypic CC	EULAR GR	6	61	0.12	1.95 (0.83–4.56)	Salazar (2014)[38]
	rs4673993	Allelic C carriers	DAS28≤3.2	6	233	**0.036**	0.27 (0.08–0.92)	Lima (2016)[1]
		Genotypic TT	DAS28≤3.2	6	120	**0.006***	3.86 (1.50–9.91)	Lee (2009)[23]
		Genotypic TT	DAS28≤3.2	6	233	0.95	0.98 (0.51–1.89)	Lima (2016)[1]
	rs7563206	Allelic T carriers	DAS28≤3.2	6	233	**<0.001***	0.20 (0.09–0.46)	Lima (2016)[1]
		Genotypic TT	DAS28≤3.2	6	233	0.56	0.81 (0.40–1.65)	Lima (2016)[1]
	rs12995526	Allelic T carriers	EULAR GR	6	233	**0.001***	0.23 (0.10–0.53)	Lima (2016)[1]
		Allelic T carriers	DAS28≤2.4	6	422	0.11	0.71 (0.47–1.07)	Kurzawski (2016)[24]
		Genotypic TT	DAS28≤2.4	6	422	0.14	0.65 (0.38–1.10)	Kurzawski (2016)[24]
		Genotypic TT	EULAR GR	6	233	0.41	0.74 (0.37–1.51)	Lima (2016)[1]
		Genotypic CC	EULAR GR	6	61	0.22	1.78 (0.70–4.52)	Salazar (2014)[38]

Table 2-1 continues on next page.

Table 2-1. ***Continued***

Gene	SNPs	Genetic model	Endpoint	Time of response evaluation (months)	N	Reported P-value	OR (95%CI)	Study
Transporters								
ABCB1	rs1045642 (C3435T)	Genotypic CT	DAS28≤3.2	6	281	**0.01**	1.97 (1.13–3.42)	Sharma (2008)[39]
		Genotypic CC	DAS28≤3.2	6	281	**0.01**	0.32 (0.13–0.80)	Sharma (2008)[39]
		Genotypic CC	DAS<2.4	6	186	0.77	-	Kooloos (2010)[40]
		Allelic C carriers	DAS<2.4	6	186	0.082	-	Kooloos (2010)[40]
ABCC1	rs246240	Allelic G carriers	DAS28≤3.2	6	233	**0.008**	5.47 (1.56–19.25)	Lima (2015)[31]
		Genotypic GG	DAS28≤3.2	6	233	0.85	0.76 (0.05–11.46)	Lima (2015)[31]
	rs3784864	Allelic A carriers	EULAR GR	6	233	0.40	0.64 (0.23–1.80)	Lima (2015)[31]
		Genotypic AA	EULAR GR	6	233	**0.015**	4.24 (1.32–13.65)	Lima (2015)[31]
SLC19A1 / RFC1	rs1051266 (G80A)	Allelic A carriers	ACR 20 & 50	12	217	**0.030**	2.20 (1.1–4.4)	Ghodke-Puranik (2015)[18]
		Allelic A carriers	EULAR GR	12	98	**0.009**	-	James (2008)[19]
		Allelic A carriers	ACR 20	6	174	**0.021***	3.32 (1.26–8.79)	Drozdzik (2007)[41]
		Allelic A carriers	DAS28≤3.2	6	233	0.67	1.23 (0.47–3.18)	Lima (2015)[31]
		Allelic A carriers	DAS28≤3.2	6	281	NS	-	Sharma (2008)[39]
		Allelic A carriers	EULAR GR	6	225	0.28	1.24 (0.85–1.81)	Muralidharan (2016-2)[42]
		Allelic A carriers	EULAR GR	4	255	0.07	1.63 (0.95–2.79)	Dervieux (2009)[30]
		Genotypic AA	EULAR GR	12	98	**0.036**	-	James (2008)[19]
		Genotypic AA	ACR 20%	6	174	**0.013***	1.78 (1.13–2.81)	Drozdzik (2007)[41]
		Genotypic AA	DAS28≤3.2	6	233	0.92	1.05 (0.36–3.09)	Lima (2015)[31]
		Genotypic AA	DAS28≤3.2	6	281	NS	-	Sharma (2008)[39]
		Genotypic GG	DAS28	4	255	0.27	-	Dervieux (2009)[30]
		Genotypic GG	DAS28	6	170	**0.0018***	2.27 (1.36–3.80)	Hayashi (2013)[37]
		Genotypic GG	EULAR GR	6	76	0.60	-	Moya (2016)[43]

		Genotypic GG	EULAR GR	6	54	NS	-	Chatzikyriakidou (2007)[44]
		Genotypic GG	DAS28≤3.2	6	240	NS	-	Świerkot (2015)[25]
		Genotypic GG	EULAR GR	6	225	0.56	0.81 (0.46–1.43)	Muralidharan (2016-2)[42]
SLC22A11	rs11231809	Genotypic AA	DAS28≤3.2	6	233	**0.031**	0.19 (0.04–0.86)	Lima (2015)[31]
		Allelic A carriers	DAS28≤3.2	6	233	0.12	0.44 (0.16–1.22)	Lima (2015)[31]
Polyglutamation								
FPGS	rs4451422	Allelic A carriers	DAS28≤3.2	6	232	0.077	0.52 (0.025–1.07)	Lima (2016)[1]
		Allelic C carriers	EULAR GR	6	457	**0.035*#**	0.73 (0.54–0.98)	Senapati (2014)[17]
		Genotypic AA	DAS28≤3.2	6	232	0.27	1.57 (0.70–3.49)	Lima (2016)[1]
		Genotypic CC	EULAR GR	6	457	**0.05#**	0.72 (0.52–1.00)	Senapati (2014)[17]
	rs1544105	Allelic A carriers	EULAR GR	6	281	**0.008**	3.47 (1.19–10.12)	Sharma (2008)[21]
		Allelic G carriers	DAS28≤3.2	6	233	0.32	1.53 (0.68–3.60)	Lima (2016)[1]
		Allelic G carriers	DAS28≤3.2	6	281	**0.043**	1.55 (1.01–2.37)	Sharma (2008)[39]
		Allelic A carriers	DAS28≤2.4	6	422	0.92	0.96 (0.65–1.43)	Kurzawski (2016)[24]
		Genotypic GG	DAS28≤3.2	6	233	0.12	0.56 (0.27–1.15)	Lima (2016)[1]
		Genotypic AA	DAS28≤2.4	6	422	0.40	0.77 (0.44–1.36)	Kurzawski (2016)[24]
	rs10106 (A1994G)	Allelic C carriers	DAS28≤2.4	6	422	0.84	0.94 (0.64–1.40)	Kurzawski (2016)[24]
		Allelic C carriers	DAS<2.4	6	352	0.9	2.90 (1.50–5.40)	van der Straaten (2007)[45]
		Allelic A carriers	DAS28≤3.2	6	233	0.32	1.50 (0.68–3.29)	Lima (2016)[1]
		Allelic A carriers	DAS≤2.4	6	186	0.64	-	Wessels (2007)[46]
		Allelic A carriers	DAS≤2.4	6	352	NS	-	van der Straaten (2007)[45]
		Genotypic AA	DAS≤2.4	6	186	0.13	-	Wessels (2007)[46]
		Genotypic AA	DAS28≤3.2	6	233	0.07	0.51 (0.25–1.06)	Lima (2016)[1]
		Genotypic TT	EULAR GR	6	76	**0.041**	-	Moya (2016)[43]
		Genotypic CC	DAS28<2.4	6	422	0.25	0.70 (0.69–1.24)	Kurzawski (2016)[24]
	rs10987742	Genotypic GG	EULAR GR	6	76	**0.033**	-	Moya (2016)[43]

Table 2-1 continues on next page.

Table 2-1. ***Continued***

Gene	SNPs	Genetic model	Endpoint	Time of response evaluation (months)	N	Reported P-value	OR (95%CI)	Study
GGH	rs2305558	Allelic A carriers	EULAR GR	6	457	**0.05**[#]	1.46 (0.98–2.17)	Senapati (2014)[17]
		Genotypic AA	EULAR GR	6	457	0.23[#]	1.51 (0.74–3.08)	Senapati (2014)[17]
	rs1800909 (C16T)	Allelic C carriers	DAS≤2.4	3	352	**0.036**	2.1 (1.0–4.7)	van der Straaten (2007)[45]
		Allelic C carriers	DAS≤2.4	6	352	NS	-	van der Straaten (2007)[45]
		Allelic C carriers	EULAR GR	4	255	0.66	1.11 (0.68–1.83)	Dervieux (2009)[30]
		Allelic T carriers	DAS≤2.4	6	186	0.71	-	Wessels (2007)[46]
		Genotypic TT	DAS≤2.4	6	186	0.31	-	Wessels (2007)[46]
Folate pathway								
DHFR	rs836788	Allelic A carriers	EULAR GR	6	457	**0.014**[*#]	1.44 (1.08–1.93)	Senapati (2014)[17]
		Genotypic AA	EULAR GR	6	457	**0.011**[*#]	1.47 (1.09–1.96)	Senapati (2014)[17]
	rs12517451	Allelic A carriers	EULAR GR	6	457	**0.05**[#]	1.35 (0.99–1.85)	Senapati (2014)[17]
		Genotypic AA	EULAR GR	6	457	**0.016**[#]	1.56 (1.07–2.26)	Senapati (2014)[17]
	rs408626 (-317)	Genotypic AA	ΔDAS	6	125	**0.050**	-	Milic (2012)[47]
		Genotypic AA	EULAR GR	6	125	0.2	-	Milic (2012)[47]
	rs1643650	Additive	EULAR GR	6	61	**0.026**	0.31 (0.10–0.96)	Salazar (2014)[38]

MTHFR	rs17421511	Additive	EULAR GR	6	61	**0.024**	3.35 (1.10–10.24)	Salazar (2014)[38]
	rs1801131 (A1298C)	Additive	EULAR GR	6	61	**0.08**	2.19 (0.89–5.37)	Salazar (2014)[38]
		Allelic A carriers	ACR 20 & 50	12	217	**0.020**	2.6 (1.1–5.8)	Ghodke-Puranik (2015)[18]
		Allelic A carriers	EULAR GR	12	98	1.00	-	James (2008)[19]
		Allelic A carriers	ACR20	6	69	0.56	-	Taraborelli (2009)[28]
		Allelic C carriers	DAS28≤3.2	6	233	**0.045**	0.51 (0.26–0.98)	Lima (2015)[31]
		Allelic C carriers	EULAR GR	4	255	0.66	0.89 (0.54–1.46)	Dervieux (2009)[30]
		Genotypic AA	ΔDAS44<1.2	6	186	**0.014**	2.30 (1.18–4.41)	Wessels (2006-1)[27]
		Genotypic AA	ACR20	6	69	0.35	-	Taraborelli (2009)[28]
		Genotypic AA	EULAR GR	12	98	0.92	-	James (2008)[19]
		Genotypic AA	DAS28≤3.2	6	240	NS	-	Świerkot (2015)[25]
		Genotypic AA	DAS28	4	48	NS	-	Dervieux (2006)[29]
		Genotypic CC	DAS28≤3.2	6	120	0.84	0.90 (0.40–2.02)	Lee (2009)[23]
		Genotypic CC	DAS28≤3.2	6	233	0.91	1.07 (0.35–3.28)	Lima (2015)[31]
		Genotypic AA	EULAR GR	6	120	0.23	-	Soukup (2015)[48]
	rs1476413	Additive	EULAR GR	6	61	**0.0086**	3.56 (1.28–9.91)	Salazar (2014)[38]

Table 2-1 continues on next page.

Table 2-1. ***Continued***

Gene	SNPs	Genetic model	Endpoint	Time of response evaluation (months)	N	Reported P-value	OR (95%CI)	Study
	rs1801133 (C677T)	Additive	EULAR GR	6	61	0.53	0.73 (0.27–1.98)	Salazar (2014)[38]
		Allelic T carriers	ACR 20 & 50	12	217	NS	-	Ghodke-Puranik (2015)[18]
		Allelic T carriers	EULAR GR	4	255	0.86	1.04 (0.63–1.72)	Dervieux (2009)[30]
		Allelic C carriers	EULAR GR	12	98	0.39	-	James (2008)[19]
		Allelic C carriers	ACR20	6	69	0.34	-	Taraborelli (2009)[29]
		Allelic C carriers	DAS28≤3.2	6	233	**0.019**	3.86 (1.25–11.89)	Lima (2016)[1]
		Genotypic CC	EULAR GR	6	113	NS	-	Aggarwal (2006)[26]
		Genotypic CC	ΔDAS44<0.6	6	186	**0.044**	2.73 (1.03–7.26)	Wessels (2006-1)[27]
		Genotypic CC	ACR20	6	69	0.26	-	Taraborelli (2009)[28]
		Genotypic CC	EULAR GR	12	98	0.64	-	James (2008)[19]
		Genotypic CC	DAS28≤3.2	6	240	**0.001***	3.4	Świerkot (2015)[25]
		Genotypic TT	DAS28	4	48	NS	-	Dervieux (2006)[29]
		Genotypic TT	EULAR GR	4	48	**<0.05**	22.2 (1.2–42.2)	Dervieux (2006)[29]
		Genotypic TT	EULAR GR	6	120	0.43	1.41 (0.51–4.55)	Soukup (2015)[48]
MTHFD1	rs17850560 (G1958A)	Genotypic GG	DAS28≤3.2	3–6	208	**0.021**	4.67 (1.27–17.26)	Grabar (2010)[20]
		Genotypic GG	DAS≤2.4	6	186	0.10	-	Wessels (2006-2)[22]
		Allelic A carriers	EULAR GR	4	255	0.11	1.62 (0.90–2.92)	Dervieux (2009)[30]
SHMT-1	rs1979277 (C1420T)	Genotypic TT	DAS28	4	48	**<0.05**	7.4 (1.0–56.4)	Dervieux (2006)[29]
		Allelic T carrier	EULAR GR	4	255	0.53	0.85 (0.52–1.40)	Dervieux (2009)[30]

Methionine pathway								
MTR (MS)	rs1805087 (A2756G)	Allelic A carriers	EULAR GR	12	98	0.06	-	James (2008)[19]
		Allelic A carriers	ACR 20 & 50	12	217	NS	-	Ghodke-Puranik (2015)[18]
		Allelic G carriers	EULAR GR	4	255	0.41	1.23 (0.73–2.10)	Dervieux (2009)[30]
		Allelic G carriers	DAS28≤3.2	6	233	**0.017**	0.42 (0.20–0.86)	Lima (2015)[31]
		Genotypic AA	EULAR GR	12	98	**0.003***	-	James (2008)[19]
		Genotypic AA	DAS28	4	255	NS	-	Dervieux (2006)[29]
		Genotypic GG	DAS28≤3.2	6	233	0.25	0.27 (0.03–2.51)	Lima (2015)[31]
MTRR	rs162040	Allelic C carriers	EULAR GR	6	457	**0.04**	1.45 (1.00–2.10)	Senapati (2014)[17]
		Genotypic CC	EULAR GR	6	457	**0.02**	2.22 (1.11–4.43)	Senapati (2014)[17]
	rs1801394 (A66G)	Allelic A carriers	DAS28≤3.2	6	233	**0.041**	2.16 (1.03–4.53)	Lima (2015)[31]
		Allelic A carriers	ACR 20 & 50	12	217	NS	-	Ghodke-Puranik (2015)[18]
		Genotypic AA	DAS28	4	48	NS	-	Dervieux (2006)[29]
		Genotypic AA	DAS28≤3.2	6	233	**0.046**	2.36 (1.01–5.52)	Lima (2015)[31]
***De novo* pyrimidine pathway**								
TYMS	rs2244500	Allelic A carriers	EULAR GR	6	457	**0.005*#**	1.48 (1.12–1.94)	Senapati (2014)[17]
		Genotypic AA	EULAR GR	6	457	**0.004*#**	1.48 (1.13–1.94)	Senapati (2014)[17]
	rs2847153	Genotypic AA	EULAR GR	6	61	0.26	1.92 (0.62–5.97)	Salazar (2014)[38]
		Allelic A carriers	EULAR GR	6	457	**0.009*#**	0.68 (0.51–0.91)	Senapati (2014)[17]
		Genotypic AA	EULAR GR	6	457	**0.04#**	0.71 (0.52–0.98)	Senapati (2014)[17]
	rs3786362	Allelic G carriers	EULAR GR	6	457	**0.011*#**	0.51 (0.30–0.86)	Senapati (2014)[17]
		Genotypic GG	EULAR GR	6	457	0.99#	-	Senapati (2014)[17]

Table 2-1 continues on next page.

Table 2-1. ***Continued***

Gene	SNPs	Genetic model	Endpoint	Time of response evaluation (months)	N	Reported P-value	OR (95%CI)	Study
Ubiquitin pathway								
CUL1	rs122571	Haplotype A-T-T	DAS28≤3.2	6	29	**0.0051***	2.83 (1.33–6.04)	Negi (2011)[32]
	rs243481	Haplotype G-C-T	DAS28≤3.2	6	74	**0.05**	1.42 (1.0–2.02)	Negi (2011)[32]
	rs243480	Haplotype G-T-T	DAS28≤3.2	6	25	**0.0045***	2.83 (1.33–6.04)	Negi (2011)[32]
Other								
KIR	2DS4 gene	Full-length	DAS28≤2.5	6	312	**0.0334***	0.43 (0.215–0.987)	Majorczyk (2014)[33]

P-values marked in bold p-values have a reported p-value below 0.05. P-values marked with an asterisk (*) were significantly associated after multiple testing correction (Bonferroni correction, $p<0.05$).
P-values marked with # have a reported p-values that was already corrected by multiple testing.
Abbreviations: BF: Bonferroni EULAR GR: European league against rheumatism good response criteria. ACR: American College of Rheumatology. OR: Odds Ratio. CI: Confidence Interval, SNPs: single nucleotide polymorphisms, NS: Not significant.

Table 2-2. Most promising SNPs that were significantly associated with MTX efficacy.

			MAF							
Gene	SNP	Location	AF	AFR	AMR	EUR	SAS	Association	OR [95% CI]	Study
SLC19A1	rs1051266*	21:45537880	0.4886	0.3268	0.5821	0.5487	0.5941	Genotypic AA	1.78 [1.13–2.81]	Drozdzik (2007)[41]
ATIC	rs7563206	2:215325931	0.4018	0.5129	0.4280	0.4871	0.3292	Allelic T carriers	0.20 [0.09–0.46]	Lima (2016)[1]
DHFR	rs836788	5:80616225	0.4235	0.5106	0.3631	0.3807	0.4335	Allelic A carriers	1.44 [1.08–1.93]	Senapati (2014)[17]
								Genotypic AA	1.47 [1.09–1.96]	Senapati (2014)[17]
TYMS	rs2244500	18:661005	0.6160	0.8101	0.4251	0.4612	0.5706	Allelic A carriers	1.48 [1.12–1.94]	Senapati (2014)[17]
								Genotypic AA	1.48 [1.13–1.94]	Senapati (2014)[17]
TYMS	rs2847153	18:661647	0.2901	0.2428	0.2305	0.2097	0.3865	Allelic A carriers	0.68 [0.51–0.91]	Senapati (2014)[17]
TYMS	rs3786362	18:662247	0.0623	0.0015	0.0490	0.0000	0.1063	Allelic G carriers	0.51 [0.30–0.86]	Senapati (2014)[17]

* Confirmed by the meta-analyses of Kung *et al*. (2014)[52] and Li *et al*. (2016)[53]
Abbreviations: AFR: African population, AMR: American population, EAS: East Asian population, EUR: European population, SAS: South Asian population, derived from the HapMap project.

DISCUSSION

This systematic review assesses the effect of genetic variation on the efficacy of MTX in RA using the validated endpoints DAS, EULAR, or ACR response criteria. After Bonferroni correction for multiple testing, we identified six genetic biomarkers related to MTX efficacy. Of these, *SLC19A1* rs1051266 had the most convincing evidence with two independent studies showing significant associations. Other potentially promising SNPs are *ATIC* rs7563206, *DHFR* rs836788, *TYMS* rs2244500, rs2847153, and rs3786362, but these lack replication studies. The six genetic biomarkers could have clinical implications for the disease outcome of RA. In fact, *SLC19A* rs1051266, *DHFR* rs836788, and *TYMS* rs2244500 showed a 40% or more increased chance of the effectiveness of MTX, and *ATIC* rs7563206 and rs378636, and *TYMS* rs2847153 showed 45% or more chance of the reduced effectiveness of MTX. Still we believe that additional studies are necessary before implementing pharmacogenetic testing for these SNPs in the treatment of RA.

A limitation of the investigated studies in this systematic review is the difference in the evaluation time points for measuring MTX efficacy. MTX is a slow-acting prodrug that becomes active when polyglutamated in the cells. The process of polyglutamation is slow and takes up to 27.5 weeks (range 6.6–62.0 weeks) to reach steady state.[34] This delay in steady-state polyglutamation explains the relatively long time to clinical response, and therefore most studies had the endpoint set to 6 months after the start of MTX therapy. However, some studies evaluated response earlier than t=6 months, while MTX may not yet have exerted its full potential. Furthermore, the genotypic or allelic genetic models were often used, when in fact the hypothesis-free driven additive genetic model seems more appropriate because the underlying genetic model is unknown.

Another limitation is that most studies tested with univariate analysis, without taking into account baseline variables (multivariate testing), such as gender, smoking status, disease severity which are known to influence response to MTX. Most drug-gene interaction studies were explorative, with the use of retrospective data and lack validation. Pharmacogenetic testing in RA remains limited mainly because the evidence for drug-gene interactions are marginal. MTX is involved in multiple pathways with different genes. Yet, most pharmacogenetic studies were candidate studies that tested only a single or a small number of SNPs, but not a combination of multiple genes or pathways.[35] To get clear evidence, additional studies with the use of a combination of multiple genes are needed. This review can show a basis, to test all suggestive SNPs together in association with the efficacy of MTX.

The strength of our study is that a systematic approach was used to identify SNPs and the selection of the articles was performed according to the PRISMA guidelines. Another

strength is that only validated outcome criteria were used and that adjustment for multiple testing by Bonferroni correction was applied for the included studies. A potential weakness of this review is that only English publications were included. This results in the exclusion of seven non-English studies, and important findings could have been missed. Another weakness was the limited sample size of some studies and the lack of power analysis to check the validity of the outcomes. Finally, a common limitation of systematic reviews is publication bias. Meaning that important – albeit negative – results were never published, which could lead to misinterpretation of the actual findings. Another limitation was that not all studies were performed with MTX monotherapy, and therefore the effect on response could be influenced by other DMARDs. Several meta-analyses have been performed on pharmacogenetics biomarkers for the efficacy or toxicity of MTX in RA. Of our promising SNPs, SLC19A1 rs1051266 with the genotypic AA (vs AG/AG) was tested in MTX efficacy in three meta-analyses. Two meta-analyses, conducted by Li *et al*.[50] and Chen *et al*.,[51] confirmed the significant association with an OR of 1.42 (95% CI: 1.04–1.93) and 1.49 (CI: 1.17–1.90), respectively. However, the third meta-analysis by Chen *et al*.[51] showed substantial heterogeneity (I^2) of 72% for the allelic model and thus represented inconsistencies of the pooled studies and affects the validity of the results. None of the other variants was evaluated in meta-analysis.

In summary, through the use of a systematic review and inclusion of studies with validated RA efficacy endpoints, we identified six SNPs for which there is substantial evidence for an association with MTX response in RA patients. For clinical application more evidence from prospective studies with multivariate testing is needed.

Acknowledgement

We are particularly grateful for the assistance for search terms given by Jan Schoones of the Walaeus library (LUMC).

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

1. Lima A, Bernardes M, Azevedo R, Seabra V, Medeiros R. Moving toward personalized medicine in rheumatoid arthritis: SNPs in methotrexate intracellular pathways are associated with methotrexate therapeutic outcome. Pharmacogenomics 17(15), 1649–1674 (2016).
2. Ulrich CM, Robien K, Sparks R. Pharmacogenetics and folate metabolism—a promising direction. Pharmacogenomics 3, 299–313 (2002).
3. Kawaida R, Yamada R, Kobayashi K, Tokuhiro S, Suzuki A, Kochi Y *et al.* CUL1, a component of E3 ubiquitin ligase, alters lymphocyte signal transduction with possible effect on rheumatoid arthritis. Genes Immun. 6, 194–202 (2005).
4. Hider SL, Bruce IN, Thomson W. The pharmacogenetics of methotrexate. Rheumatology 46, 1520–1524 (2007).
5. Malik F, Ranganathan P. Methotrexate pharmacogenetics in rheumatoid arthritis: a status report review. Pharmacogenomics 14, 305–314 (2013).
6. Plant D, Wilson AG, Barton A. Genetic and epigenetic predictors of responsiveness to treatment in RA. Nat. Rev. Rheumatol. 10, 329–337 (2014).
7. Ranganathan P, McLeod HL. Methotrexate pharmacogenetics: the first step toward individualized therapy in rheumatoid arthritis. Arthritis Rheum. 54, 1366–1377 (2006).
8. Brinker RR, Ranganathan P. Methotrexate pharmacogenetics in rheumatoid arthritis. Clin. Exp. Rheumatol. 28, S33–S39 (2010).
9. Stamp L, Roberts R, Kennedy M, Barclay M, O'Donnell J, Chapman P. The use of low dose methotrexate in rheumatoid arthritis—are we entering a new era of therapeutic drug monitoring and pharmacogenomics? Biomed. Pharmacother. 60, 678–687 (2006).
10. Zhu H, Deng F-Y, Mo X-B, Qiu Y-H, Lei S-F. Pharmacogenetics and pharmacogenomics for rheumatoid arthritis responsiveness to methotrexate treatment: the 2013 update. Pharmacogenomics 15, 551–566 (2014).
11. Dervieux T, Furst D, Lein DO, Capps R, Smith K, Caldwell J *et al.* Pharmacogenetic and metabolite measurements are associated with clinical status in patients with rheumatoid arthritis treated with methotrexate: results of a multicentred cross sectional observational study. Ann. Rheum. Dis. 64, 1180–1185 (2005).
12. Ranganath VK, Khanna D, Paulus HE. ACR remission criteria and response criteria. Clin. Exp. Rheumatol. 24, 1909 (2006).
13. Fransen J, van Riel PL. The disease activity score and the EULAR response criteria. Rheum. Dis. Clin. North Am. 35, 745–757 (2009).
14. Counsell C. Formulating questions and locating primary studies for inclusion in systematic reviews. Ann. Intern. Med. 127, 380–387 (1997).
15. Moher D, Shamseer L, Clarke M, Ghersi D, Liberati A, Petticrew M *et al.* Preferred reporting items for systematic review and metaanalysis protocols (PRISMA-P) 2015 statement. Syst. Rev. 4, 1 (2015).
16. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, De Bakker PIW. SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. Bioinformatics 24, 2938–2939 (2008).
17. Senapati S, Singh S, Das M, Kumar A, Gupta R, Kumar U *et al.* Genome-wide analysis of methotrexate pharmacogenomics in rheumatoid arthritis shows multiple novel risk variants and leads for TYMS regulation. Pharmacogenet. Genomics. 24, 211–219 (2014).

18. Ghodke-Puranik Y, Puranik AS, Shintre P, Joshi K, Patwardhan B, Lamba J *et al*. Folate metabolic pathway single nucleotide polymorphisms: a predictive pharmacogenetic marker of methotrexate response in Indian (Asian) patients with rheumatoid arthritis. Pharmacogenomics 16, 2019–2034 (2015).

19. James HM, Gillis D, Hissaria P, Lester S, Somogyi AA, Cleland LG *et al*. Common polymorphisms in the folate pathway predict efficacy of combination regimens containing methotrexate and sulfasalazine in early rheumatoid arthritis. J. Rheumatol. 35, 62–71 (2008).

20. Grabar PB, Rojko S, Logar D, Dolzan V. Genetic determinants of methotrexate treatment in rheumatoid arthritis patients: a study of polymorphisms in the adenosine pathway. Ann. Rheum. Dis. 69, 931–932 (2010).

21. Sharma Shruti, Das Mitashree, Kumar Ashok, Vishal Marwaha, Subramanian Shankar, Paramjeet Singh *et al*. Purine biosynthetic pathway genes and methotrexate response in rheumatoid arthritis patients among north Indians. PubMed commons. Pharmacogenet. Genomics 19, 823–828 (2009).

22. Wessels JAM, Kooloos 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. 54, 2830–2839 (2006).

23. Lee YC, Cui J, Costenbader KH, Shadick NA, Weinblatt ME, Karlson EW. Investigation of candidate polymorphisms and disease activity in rheumatoid arthritis patients on methotrexate. Rheumatology 48, 613–617 (2009).

24. Kurzawski M, Malinowski D, Szarmach N, Nowak A, Goryniak A, Pawlik A *et al*. ATIC missense variant affects response to methotrexate treatment in rheumatoid arthritis patients. Pharmacogenomics 17, 1971–1978 (2016).

25. Swierkot J, Bogunia-Kubik K, Nowak B, Bialowas K, Korman L, Gebura K *et al*. Analysis of associations between polymorphisms within genes coding for tumour necrosis factor (TNF)-alpha and TNF receptors and responsiveness to TNF-alpha blockers in patients with rheumatoid arthritis. Jt. Bone Spine. 82, 94–99 (2015).

26. Aggarwal P, Naik S, Mishra KP, Aggarwal A, Misra R. Correlation between methotrexate efficacy & toxicity with C677T polymorphism of the methylenetetrahydrofolate gene in rheumatoid arthritis patients on folate supplementation. Indian J. Med. Res. 124, 521–526 (2006).

27. Wessels JAM, De Vries-Bouwstra JK, Heijmans BT, Slagboom PE, Goekoop-Ruiterman YPM, 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. 54, 1087–1095 (2006).

28. Taraborelli M, Andreoli L, Archetti S, Ferrari M, Cattaneo R, Tincani A. Methylenetetrahydrofolate reductase polymorphisms and methotrexate: no association with response to therapy nor with drug-related adverse events in an Italian population of rheumatic patients. Clin. Exp. Rheumatol. 27, 499–502 (2009).

29. Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum. 54, 3095–3103 (2006).

30. Dervieux T, Wessels JaMM, van der Straaten T, Penrod N, Moore JH, Guchelaar H-J *et al*. Gene–gene interactions in folate and adenosine biosynthesis pathways affect methotrexate efficacy and tolerability in rheumatoid arthritis. Pharmacogenet. Genomics 19, 935–944 (2009).

31. Lima A, Bernardes M, Azevedo R, Medeiros R, Seabra V. Pharmacogenomics of methotrexate membrane transport pathway: can clinical response to methotrexate in rheumatoid arthritis be predicted? Int. J. Mol. Sci. 16, 13760–13780 (2015).

32. Negi S, Kumar A, Thelma BK, Juyal RC. Association of Cullin1 haplotype variants with rheumatoid arthritis and response to methotrexate. Pharmacogenet. Genomics 21, 590–593 (2011).

33. Majorczyk E, Pawlik A, Gendosz D, Kusnierczyk P. Presence of the full-length KIR2DS4 gene reduces the chance of rheumatoid arthritis patients to respond to methotrexate treatment. BMC Musculoskelet. Disord. 15, 256 (2014).

34. Dalrymple JM, Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Barclay ML. Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. Arthritis Rheum. 58, 3299–3308 (2008).

35. Eektimmerman F, Swen JJ, Böhringer S, Huizinga TW, Kooloos WM, Allaart CF *et al*. Pathway analysis to identify genetic variants associated with efficacy of adalimumab in rheumatoid arthritis. Pharmacogenomics 18, 945–953 (2017).

36. Muralidharan N, Mariaselvam CM, Jain VK, Gulati R, Negi VS. ATIC 347C>G gene polymorphism may be associated with methotrexate-induced adverse events in south Indian Tamil rheumatoid arthritis. Pharmacogenomics 17, 241–248 (2016).

37. Hayashi H, Tazoe Y, Tsuboi S, Horino M, Morishita M, Arai T *et al*. A single nucleotide polymorphism of reduced folate carrier 1 predicts methotrexate efficacy in Japanese patients with rheumatoid arthritis. Drug Metab. Pharmacokinet. 28, 164–168 (2013).

38. Salazar J, Moya P, Altés A, Díaz-Torné C, Casademont J, Cerdà-Gabaroi D *et al*. Polymorphisms in genes involved in the mechanism of action of methotrexate: are they associated with outcome in rheumatoid arthritis patients? Pharmacogenomics 15, 1079–1090 (2014).

39. Sharma S, Das M, Kumar A, Marwaha V, Shankar S, Aneja R *et al*. Interaction of genes from influx-metabolism-efflux pathway and their influence on methotrexate efficacy in rheumatoid arthritis patients among Indians. Pharmacogenet. Genomics 18, 1041–1049 (2008).

40. Kooloos WM, Wessels JA, van der Straaten T, Allaart CF, Huizinga TW, Guchelaar H-J. Functional polymorphisms and methotrexate treatment outcome in recent-onset rheumatoid arthritis. Pharmacogenomics 11, 163–175 (2010).

41. Drozdzik M, Rudas T, Pawlik A, Gornik W, Kurzawski M, Herczynska M. Reduced folate carrier-1 80G>A polymorphism affects methotrexate treatment outcome in rheumatoid arthritis. Pharmacogenomics J. 7, 404–407 (2007).

42. Muralidharan N, Mariaselvam CM, Mithun CB, Negi VS. Reduced folate carrier-1 80G>A gene polymorphism is not associated with methotrexate treatment response in South Indian Tamils with rheumatoid arthritis. Clin. Rheumatol. 35, 879–885 (2016).

43. Moya P, Salazar J, Arranz MJ, Díaz-Torné C, del Río E, Casademont J *et al*. Methotrexate pharmacokinetic genetic variants are associated with outcome in rheumatoid arthritis patients. Pharmacogenomics 17, 25–29 (2016).

44. Chatzikyriakidou A, Georgiou I, Voulgari PV, Papadopoulos CG, Tzavaras T, Drosos AA. Transcription regulatory polymorphism -43T>C in the 5′-flanking region of SLC19A1 gene could affect rheumatoid arthritis patient response to methotrexate therapy. Rheumatol. Int. 27, 1057–1061 (2007).

45. van der Straaten R, Wessels JA, de Vries-Bouwstra JK, Goekoop- Ruiterman YP, Allaart CF, Bogaartz J *et al*. Exploratory analysis of four polymorphisms in human GGH and FPGS genes and their effect in methotrexate-treated rheumatoid arthritis patients. Pharmacogenomics 8, 141–150 (2007).

46. Wessels JAM, Van Der Kooij SM, Le Cessie S, Kievit W, Barerra P, Allaart CF *et al*. A clinical pharmacogenetic model to predict the efficacy of methotrexate monotherapy in recent-onset rheumatoid arthritis. Arthritis Rheum. 56, 1765–1775 (20070.

47. Milic V, Jekic B, Lukovic L, Bunjevacki V, Milasin J, Novakovic I *et al*. Association of dihydrofolate reductase (DHFR)-317AA genotype with poor response to methotrexate in patients with rheumatoid arthritis. Clin. Exp. Rheumatol. 30, 178–183 (2012).

48. Soukup T, Dosedel M, Pavek P, Nekvindova J, Barvik I, Bubancova I *et al*. The impact of C677T and A1298C MTHFR polymorphisms on methotrexate therapeutic response in East Bohemian region rheumatoid arthritis patients. Rheumatol. Int. 35, 1149–1161 (2015).

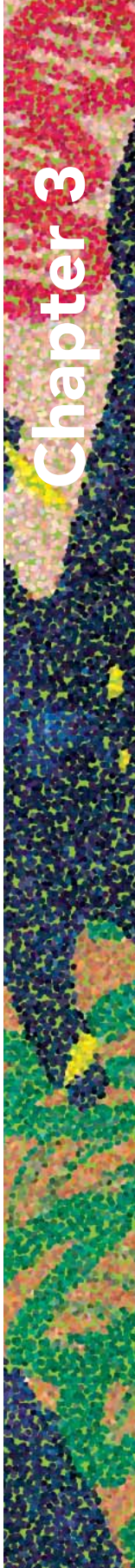
49. Kung TN, Dennis J, Ma Y, Xie G, Bykerk V, Pope J *et al*. RFC1 80G>A is a genetic determinant of methotrexate efficacy in rheumatoid arthritis: a human genome epidemiologic review and meta-analysis of observational studies. Arthritis Rheumatol. 66, 1111–1120 (2014).

50. Li X, Hu M, Li W, Gu L, Chen M, Ding H *et al*. The association between reduced folate carrier-1 gene 80G/A polymorphism and methotrexate efficacy or methotrexate related-toxicity in rheumatoid arthritis: A meta-analysis. Int. Immunopharmacol. 38, 8–15 (2016).

51. Chen Y, Zou K, Sun J, Yang Y, Liu G. Are gene polymorphisms related to treatment outcomes of methotrexate in patients with rheumatoid arthritis? A systematic review and meta-analysis. Pharmacogenomics 18, 175–195 (2016).

Supplementary File S2-1. Full search string.

((“Arthritis, Rheumatoid”[Majr:NoExp] OR “Rheumatoid Arthritis”[ti]) AND (“Methotrexate” [Majr] OR methotrexat*[ti] OR “Amethopterin”[ti]) AND (“Pharmacogenetics”[Mesh] OR pharmacogenet*[tw] OR pharmacogenom*[tw] OR “Epigenomics”[Mesh] OR epigenet*[tw] OR epigenom*[tw] OR “Polymorphism, Single Nucleotide”[Mesh] OR “SNPs”[tw] OR “Single Nucleotide Polymorphism”[tw] OR “Single Nucleotide Polymorphisms”[tw]))



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

Frank Eektimmerman
Jesse J. Swen
Stefan Böhringer
Stella Aslibekyan
Cornelia F. Allaart
Henk-Jan Guchelaar

Pharmacogenomics 19(7), 613–619 (2018)

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.

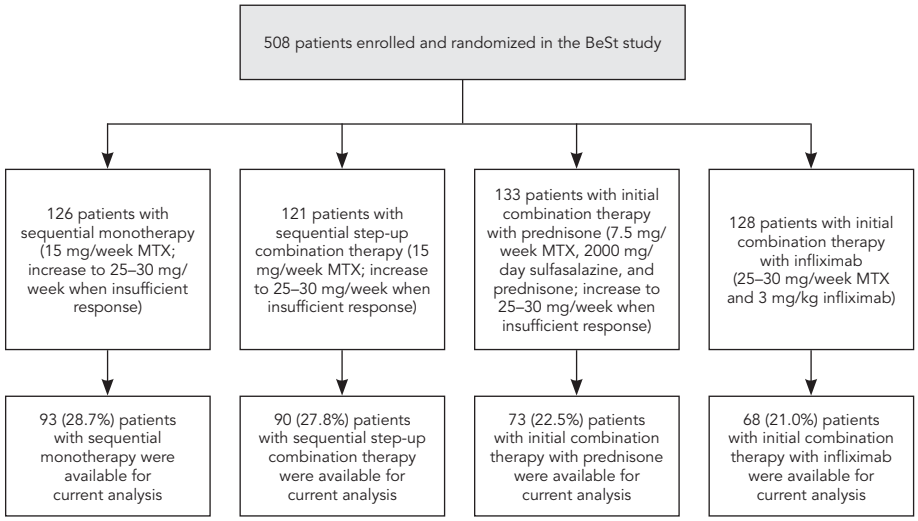


Figure 3-1. Flowchart of the enrolled BeSt patients.
Abbreviations: MXT: Methotrexate.

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.

Acknowledgement

The authors gratefully acknowledge the contributions of the investigators on the Treatment of Early Aggressive Rheumatoid Arthritis Trial: L.W. Moreland, J.R. O'Dell, H.E. Paulus, J.R. Curtis, J.M. Bathon, E.W. St Clair, G. Howard, D. van der Heijde and S.S. Cofield. We would also show our gratitude to D. Odijk, R. Baak-Pablo, D. Klootwijk and R. Schaap for designing and conducting the genotyping of the BeSt participants.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

REFERENCES

1. Romão VC, Lima A, Bernardes M, Canhão H, Fonseca JE. Three decades of low-dose methotrexate in rheumatoid arthritis: can we predict toxicity? Immunol. Res. 60(2–3), 289–310 (2014).
2. Anderson JJ, Wells G, Verhoeven AC, Felson DT. Factors predicting response to treatment in rheumatoid arthritis: the importance of disease duration. Arthritis Rheum. 43(1), 22–29 (2000).
3. Hider SL, Bruce IN, Thomson W. The pharmacogenetics of methotrexate. Rheumatology 46(10), 1520–1524 (2007).
4. Chen Y, Zou K, Sun J, Yang Y, Liu G. Are gene polymorphisms related to treatment outcomes of methotrexate in patients with rheumatoid arthritis? A systematic review and meta-analysis. Pharmacogenomics 18(2), 175–195 (2016).
5. Lee YH, Bae S-C. Association of the ATIC 347 C/G polymorphism with responsiveness to and toxicity of methotrexate in rheumatoid arthritis: a meta-analysis. Rheumatol. Int. 36(11), 1591–1599 (2016).
6. Kung TN, Dennis J, Ma Y *et al*. RFC1 80G > A is a genetic determinant of methotrexate efficacy in rheumatoid arthritis: a human genome epidemiologic review and meta-analysis of observational studies. Arthritis Rheumatol. 66(5), 1111–1120 (2014).
7. Li X, Hu M, Li W *et al*. The association between reduced folate carrier-1 gene 80G/A polymorphism and methotrexate efficacy or methotrexate related-toxicity in rheumatoid arthritis: a meta-analysis. Int. Immunopharmacol. 38, 8–15 (2016).
8. Owen SA, Hider SL, Martin P, Bruce IN, Barton A, Thomson W. Genetic polymorphisms in key methotrexate pathway genes are associated with response to treatment in rheumatoid arthritis patients. Pharmacogenomics J. 13(3), 227–234 (2012).
9. Qiu Q, Huang J, Shu X, Fan H, Zhou Y, Xiao C. Polymorphisms and pharmacogenomics for the clinical efficacy of methotrexate in patients with rheumatoid arthritis: a systematic review and meta-analysis. Sci. Rep. 7, 44015 (2017).
10. Lima A, Bernardes M, Azevedo R *et al*. SLC19A1, SLC46A1 and SLCO1B1 polymorphisms as predictors of methotrexate-related toxicity in Portuguese rheumatoid arthritis patients. Toxicol. Sci. 142(1), 196–209 (2014).
11. Aslibekyan S, Brown EE, Reynolds RJ *et al*. Genetic variants associated with methotrexate efficacy and toxicity in early rheumatoid arthritis: results from the treatment of early aggressive rheumatoid arthritis trial. Pharmacogenomics J. 14(1), 48–53 (2014).
12. Goekoop-Ruiterman YPM, De Vries-Bouwstra JK, Allaart CF *et al*. Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the BeSt study): a randomized, controlled trial. Arthritis Rheum. 52(11), 3381–3390 (2005).
13. Moreland LW, O'Dell JR, Paulus HE *et al*. A randomized comparative effectiveness study of oral triple therapy versus etanercept plus methotrexate in early aggressive rheumatoid arthritis: the treatment of early aggressive rheumatoid arthritis trial. Arthritis Rheum. 64(9), 2824–2835 (2012).
14. Purcell S, Neale B, Todd-Brown K *et al*. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81(3), 559–575 (2007).
15. Clarke L, Zheng-Bradley X, Smith R *et al*. The 1000 Genomes Project: data management and community access. Nat. Methods 9(5), 459–462 (2012).

16. Filipski KK, Loos WJ, Verweij J, Sparreboom A. Interaction of cisplatin with the human organic cation transporter 2. Clin. Cancer Res. 14(12), 3875–3880 (2008).

17. Tkáč I, Klimčáková L, Javorský M *et al*. Pharmacogenomic association between a variant in SLC47A1 gene and therapeutic response to metformin in Type 2 diabetes. Diabetes Obes. Metab. 15(2), 189–191 (2013).

18. Bergen AW, Javitz HS, Krasnow R *et al*. Organic cation transporter variation and response to smoking cessation therapies. Nicotine Tob. Res. 16(12), 1638–1646 (2014).

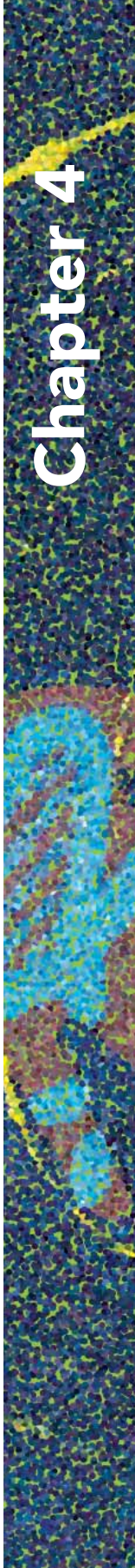
19. Sallinen R, Kaunisto MA, Forsblom C *et al*. Association of the SLC22A1, SLC22A2, and SLC22A3 genes encoding organic cation transporters with diabetic nephropathy and hypertension. Ann. Med. 42(4), 296–304 (2010).

20. Allegra S, Cusato J, De Nicolò A *et al*. Role of pharmacogenetic in ribavirin outcome prediction and pharmacokinetics in an Italian cohort of HCV-1 and 4 patients. Biomed. Pharmacother. 69, 47–55 (2015).

21. Tibshirani R. Regression selection and shrinkage via the Lasso. J. R. Stat. Soc. B 58(1), 267–288 (1996).

22. Friedman J, Hastie T, Tibshirani R. Regularization paths for generalized linear models via coordinate descent. J. Stat. Softw. 33(1), 1–20 (2010).

23. Simon N, Friedman J, Hastie T, Tibshirani R. A sparse-group lasso. J. Comput. Graph. Stat. 22(2), 231–245 (2013).



Validation of a clinical pharmacogenetic model to predict methotrexate non-response in rheumatoid arthritis patients

Frank Eektimmerman
Cornelia F. Allaart
Johanna M.W. Hazes
Moenira B. Madhar
Alfons A. den Broeder
Jaap Fransen
Jesse J. Swen
Henk-Jan Guchelaar

Pharmacogenomics 20(2), 85–93 (2019)

Abstract

Aim: To study the performance of a clinical pharmacogenetic model for the prediction of non-response to methotrexate in rheumatoid arthritis patients treated with combination therapy.

Methods: Prediction model risk scores were calculated and compared with non-response (DAS>2.4). Regression and ROC curve analyses of the prediction model were performed. Also, the sensitivity, specificity, and the positive and negative predictive values (PPV and NPV) were determined.

Results: The ROC AUC was 75% at first and 70% after second evaluation. At the second evaluation, prediction non-response had a sensitivity of 67% (CI: 54–78%), specificity of 69% (CI: 60–77%), PPV of 52% (CI: 45–60%) and NPV of 80% (CI: 73–85%).

Conclusion: The clinical pharmacogenetic model could not predict non-response in RA patients treated with methotrexate combination therapies.

INTRODUCTION

Rheumatoid arthritis (RA) is the most common form of autoimmune arthritis, affecting 0.5–1.0% of the adult population in the Western World.[1] Much of the joint damage that ultimately results in disability begins early in the course of the disease. Thus, early disease recognition, prompt diagnosis with early (intensive) treatment is critical to quickly achieve and maintain control of the inflammation and the underlying disease process. The vast majority of patients with RA start with methotrexate (MTX),[2] with the treatment goal of remission or low disease activity (Disease Activity Score [DAS] ≤2.4). MTX has been used for decades, but a considerable proportion of patients experience an inadequate response. Temporary treatment with corticosteroids has shown to increase early response rates, but after discontinuation of this, MTX can still prove insufficient response. On the other hand, some patients achieve lasting clinical remission on MTX monotherapy. To date, it remains a process of trial and error to choose the best initial treatment for newly diagnosed RA patients, although attempts have been made to identify clinical and genetic risk factors for response to MTX.

A clinical pharmacogenetic model was prior developed to predict non-response (DAS>2.4) of monotherapy MTX in early RA patients.[3] This predictive model combines clinical predictors with genetic variants related to the mechanism of action of MTX (Table 4-1). Based on the summed score in the model, patients are divided into predicted responders (summed score of ≤3.5), intermediate responders (summed score between 3.5 and 6.0) or non-responders (summed score ≥6.0). The predicted non-responders and predicted responders were used to calculate the predictive parameters for the clinical outcome low disease activity (DAS<2.4).

The originally derived prediction model, in patients treated with MTX monotherapy (n=205), showed a sensitivity of 86% (95% confidence interval (CI:) 76–93%) and specificity of 95% (CI: 82–99%) with an AUC of 85% (CI: 80–91%) for the prediction of MTX non-responders. Cross-validation in a small group of 38 early RA patients treated with MTX monotherapy supported the obtained results, although with worse sensitivity and specificity of respectively 70% (CI: 35–93%) and 72% (CI: 47–90%). A subsequent study (n=71) in MTX treated patients with preceding DMARD failure confirmed that the model performs modestly well in predicting MTX non-response, with a sensitivity of 81% (CI: 61–94%), a specificity of 47% (28–66%) and AUC of 77% (CI: not available).[4] Also, a recent replication study, that combined predicted intermediate responders with predicted responders, showed in a large number of MTX monotherapy treated RA patients (n=720) a sensitivity of 50% (CI: 45–55%), a specificity of 75% (CI: 69–80%) and an AUC of 66% (CI: not available).[5]

Since in daily clinical practice RA patients are frequently treated with MTX based (sometimes temporary) combination therapies at an early disease stage – although debate remains

whether these combinations are superior to MTX alone – the performance of the prediction model in these patients is of importance. This study aimed to evaluate the test characteristics of the pharmacogenetic model to predict MTX non-response in RA patients treated with combination therapies.

PATIENTS & METHODS

The recommendations in the TRIPOD statements[6] and the STARD guidelines[7] were used for the describing of the methods and results of the study.

Study participants

Retrospective data of 314 patients were collected from three academic hospitals in the Netherlands: Radboud University Medical Centre, Nijmegen (RUMC), Erasmus Medical Centre, Rotterdam (EMC) and Leiden University Medical Center, Leiden (LUMC). Included patients derived from the tREACH trial[8] (EMC), the IMPROVED study[9] (LUMC), and the early RA inception cohort[10] (Radboud UMC). The period for patient recruitment was between 1989 and 2009, 2007 and 2010, and 2007 and 2011 for respectively the early inception cohort, the IMPROVED study, and the tREACH trial.

Eligible patients were diagnosed with RA, based on the ACR 1987 or EULAR/ACR 2010 classification criteria for RA. Included patients had a treatment duration with MTX and follow-up for at least two study evaluation visits, were 18 years or older, and had not used any DMARD before the start of MTX. Further, DNA samples and clinical data included in the prediction model must be available (complete-case analysis). All patients provided written consent for participation in this study, and the institutional ethics committees approved the study protocol.

IMPROVED patients started their treatment with MTX and tapered prednisone in 7 weeks from 60 mg/day to 7.5 mg/day. At four months, patients with DAS<1.6 received tapered prednisolone to zero in 3 weeks. Patients not in remission (DAS>1.6) at four months were randomized in either 1) MTX + hydroxychloroquine + sulfasalazine and prednisolone, or 2) MTX and adalimumab. The given doses in IMPROVED were: MTX 25 mg/week, hydroxychloroquine 400 mg/day, sulfasalazine 2 g/day, prednisolone 7.5 mg/day, and adalimumab 40 mg/2 weeks.

tREACh patients started their treatment with either 1) MTX + sulfasalazine and hydroxychloroquine with glucocorticosteroids intramuscularly, 2) MTX + sulfasalazine and hydroxychloroquine with oral glucocorticosteroids, or 3) MTX + tapered oral glucocorticosteroids.

Glucocorticosteroids were either given as a single intramuscular dose (either methylprednisolone 120 mg or triamcinolone 80 mg) or an oral tapering scheme of prednisolone in 9 weeks from 15 mg/day to 2.5 mg/day. Patients that not achieved low disease activity (DAS>2.5) at three months switched to MTX with etanercept. The given doses in tREACH were: MTX 25 mg/week, sulfasalazine 2 g/day, hydroxychloroquine 400 mg/day, etanercept 50 mg/week.

RUMC patients were asked in the outpatient clinic to participate in follow-up research (early RA inception cohort). Most RUMC patients started with MTX monotherapy. Also, circa one-fourth of patients were treated with a combination of MTX with either leflunomide or with sulfasalazine. Typically, few patients received oral corticosteroids or biological DMARDs as first-line treatment. However, often intra-articular corticosteroids are used to offer temporary relief, and in a later stage, the combination of MTX and biological DMARDs are sometimes required for adequate disease control.

Outcome and predictors

The primary endpoint was non-response set as not achieving low disease activity (DAS>2.4) at first or second evaluation visit after 3–4 months and 6–8 months after the start of therapy, respectively. The secondary endpoint was EULAR good response criteria, defined as a DAS improvement of >1.2 from baseline and with a DAS of ≤2.4 attained during the first or second evaluation.[11]

Genotyping

Four genetic variants in four genes – *MTHFD1* rs17850560, *AMPD1* rs17602729, *ITPA* rs1127354, *ATIC* rs2372536 – were genotyped in all patients using the TaqMan technique. A TaqMan assay performed quantitative genotyping with a real-time polymerase chain reaction using the LightCycler® 480 (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol. The program LightCycler® 480 Endpoint Genotyping analysis software (Roche Diagnostics, Mannheim, Germany) was used to call the genotype results. Each variant was tested for Hardy-Weinberg equilibrium, and a $p<0.05$ was considered as deviance.

Statistical analysis

On baseline, first and second evaluation, the variables between the three cohorts were evaluated. To test differences between the observed responders (attained low disease activity; DAS≤2.4) and non-responders (DAS>2.4), variables at the second evaluation were compared. The variables in the prediction model (Table 4-1) at the first and second evaluation were entered into a logistic regression model and checked if those variables

showed the same effect as in the discovery study. The included variables with the same weighted scores were associated with actual response (low disease activity, DAS>2.4). The associations were reported as betas and OR with the corresponding p-values.

Receiver Operating Characteristic (ROC) curves of the prediction model with the four pharmacogenetic variants (pharmacogenetic model) and without (clinical model) were plotted and the area under the curve (AUC) was calculated.

Based on the summed score in the model, patients are divided into predicted responders (summed score of ≤3.5), intermediate responders (summed score between 3.5 and 6.0) or non-responders (summed score ≥6.0). To assess the performance of the prediction model the sensitivity, specificity, PPV and NPV were calculated. The intermediate responders were ignored in the calculation of the predictive parameters, but were used in the calculation of the AUC of the ROC curve.

Table 4-1. The pharmacogenetic model to predict non-response to methotrexate

Score	Variable
0	Male gender DAS at baseline ≤3.8 RF-negative non-smoker
1	Female gender RF-negative smoker RF-positive non-smoker *MTHFD1 1958* AA genotype *AMPD1 34* CC genotype *ATIC* 347 G-allele carrier
2	RF-positive smoker *ITPA* 94 A-allele carrier
3	DAS at baseline >3.8 and ≤5.1
3.5	DAS at baseline >5.1

A higher summed scores indicate a higher probability of non-response to methotrexate.
Abbreviations: DAS: Disease Activity Score, RF: Rheumatoid Factor.

Time evaluation of the different cohorts

The evaluation time differed intra-and interstudy. For instance, the tREACH study evaluation points were planned quarterly, while the IMPROVED study assessment was planned every four months. To check if those evaluation points influence the DAS and the prediction model, association between visiting times and the DAS were tested using Chi-square test and additionally, visually inspected for a pattern by a scatterplot.

Intended sample size

Based on the development study, the amount of minimal included patients was guided to an expected 40% prevalence of non-responders and a point estimate of 85% sensitivity. As a result, at least 264 patients required to be included to achieve a confidence limit of >75 with 0.95 probability.[12] We planned to include 320 patients to achieve some margins of error and misjudgment of the frequency of non-responders. This sample would allow 80% power to detect differences in sensitivity between responders and non-responders.

All statistical analyses were performed using RStudio version 1.0.136 (RStudio, Boston, MA) and IBM®. SPSS® Statistics 24.0 version (SPSS INC, Chicago, Illinois, USA). P-values lower than 0.05 were considered significant.

RESULTS

Cohort differences

Patient baseline characteristics were similar between the three study cohorts, except for age, smoking, ESR, CRP, VAS, and drug treatment (Table 4-2). The mean DAS at baseline was 3.49 (SD±0.98, range 0.67–6.77), 34 patients (11%) had a DAS below 2.4, patients median age was 54 years (range 18–87 years), the majority was female (69%), rheumatoid factor and anti-citrullinated protein antibodies were positive in 70 and 67% of patients, respectively. At first and second evaluation respectively, the mean MTX dosage of all included patients was 23.3±4.1 and 22.1±5.2 mg/week, and the given weekly MTX dosage was approximately the same between the cohorts. On the contrary, concomitant drug treatment differed between the groups, for example, RUMC patients started their treatment with fewer oral corticosteroids and less concomitant DMARDs than EMC and LUMC, on both evaluation points.

Study outcomes

After the first and second evaluation respectively, 215 (68%) and 223 patients (71%) achieved low disease activity (DAS≤2.4). EULAR good response (DAS<2.4 and DAS improvement >1.2 from baseline) was attained at the first and second visit, in respectively 165 (53%) and 169 (64%) patients. Genotype distribution of all four genetic variants were in Hardy-Weinberg equilibrium (p-value >0.05).

The patient baseline characteristics of the actual responders (DAS<2.4) and non-responders at the second evaluation are shown in Table 4-3. At baseline (start of therapy), significant differences were observed for the use of concomitant DMARDs and corticosteroids, gender,

Table 4-2. Patients characteristics at baseline, first and second evaluation.

	EMC (n=142)	LUMC (n=135)	RUMC (n=37)	Combined (n=314)
At first visit (baseline)				
Age, mean ±SD years	55.2±14.4	52.3±13.6	58.8±14.2	54.4±14.1
Female, n (%)	92 (64.8)	95 (70.4)	29 (78.4)	216 (68.8)
Smoker, n (%)	52 (36.6)	34 (25.2)	6 (16.2)	92 (29.3)
RF-positive, n (%)	102 (71.8)	90 (66.7)	27 (73.0)	219 (69.8)
ACPA positive, n (%)	101 (71.6)[#]	86 (63.7)	16 (61.5)[#]	203 (67.2)[#]
DAS, mean ±SD	3.5±0.9	3.4±1.0	3.6±1.2	3.5±1.0
ESR, mean ±SD	32.0±22.4	33.6±25.9	22.8±19.2	31.6±23.8
CRP, mean ±SD	20.4±27.9	23.1±32.0	19.0±30.4	21.5±30.0
VAS, mean ±SD	51.2±24.2	43.2±24.1	56.1±24.7	47.8±24.5
MTX doses, mean ±SD	25.0±0.0	25.0±0.0	14.24±6.88	23.72±3.89
Concomitant DMARDs, n (%)	138 (97.2)	135 (100.0)	8 (21.6)	281 (89.5)
Concomitant NSAIDs, n (%)	4 (2.8)	93 (68.9)	27 (73.0)	124 (39.5)
Concomitant corticosteroid, n(%)	136 (95.8)	135 (100.0)	3 (8.3)	247 (87.3)
Concomitant biologicals, n (%)	0 (0.0)	23 (17.0)	1 (2.7)	24 (7.6)
At first evaluation (t=3–4 months)				
DAS, mean ±SD	2.0±1.0	1.6±0.9	3.0±1.2	1.9±1.0
ΔDAS from baseline, mean ±SD	1.5±1.1	1.9±1.1	0.6±1.2	1.6±1.2
ESR, mean ±SD	17.3±14.2	12.4±10.1	21.9±22.7	15.7±14.3
CRP, mean ±SD	8.5±12.9	7.7±11.1	13.8±27.3	8.7±14.4
VAS, mean ±SD	31.1±23.0	21.7±20.5	40.6±27.0	28.2±23.3
MTX doses, mean ±SD	24.0±3.2	24.5±2.1	22.2±5.3	23.3±4.1
Concomitant DMARDs, n (%)	138 (97.2)	135 (100.0)	9 (24.3)	282 (89.9)
Concomitant NSAIDs, n (%)	18 (12.7)	65 (48.1)	24 (64.9)	107 (34.1)
Concomitant corticosteroid, n (%)	9 (6.3)	34 (25.2)	4 (10.8)	47 (15.0)
Concomitant biologicals, n (%)	0 (0.0)	23 (17.0)	1 (2.7)	24 (7.6)
At second evaluation (t=6–8 months)				
DAS, mean ±SD	1.9±0.9	1.6±0.8	2.8±0.9	1.9±1.0
ΔDAS from baseline, mean ±SD	1.7±1.1	1.8±1.1	0.8±1.0	1.6±1.1
ESR, mean ±SD	14.9±13.2	13.2±14.7	15.5±12.5	14.2±13.7
CRP, mean ±SD	7.4±11.6	7.7±16.1	6.4±10.0	7.4±13.5
VAS, mean ±SD	28.6±20.7	24.7±20.8	24.8±13.8	21.7±8.7
MTX doses, mean ±SD	22.7±4.4	22.5±5.4	21.6±5.6	22.2±5.2
Concomitant DMARDs, n (%)	141 (99.3)	134 (99.3)	10 (27.0)	285 (90.8)
Concomitant NSAIDs, n (%)	19 (13.4)	26 (19.3)	24 (64.9)	69 (22.0)
Concomitant corticosteroid, n (%)	9 (6.3)	34 (25.2)	3 (8.1)	46 (14.6)
Concomitant biologicals, n (%)	13 (9.2)	35 (25.9)	1 (2.7)	49 (15.6)

[#] Missing data.

Abbreviations: EMC: Erasmus Medical Center, LUMC: Leiden University Medical Center, RUMC: Radboud University Medical Center. RF: Rheumatoid Factor, ACPA: Anti-citrullinated protein antibodies, DAS: Disease activity score, ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein, VAS: visual analogue score, DMARDs: Disease-modifying antirheumatic drugs, NSAIDs: nonsteroidal anti-inflammatory drugs.

Table 4-3. Variables at baseline of the responders and non-responders (according the second evaluation)

Baseline variables	Responders (n=223)	Non-responders (n=91)	p-value
Age, mean ±SD	53.6±14.3	56.3±13.5	0.187
Female gender, n (%)	142 (63.7)	74 (81.3)	$3.42*10^{-3}$ **
RF-positive, n (%)	159 (71.3)	60 (65.9)	0.422
Current smoker, n (%)	64 (28.7)	28 (30.8)	0.819
DAS, mean ±SD	3.3±0.9	3.9±1.0	0.056
ESR, mean ±SD	30.9±23.5	33.5±24.6	0.795
VAS, mean ±SD	44.1±24.0	56.9±23.2	$2.30*10^{-5}$ ***
CRP, mean ±SD	20.4±26.8	24.2±36.8	0.315
MTX dose, mean ±SD	24.4±2.4	21.9±5.8	0.324
Concomitant NSAIDs, n (%)	89 (39.9)	35 (38.5)	0.912
Concomitant DMARDs, n (%)	211 (94.6)	70 (76.9)	$9.17*10^{-6}$ ***
Concomitant corticosteroids, n (%)	208 (93.3)	66 (72.5)	$1.47*10^{-6}$ ***
ITPA 94 A-allele carrier, n (%)	22 (9.8)	16 (17.6)	0.087
ATIC 347 G-allele carrier, n (%)	120 (53.8)	50 (54.9)	0.954
AMPD1 34 CC genotype, n (%)	187 (83.9)	70 (76.9)	0.199
MTHFD1 1985 AA genotype, n (%)	42 (18.8)	21 (22.1)	0.486

Responders were defined as DAS≤2.4 at 6 months.
Abbreviations: RF: Rheumatoid Factor, DAS: Disease Activity Score 28, ESR: Erythrocyte Sedimentation Rate, VAS: Visual Analogue Score, CRP: C-reactive protein, MTX: methotrexate, NSAIDs: non-steroidal anti-inflammatory drugs,
DMARDs: Disease-modifying antirheumatic drugs.
* p<0.05, ** p<0.01, *** p<0.001. # Including missing data.

and the VAS. RUMC patients less often started on combination therapy with corticosteroids, less often had an EULAR response than patients in the other cohorts.

Performance of the pharmacogenetic model

Table 4-4 shows the distribution of the patients into non-responders, intermediate and responders according to the cut-off values of the pharmacogenetic model, divided into patients that achieved response (DAS<2.4) or non-response (DAS≥2.4). At first evaluation, the model for prediction non-response had a sensitivity of 67% (CI: 54–78%), specificity of 70% (CI: 61–78%), PPV of 55% (CI: 47–63%) and NPV of 79% (73–85%). At the second evaluation, the model for prediction non-response had a sensitivity of 67% (CI: 54–78%), specificity of 69% (CI: 60–77%), PPV of 52% (CI: 45–60%) and NPV of 80% (73–85%).

Table 4-4. Pharmacogenetic model at first and second evaluation with observed and predicted MTX response (n=314)

	Predicted response according to the prediction model			
	Non-responders	Intermediate	Responder	
	Score ≥6	Score 3.5–6	Score ≤3.5	Total
Observed response at first evaluation				
Non-responder	46	30	23	99
Responder	38	89	88	215
Total	84	119	111	314
Observed response at second evaluation				
Non-responder	44	25	22	91
Responder	40	94	89	223
Total	84	119	111	314

Non-responders were classified as DAS>2.4 and responders as DAS≤2.4

Regression analysis of the prediction model

Regression analyses of the variables in the prediction are shown in Supplementary Table S4-1. At both time points (first and second evaluation) only the variables female gender and DAS at baseline were significantly associated ($p<0.05$) with MTX response (DAS≤2.4). At first evaluation, RF-positive smoker, *MTHFD1*, and *ATIC* were associated with non-response, while at second evaluation this was only seen for *AMPD1* (OR<1.0). Also, the confidence intervals of most included variables cross 1.0, and this implies that those variables show no difference between the responders and non-responders.

Figure 4-1 plots the ROC curves of the pharmacogenetic and clinical model (without the four genetic variants). The AUC of the ROC curves were 74.6% and 71.5% for the pharmacogenetic model and the clinical model respectively at the first evaluation. The AUC of the second evaluation was lower than that of the first evaluation, with 69.1% and 67.1%, for the pharmacogenetic and clinical model respectively. Taken LUMC and EMC together (without the 32 RUMC patients); the AUC of the ROC were similar to the group consisting of the three cohorts.

Using the EULAR response criteria as an endpoint instead of low disease activity (DAS<2.4) leads to worse performance of the prediction model. The AUC of the ROC curves with EULAR response were AUC of 62.9 and 63.4 (pharmacogenetic), and AUC of 57.7 and 62.3 (clinical model), respectively for the first and second visit.

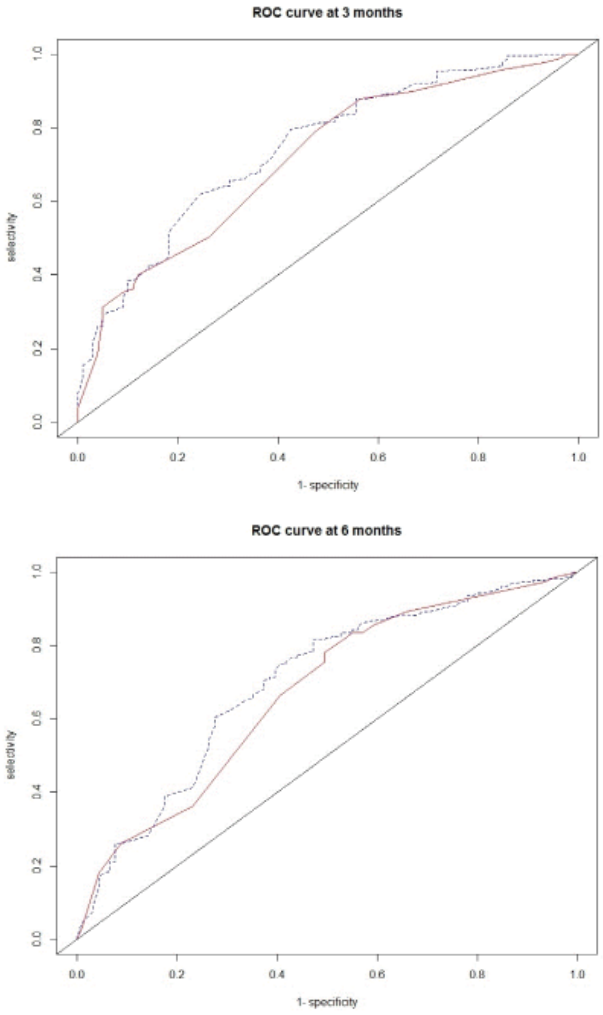


Figure 4-1. The receiver operating characteristic (ROC) curves of the pharmacogenetic model and clinical model.
The ROC curve was expressed as 1-specificity with sensitivity at first evaluation (t = 3 mo) and second evaluation (t = 6 mo). The pharmacogenetic model (blue line) contained the variables: gender, DAS28 at baseline, RF, smoking status and the genetic variants *ATIC* 347G, *IPTA* 94A, *MTHFD1* 1985AA and *AMPD1* 34CC. The clinical model (red line) contained the variables: gender, DAS28 at baseline, RF and smoking status.

Evaluation time differences

Analysis of the time after the start of MTX (evaluation time) to the DAS showed no pattern in the scatterplots in both evaluation points (Supplementary Figure S4-1). Also, no statistical difference was found between the DAS and the time visits: p-values were 0.08 and 0.56 at the first and second evaluation, respectively.

DISCUSSION

This study shows that the pharmacogenetic model, originally derived in early RA patients treated with MTX monotherapy, could not predict non-response of RA patients treated with MTX based combination therapies. Although the AUCs of the ROC curves were weak to modest (approximately 70%), the PPV, specificity and sensitivity were inadequate to predict non-response. For instance, the PPV, the complement of the false discovery rate, showed that approximately 50% of the actual non-responders (47 out of 91) were predicted as responders. Interestingly, while in MTX monotherapy the model had a sensitivity of 86% in predicting non-response, in patients treated with MTX combination therapy the PPV had a decrease to 70%. Therefore, the prediction model is not clinically applicable to predict non-response in patients treated with MTX combination therapy.

There are several possible explanations for the underperformance of the prediction model. One reason is that the included pharmacogenetic variants showed a minimal additive value in the prediction model with an AUC increase of 2.0 and 3.1% of the ROC curves, for the first and second evaluation visit respectively. The reason may be that the pharmacogenetic variants are related to the mechanism of action of MTX and adding other DMARDs as is the case in our replication cohort compared to the discovery cohort could dilute the predictive effect. While the weak predictive value of the pharmacogenetic variants was confirmed in the replication studies with MTX monotherapy treated RA patients, the variants showed a better prediction and therefore makes it a necessary component in the pharmacogenetic model.

Another potential explanation for the underperformance of the prediction model may be the baseline DAS in the prediction model. Patients in the development cohort had a high mean baseline DAS of 4.4, and as a consequence, a DAS of 3.8 was a modifier for response in the prediction model. In our cohort, however, the baseline DAS was 3.5 and showed a small contribution in the prediction model. Because the low baseline DAS, and because the use of combination therapies, the majority of the patients achieved low disease activity on both evaluation visits (circa 70%). Yet, using the EULAR response criteria, that takes the baseline DAS into account and showed ~50% responders, still results in poor prediction and is not applicable in the clinical setting. This study showed that the predictive value of the model exists mainly on the clinical values: gender, rheumatoid factor positivity, and smoking status. The use of different RA classification criteria (1987 or 2010 criteria) could also play a role in the underperformance of the prediction model. For instance, the 2010 criteria were broader, and patients could be indicated with RA in an earlier disease stage. However, no difference was found between the classification criteria of RA in baseline DAS scores.

The frequency of the predicted intermediate responders is an important indicator of the feasibility of the prediction model and could limit the clinical usefulness, as it increases the number needed to diagnose. In our study, a large group of patients (approximately 40%) were predicted intermediate responders, and for this group, no drug advice (MTX or alternative drug treatment) could be given. Therefore, it may be better to use a single cutoff value in the prediction model to get a clear distinguishment between two groups: predicted responders and predicted non-responders. For instance, this was performed in the large replication study, where responders and intermediate responders were combined into one group.

Our study has a few strengths. First, with 314 patients the study is one of the largest MTX pharmacogenetics studies published so far. Also, the estimations of the diagnostic parameters were precise, with small CIs around them. Second, patients were treated with mainly combination therapies of MTX with either another DMARD or tapered corticosteroids and thus represents treatment according to daily clinical practice. Third, the use of the TRIPOD and STARD reporting criteria ensures a full and transparent way of reporting.

The prediction of efficacy in RA seems challenging with still today no clear indicators for routine daily practice. Multiple studies tried to find predictors for the response to MTX or the discontinuation of MTX in RA patients,[13–16] but those studies lack or failed replication. Subsequently, a review on biological DMARDs showed 65 potential (bio)markers, but as well no validation studies were performed.[17] Probably, even a reasonably accurate prediction of response will not have a substantial impact on the treatment outcome. One explanation was that hospitals increasingly used the treat-to-target approach (with the DAS steered therapy) and the use of temporary corticosteroids treatment. This results in the finding of current trials that >80% of the patients are in a state of remission after one year of drug treatment. Also, the prediction models include variables that also predict to some extent non-response for alternatives for MTX. For example, sex, RF as acute phase reactants have weak predictive effects also for other (b)DMARDs. Therefore, overall, there seems little room to improve the treat-to-target and trial and error RA care vastly.

In summary, a prediction model developed to predict response to MTX monotherapy was tested in three other cohorts starting with MTX combination therapy and performed poorly. Based on patients with the treat-to-target approach, prediction models offer no added value for daily clinical practice.

Acknowledgement

The authors would like to thank the following persons for their assistance with the collection of the data or receiving the DNA: Thea van Gaalen, Els van den Enden, Ron Buijs, and Marieke Coenen. We would also like to extend our thanks to Renée Baak-Pablo, Daniëlle Klootwijk, and Rowena Schaap, for their help in genotyping.

REFERENCES

1. Scott DL, Wolfe F, Huizinga TWJ. Rheumatoid arthritis. Lancet 376, 1094–1108 (2010).
2. Singh JA, Saag KG, Bridges SL *et al*. 2015 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis. Arthritis Care Res. (Hoboken). 68, 1–25 (2016).
3. Wessels JAM, van der Kooij SM, le Cessie S *et al*. A clinical pharmacogenetic model to predict the efficacy of methotrexate monotherapy in recent-onset rheumatoid arthritis. Arthritis Rheum. 56, 1765–1775 (2007).
4. Fransen J, Kooloos WM, Wessels JAM *et al*. Clinical pharmacogenetic model to predict response of MTX monotherapy in patients with established rheumatoid arthritis after DMARD failure. Pharmacogenomics 13, 1087–1094 (2012).
5. López-Rodríguez R, Ferreiro-Iglesias A, Lima A *et al*. Evaluation of a clinical pharmacogenetics model to predict methotrexate response in patients with rheumatoid arthritis. Pharmacogenomics J. 18, 539–545 (2018).
6. Collins GS, Reitsma JB, Altman DG, Moons KGM. Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD): the TRIPOD statement. BMJ 350, g7594 (2015).
7. Cohen JF, Korevaar DA, Altman DG *et al*. STARD 2015 guidelines for reporting diagnostic accuracy studies: Explanation and elaboration. BMJ Open 6, 1–17 (2016).
8. de Jong PH, Hazes JM, Kan HK *et al*. Randomised comparison of initial triple DMARD therapy with methotrexate monotherapy in combination with low-dose glucocorticoid bridging therapy; 1-year data of the tREACH trial. Ann. Rheum. Dis. 73, 1331–9 (2014).
9. Heimans L, Wevers-de Boer KVC, Visser K *et al*. A two-step treatment strategy trial in patients with early arthritis aimed at achieving remission: the IMPROVED study. Ann. Rheum. Dis. 1356–1361 (2013). doi:10.1136/annrheumdis-2013-203243
10. Kievit W, Fransen J, de Waal Malefijt MC, den Broeder AA, van Riel PLCM. Treatment changes and improved outcomes in RA: An overview of a large inception cohort from 1989 to 2009. Rheumatol. (United Kingdom) 52, 1500–1508 (2013).
11. van Gestel AM, Prevoo ML, van 't Hof MA *et al*. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis: Comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism Criteria. Arthritis Rheum. 39, 34–40 (1996).
12. Flahault A, Cadilhac M, Thomas G. Sample size calculation should be performed for design accuracy in diagnostic test studies. J. Clin. Epidemiol. 58, 859–862 (2005).
13. Jenko B, Tomsic M, Jekic B *et al*. Clinical Pharmacogenetic Models of Treatment Response to Methotrexate Monotherapy in Slovenian and Serbian Rheumatoid Arthritis Patients: Differences in Patient's Management May Preclude Generalization of the Models. Front. Pharmacol. 9, 20 (2018).
14. Jenko B, Lusa L, Tomsic M, Praprotnik S, Dolzan V. Clinical-pharmacogenetic predictive models for MTX discontinuation due to adverse events in rheumatoid arthritis. Pharmacogenomics J. 1–7 (2016). doi:10.1038/tpj.2016.36

15. Hider SL, Silman AJ, Thomson W *et al*. Can clinical factors at presentation be used to predict outcome of treatment with methotrexate in patients with early inflammatory polyarthritis? Ann. Rheum. Dis. 68, 57–62 (2009).

16. de Rotte MCFJ, de Jong PHP, Pluijm SMF *et al*. Association of Low Baseline Levels of Erythrocyte Folate With Treatment Nonresponse at Three Months in Rheumatoid Arthritis Patients Receiving Methotrexate. Arthritis Rheum. 65, 2803–2813 (2013).

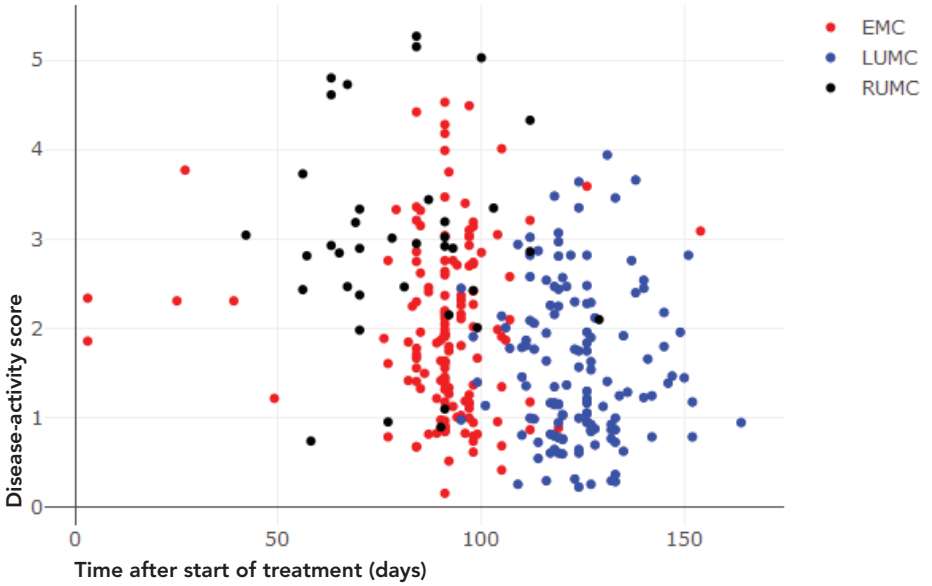
17. Cuppen BVJ, Welsing PMJ, Sprengers JJ *et al*. Personalized biological treatment for rheumatoid arthritis: a systematic review with a focus on clinical applicability. Rheumatology 55, 826–839 (2016).

Supplementary Table S4-1. Regression coefficients and odds ratios of the logistic regression models to predict MTX response

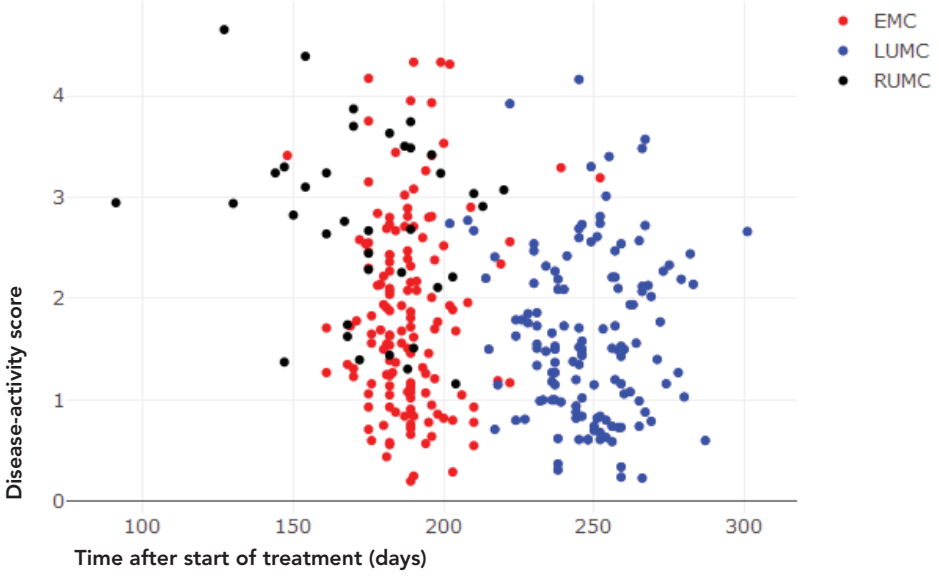
	At first evaluation visit (3–4 months)		
Variable	β	OR [95% CI]	p-value
(intercept)	2.53	12.54 [4.59–37.08]	$1.92 * 10^{-06}$ ***
Female gender	-1.72	0.18 [0.09–0.35]	$1.55 * 10^{-06}$ ***
DAS at baseline	-0.43	0.65 [0.55–0.78]	$2.01 * 10^{-06}$ ***
RF positive smoker	0.01	1.01 [0.68–1.50]	0.960
MTHFD1 1958 AA genotype	0.09	1.09 [0.57–2.14]	0.795
AMPD1 34 CC genotype	-0.03	0.97 [0.48–1.90]	0.929
ITPA 954 A-allele carrier	-0.33	0.72 [0.49–1.05]	$8.39 * 10^{-2}$ *
ATIC 347 G-allele carrier	0.21	1.23 [0.73–2.08]	0.434
	At second evaluation visit (6–8 months)		
Variable	β	OR [95% CI]	p-value
(intercept)	1.94	6.97 [2.73–18.89]	$7.92 * 10^{-05}$ ***
Female gender	-0.94	0.39 [0.20–0.71]	$2.93 * 10^{-3}$ **
DAS at baseline	-0.40	0.67 [0.57–0.80]	$6.02 * 10^{-6}$ ***
RF positive smoker	-0.04	0.96 [0.65–1.43]	0.857
MTHFD1 1958 AA genotype	-0.16	0.86 [0.45–1.65]	0.637
AMPD1 34 CC genotype	0.38	1.46 [0.75–2.79]	0.256
ITPA 954 A-allele carrier	-0.34	0.71 [0.49–1.04]	$7.56 * 10^{-2}$ *
ATIC 347 G-allele carrier	-0.09	0.92 [0.54–1.54]	0.742

Abbreviations: DAS: disease activity score, RF: rheumatoid factor, β: regression coefficient, OR: Odds ratio, CI: confidence interval.
* p<0.10, ** p<0.01, *** p<0.001.

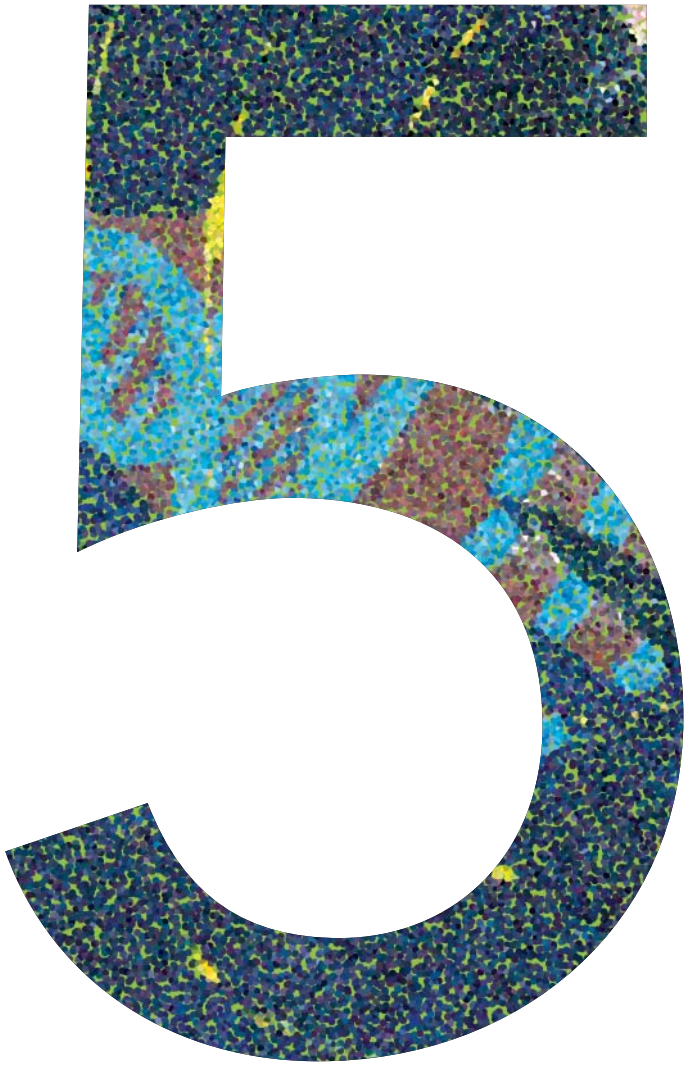
A. First evaluation



B. Second evaluation



Supplementary Figure S4-1. Scatterplot of the time visits of the first and second evaluation (days) versus the disease-activity score.



Genome-wide association analysis of methotrexate-induced liver injury in rheumatoid arthritis patients

Frank Eektimmerman
Jesse J. Swen
Alfons A. den Broeder
Johanna M.W. Hazes
Fina A.S. Kurreeman
Suzanne Verstappen
Andrzej Pawlik
Michael T. Nurmohamed
Vita Dolžan
Stefan Böhringer
Cornelia F. Allaart
Henk-Jan Guchelaar

Submitted

Abstract

Introduction: Approximately 16% of the patient discontinue low-dose methotrexate (MTX) treatment due to adverse drug reactions (ADRs), such as hepatic toxicity. However, the cause of drug-induced liver injury (DILI) is unclear and unpredictable and may have a genetic origin. Therefore, we performed a nested case control genome-wide association study (GWAS) to explore genetic factors that are associated with MTX DILI.

Methods: Seven international groups contributed blood samples and data of RA patients who started MTX. MTX-induced DILI was defined as an ALT level of ≥3X the upper limit of normal (ULN), while controls had no ALT levels above 3X ULN. Controls (ratio 3:1) were matched per study site for age, gender, and duration of MTX use. GWAS with MTX DILI and individual SNPs (693,931) was performed using the additive genetic model, corrected for sex, country, and age. A $p \leq 5x10^{-8}$ was considered significant, while a $p \leq 5x10^{-6}$ was considered suggestive.

Results: 104 MTX-induced DILI cases and 315 controls were included for association analysis. None of the SNPs were significantly associated with MTX DILI. However, we found seven genetic variants that were suggestive of association with MTX DILI (p-value $7.43*10^{-8}$ to $4.86*10^{-6}$). Of those, five SNPs are in the intronic protein-coding regions of *FTCDNL1*, *BCOR*, *FGF14*, *RBMS3*, and *PFDN4*/*DOK5*. Further investigation into *MTHFR* C677T and the HLA region did not lead to significant findings.

Conclusion: We were not able to find clear genetic variants associated with MTX-induced DILI.

INTRODUCTION

Low-dose methotrexate (MTX) is the cornerstone of antirheumatic drug treatment. Although MTX is an effective and safe antirheumatic drug, adverse drug reactions (ADRs) are the reason for discontinuation in about 16% of the patients.[1,2] Common ADR of MTX includes bone marrow suppression, pulmonary, gastrointestinal, and hepatic toxicity. Drug-induced liver injury (DILI) is among the most important ADR, but the cause of liver damage from MTX use is still unclear and MTX-induced liver toxicity remains largely unpredictable.[6] A potential cause of DILI is the depletion of folate and accumulation of MTX polyglutamates in the liver.[3] Another possible mechanism is the release of adenosine, which stimulates the matrix proteins by fibrogenic activation of stellate cells in the liver and thus fibrosis formation.[4] Additional risk factors for MTX-induced hepatotoxicity are concomitant use of NSAIDs, obesity, and excessive alcohol consumption.[5–8]

Elevated levels of the hepatic transaminase enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) occurs in 7.5 to 26% of the RA patients treated with MTX, and the incidence of high-level enzyme elevation (at least 2–3 times the upper limit of the normal range [ULN]) has been found in 3.5% of patients.[9,10] Persistently elevated levels correlate with histopathologic abnormalities and changes in fibrosis assessed by liver biopsy samples, although relation with the clinical outcome of interest – clinically overt liver cirrhosis – has not been well established. Therefore, monitoring of the transaminase levels in RA patients treated with MTX is important and combined with liver biopsy are recommended in the American College of Rheumatology Guideline for the Management of Rheumatoid Arthritis. According to these guidelines it is recommended to initially monitor transaminases every 2–4 weeks, then every 12 weeks in patients on stable therapeutic doses.[11] MTX therapy must be discontinued when two subsequent ALT/AST levels are higher than three times the ULN. After normalization of the liver enzyme levels, MTX therapy may be resumed at a lower dose.

A large meta-study by Owen *et al*.[12] showed that the polymorphism MTHFR C677T is a promising genetic variant predictive for increased risk of MTX-induced liver toxicity. RA patients treated with MTX who are a carrier of C677T have a mildly increased risk of hepatotoxicity (OR 1.71, 95% CI: 1.32–2.21, $p<0.001$) compared to patients with the wildtype genotype. Interestingly, genetic variants in the human leukocyte antigen (HLA) gene have been related to DILI for a variety of drugs, albeit not with MTX-induced DILI.[13] For example, HLA-B*14:01 was associated with trimethoprim-sulfamethoxazole-induced liver injury in European Americans, and HLA-B*5701 was associated with flucloxacillin DILI.[14,15] In the field of rheumatology, however, no association was found between HLA antigens and MTX DILI RA.

Since the role of genetic predisposition of MTX liver injury remains unclear, we hypothesize that genetic polymorphisms could unravel the mechanism or cause of MTX-induced DILI. Therefore, we performed a genome-wide association study (GWAS) in a case-control design to explore genetic loci associated with MTX DILI in RA patients.

METHODS

Study population

In the literature, we searched for publications on biomarker studies in RA on MTX monotherapy and invited principal investigators to participate in this GWAS MTX DILI study. MTX DILI cases were defined as RA patients on MTX having a single event of an ALT level of >3x ULN, while controls had an ALT within the range 0–3x ULN.

A total of seven international research groups from Poland, the United Kingdom, Slovenia, and The Netherlands provided blood or DNA samples and clinical data for the GWAS. The included patients (n=430) were from previously described clinical cohorts: RAMS study (Manchester, United Kingdom, n=24),[16] BeST study[17] (Leiden, The Netherlands, n=114), tREACH trial (Rotterdam, The Netherlands, n=48),[18] DREAM registry[19] (Nijmegen, the Netherlands, n=84), or were derived from daily clinical practice in Slovenia (University Medical Centre Ljubljana, Ljubljana, Slovenia, n=76), The Netherlands (Reade, Amsterdam, the Netherlands, n=48) or Poland (Pomeranian Medical University, Szczecin, Poland, n=36).

All included patients were diagnosed with RA according to the 1987 ACR and/or 2010 ACR/EULAR criteria and used MTX with folic acid for at least 6 months. Controls were matched per study site to cases (3:1) for age (±5 years difference), gender (male/female), and the duration of MTX use (±50 days). Data collected for each patient were baseline laboratory measurements at the start of MTX, age, gender, concomitant drug treatment (NSAIDs, corticosteroids, other DMARDs). According the study protocol or the standard treatment procedure, regular laboratory measurements were included during the follow-up time (until ALT >3 ULN of the included cases or the related control).

At each site, investigators obtained written informed consent from the patients. Every participating study group provided consent for the use of these samples for this study. Each study site obtained approval from the local ethics committee or institutional research ethics board.

Sample size

Prior to the inclusion of the cases/controls the required sample size was estimated. A logistic regression of the binary response variable (ALT >3X ULN) on a binary independent variable (genotype of the individual SNP) with a sample size of 320 observations (ratio case/control groups of 3:1) achieves 80% power at a 0.05 significance level to detect a change from the baseline value of 0.250 to 0.417. This change corresponds to an odds ratio of 2.146.

Validation cohort

Another research group was asked to confirm our findings using an independent validation cohort. Validation was performed only if there were significant hits.

Genotyping and quality control

Full blood samples or germline DNA were collected at each site and sent to the LUMC for preparation according to the manufacturer's recommended protocol. Samples were prepared to a DNA concentration of 4 µg (50 ng/µl) and were assessed by a spectrophotometer (Nanodrop; Wilmington, DE, USA). GWAS genotyping was conducted with the Illumina GSA Beadchip Illumina GSA MD-24v1-0 in the Human Genotyping Facility Genetic Laboratory at the Erasmus MC, Rotterdam, The Netherlands. This array contains 693,931 SNPs. The RAMS study had previously performed a GWAS using GRCh37/hg19 imputed with the 1000 genomes V3 reference panel. For the analysis, the SNPs corresponding to the GSA Beadchip were extracted from the RAMS GWAS and merged with the GWAS data.

Quality control (QC) checks were performed using software R version 3.5.0[28] and PLINK-software, version 1.07[20,29,30] Patients were excluded from analyses based on an individual genotype call rate <97%, gender mismatch between reported and estimated sex based on genotypes of the X-chromosome (using PLINK), or excess of heterozygous genotypes as measured by the inbreeding coefficient. The imbreeding F-statistic was used to detect excess of heterocygosity based on outlier detection. Genetic markers were excluded based on a SNP call rate <97% and a $p \leq 10^{-7}$ for the Hardy-Weinberg equilibrium (HWE) goodness-of-fit test.

After exclusion of patients and markers in these marginal QCs, the remaining set was used for integrative QC assessment. To evaluate the possibility of population stratification or outliers, multidimensional scaling (MDS) analysis was performed using PLINK. Additionally, pairwise identity by state (IBS) statistics were calculated to identify potential duplicates. MDS and IBS were computed using PLINK. Patients who were identified as outliers based on IBS clustering were excluded from the analysis. MDS coordinates were extracted and used as

covariates in the association analysis. SNP imputation was performed using the programs "shapeit" and "impute2" with default parameters using the reference panel 1000Genomes build version 3 with the total, 'cosmopolitan', set of individuals.[21] An MDS plot was used to compare the self-reported and genetic ethnicity of patients.

Data analysis

Association analysis with MTX DILI and individual SNPs was performed using an additive genetic model, with sex, country, and age as covariates. Specifically, *MTHFR* C677T (rs1801133) and the HLA gene region were explored to investigate associations between MTX DILI and these SNPs, using the additive genetic model, with covariates sex, country, and age.

Statistical analyses were performed in R statistics version 3.5.0. Associations with a $p \leq 5x10^{-8}$ were considered statistically genome-wide significant and associations with a p-value between $5x10^{-8}$ and $5x10^{-6}$ were considered suggestive.[22] Post association QC was performed by visual inspection of Quantile-Quantile (QQ) plots of p-values of association tests and computation of the inflation factor.[23] To improve readability, and account for high correlation between neighbouring SNPs, the list of top-SNPs according to p-values, only contains the best association in a window of 100kb. The full list is given as supplementary material.

RESULTS

Quality control

Eleven patients were excluded based on missing data (n=8, due to insufficient amount of DNA) or gender check using the heterozygosity/inbreeding coefficient (n=8, Figure 5-1A). 196,650 SNPs were excluded due to low allele frequency, missing data analysis, or not meeting the HWE criterion (Figure 5-1B). No outliers were detected on IBS clustering. After applying the quality control criteria, a total of 502,291 NPs in 419 RA patients, 104 cases, and 315 controls, were available for association analysis.

Study population

The demographic and clinical characteristics of the study population are shown in Table 5-1. The mean dose of MTX was 18.8 mg/week (SD: 6.0, range 7.5–30 mg/week), mean age was 54.8 years (SD: 13.0, range 20–87 years), with a disease duration of 34.3±84.2 weeks. All patients used folic acid.

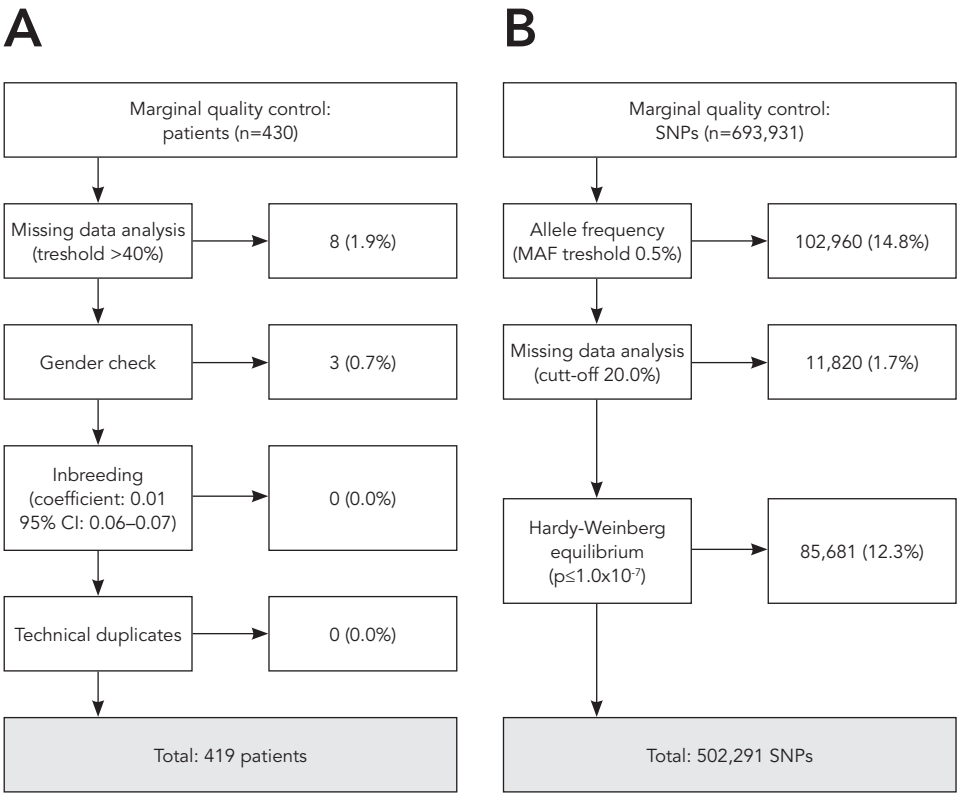


Figure 5-1. Flow chart of the quality controls for patients (A) and SNPs (B).

GWAS results

None of the SNPs reached the genome-wide level of statistical significance ($p \leq 5x10^{-8}$) in the analysis (multivariate corrected for gender, age, and country) and therefore no external validation was used. Seven SNPs were found to meet the threshold of $p \leq 5x10^{-6}$ and are therefore considered suggestive for an association with MTX DILI. From those, five SNPs were related to genes in *FTCDNL1* rs12693892, *BCOR* rs4827191, *FGF14* rs75805413, *RBMS3* rs73044680 and *PFDN4/DOK5* rs67738640. After merging with the RAMS GWAS 693,931 SNPs were tested. Also, imputation of genetic markers using the 1000Genomes dataset lead to 932,8966 SNPs, but did not lead to hits after Bonferroni correction ($p > 5x10^{-8}$).

The *MTHFR* C677T (rs1801133) genetic variant was not significantly associated with MTX DILI (p=0.4). Also, the result of the SNPs in the HLA region (chromosome 6 29941260 to 33143325 base pairs) found no significant association ($p > 10^{-4}$).

Table 5-1. Demographic and clinical characteristics of the included patients (n=419)*

Characteristic	
Age, years, mean	54.8±13.0
Female gender, no (%)	325 (77.8%)
Smoker, no (%)	96 (32%)
Alcohol consumption, no (%)#	128 (50%)
Disease duration, weeks	34.3±84.2
DAS28 start of MTX	4.6±1.39
Rheumatoid factor positive, no (%)	233 (71.3%)
C-reactive protein, mean	20.9±23.9
MTX maintenance doses, mg/week	18.8±6.0
Folic acid doses, mg/week	6.5±3.2
Other DMARD use, no (%)	72 (35.5%)
NSAID use, no (%)	32 (30.2%)
Corticosteroid use, no (%)	97 (75.8%)
Laboratory values at start MTX	
ASAT (U/L)	26.1±24.4
ALAT (U/L)	25.0±17.3
Creatinine	56.7±36.4
Hemoglobin (mmol/L)	8.2±2.2
Gamma-GT (U/L)	32.4±19.7

Abbreviations: AST: aspartate transaminase, ALT: alanine transaminase, DAS28: Disease Activity Score of 28 joints. DMARD: disease-modifying antirheumatic drugs, MTX: methotrexate.
* Plus-minus values are means ±SD.
# Alcohol consumption is defined as at least three alcoholic units per week.
Continuous values as mean ± standard deviation.

Table 5-2. Suggestive biomarkers related to MTX toxicity using the model corrected for sex, country, age, and SNP ($p<5.0*10^{-6}$)

Marker	Gene	Allele	Chromosome (GRCh37.p13)	MAF	1000 genome MAF	p-value
rs12693892	LINC01877 FTCDNL1[1)]	A>G	2:200483842	0.4964	0.3764	$7.43*10^{-8}$
rs4827191	- BCOR	A>C	23:39901078	0.1646	0.1891	$2.51*10^{-6}$
rs75805413	FGF14	T>C	13:102917882	0.0152	0.0042	$3.50*10^{-6}$
rs73044680	RBMS3	A>G	3:295756660	0.0426	0.0176	$4.26*10^{-6}$
rs7447381	LINC01170	C>T	5:123401074	0.4839	0.4820	$4.33*10^{-6}$
rs12693889	LINC01877	T>A,C	2:200473658	0.1975	0.2456	$4.53*10^{-6}$
rs67738640	- PFDN4 DOK5	T>C	20:52931326	0.0589	0.1336	$4.86*10^{-6}$

[1)] 156,200 base pairs away from the FTCDNL11 gene.

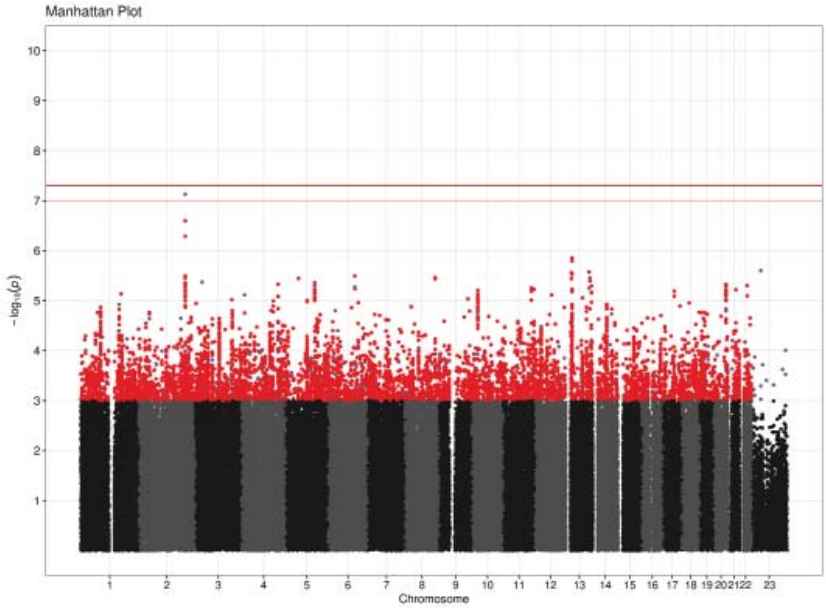


Figure 5-2. Manhattan plot for the model MTX DILI corrected for gender, age, and country.
The significance level (red horizontal line) is set to $5.0*10^{-8}$. The red dots are the imputed data and the dark blue dots are the measured data.

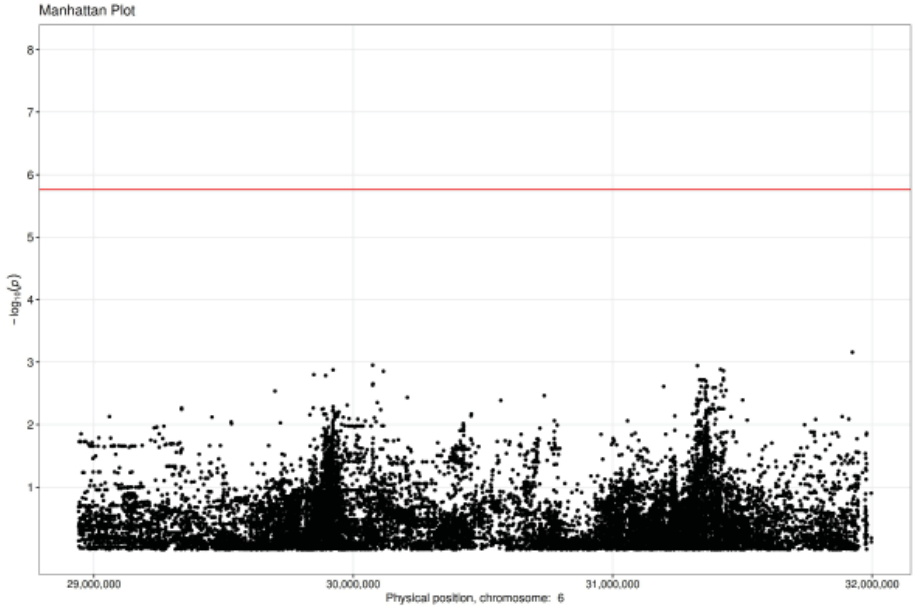


Figure 5-3. Manhattan plot of the HLA region for the model MTD DILI corrected for gender, age, and country.
The significance level (red horizontal line) is set to the Bonferroni boundary for the HLA region ($p=1.73*10^{-6}$).

DISCUSSION

To the best of our knowledge, this study is the first GWAS to investigate genetic variants associated with MTX DILI. In this exploratory GWAS, we identified seven suggestive variants associated with MTX DILI in LINC01877 (*FTCDNL1*) *rs12693892*, *BCOR rs4827191*, *FGF14 rs75805413*, *RBMS3 rs73044680*, LINC01170 rs7447381, LINC01877 rs12693889 and *PFDN4/DOK5*. While no SNPs with genome-wide significance ($p<5.0*10^{-8}$) were found, the most promising SNP was *LINC01877* rs12693892 with $p=7.43*10^{-8}$.

Our findings point toward genes encoding proteins related to the mechanism of action of MTX. Albeit *LINC01877* rs12693892 is a non-protein-coding RNA sequence, it is close within the *FTCDNL1* gene (156,200 base pair difference). Protein expression of *FTCDNL1* was mainly found in the brain and liver tissue, whereas *FTCDNL1* provides a transferase and binding activity of folic acid.[24] The use of MTX and a deficiency of *FTCDNL1* could lead to a reduction of the protective effects of folic acid and could therefore cause liver injury. No other studies found an association of liver injury with rs12693892, while two other SNPs in *FTCDNL1* (rs10203122 and rs7605378) were previously associated with osteoporosis.[25]

BCOR is an epigenetic regulator that binds to BCL-6 and takes part in the polycomb repressive complex (PRC) 1.1. PRC1.1 silences genes through ubiquitination of Lys119 in histone H2A. *BCOR* germinal loss-of-function mutations determine oculo-facio-cardio-dental syndrome and mutations in BCOR have been associated with different types of cancer (CNS tumors, sarcomas, hemolymphopoietic system tumors, and carcinomas).[26] Hypothetically, mutations in *BCOR* could change cell functioning and cell survival. The SNP identified in this GWAS (rs4827191) is close within the BCOR gene effect allele and is located on the X-chromosome. Due to the haploid nature of males, the power to detect a significant association with rs4827191 are halved for males compared to females. To take account of this haploid effect, we matched on gender.

Fibroblast growth factor (FGF) family members possess broad mitogenic and cell survival activities and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth, and invasion.[27] A hypothetic mechanism could be that alterations in the production of the protein-coding *FGF14* rs75805413 cause less liver cell recovery by the hepatotoxic effects of MTX and could explain the increase of the level of transaminases. Also, multiple *FGF* SNPs have been associated with Spinocerebellar Ataxia 27[28] and Autosomal Dominant Cerebellar Ataxia.[29]

RBMS3 is a protein-coding gene that has been implicated in diverse functions, like DNA replication, gene transcription, cell cycle progression, and apoptosis. Previous studies found that RBMS3 is a risk factor related to bisphosphonate-related osteonecrosis,[30] systemic

sclerosis,[31] and different cancer types.[32] *RBMS3* A>G (rs73044680) could influence the liver cell, which can trigger MTX DILI.

rs67738640 is a non-coding SNP located between the *PFDN4* and *DOK5*. *PFDN4* is a transcription factor that regulates the cell cycle and ensures the binding and stabilizing of new proteins by correct folding. Prognostic markers have been associated with B-cell non-Hodgkin's lymphoma, breast cancer,[33] colorectal,[34,35] and liver cancer and hepatocellular carcinoma.[36] *DOK5* is essential in signal transduction, including MAP kinase (inflammatory cascade) activity. DOK5 has been associated with multiple auto-immune diseases, such as diabetes type II,[37] systemic sclerosis,[38] and also with different cancer types.[39] Genetic alterations in *PFDN4* or *DOK5* could cause dissection of the folding and inflammation mechanism and subsequent lead to hepatotoxicity.

The MTHFR enzyme is important in folate metabolism, an integral process for cell metabolism in the DNA, RNA, and protein methylation.[40] Previous studies showed that *MTHFR* C677T (rs1801133) was associated with MTX DILI.[12] In our study, analysis on the *MTHFR C677T* showed no association with MTX DILI ($p<10^{-3}$). Previous studies showed associations of drug-induced liver toxicity with SNPs in the HLA region, but this was not yet explored for MTX DILI. HLA is responsible for the regulation of the immune system and it may protect against or fail to protect against external pathogens and is also linked to different auto-immune diseases, such as RA and coeliac disease. In our study, we found no evidence of an association between the HLA SNPs and MTX DILI. However, if HLA genetic variants predispose for RA, choosing the control group is of great importance. Thereby, the effect of MTX DILI could be missed, and another study with a second non-RA patient group had to be selected that used MTX without having DILI.

DILI was defined as at least three times the ULN of the ALT. A limitation is that we included RA patients that used MTX and had a single event of an increase of ALT of 3 times the ULN. Obviously, ALT elevation is not specific for DILI as other factors may also cause the increase of ALT, such as concomitant non-alcoholic fatty liver disease or alcoholic liver disease.[41–46] For instance, in our study 50% of the included patients used regularly alcohol (minimal 3 units per week) that could contribute to the emerging of MTX DILI. Of note, we did not prospectively match alcohol consumption between the case and control groups, but post hoc analysis showed that alcoholic use was evenly contributed amongst cases and controls.

Obesity might be associated with more severe inflammation through elevated or reduced levels of secretory adipocytes products, such as resistin and adipocytokines leptin. Those inflammatory processes may exacerbate chemical-induced hepatotoxicity.[47] These mechanisms might be involved with MTX DILI, but further research is needed for a clear

understanding of the possible association between obesity and MTX DILI. Unfortunately, we did not have the data on BMI, and could not further investigate the effect of obesity and the emerge of DILI and also could not correct for it in our association analysis. In addition to the BMI, other potential factors for MTX DILI are the lack of folate supplementation, weekly dose of MTX, exposure duration of MTX (MTX cumulative dose), the disease duration, gender, and age.[48,49] In our study, all patients used folic acid, and other risk factors like gender, age, and MTX dose, and disease duration were matched with the case-control design or were corrected in our data analysis.

As reported previously, MTX acts on multiple pathways, including the adenosine, *de novo* purine synthesis, folate, methionine, and *de novo* pyrimidine synthesis pathways.[50] In this study, we tested if specific SNPs with a large effect size were associated with MTX DILI. However, it could be possible that multiple SNPs with small effect sizes are involved in the development of DILI. Another limitation is that no replication cohort was used to validate our multiple findings. Furthermore, our study was performed on patients of European ancestry (mostly Caucasian). Those findings could have a disparity on association analysis to other races, like African or Asian races.

In conclusion, we identified no clear genetic variants related to MTX DILI in RA patients of European ancestry.

Acknowledgement

The authors would like to thank Yavuz Ariyureka for his technical advice concerning performing the GWAS. In addition, we would show our gratitude to Jip Linthorst for the preliminary research for causes of DILI and to Renée Baak-Pablo, Daniëlle Klootwijk and Rowena Schaap for the preparation of the GWAS.

REFERENCES

1. Bluett J, Sergeant JC, MacGregor AJ *et al*. Risk factors for oral methotrexate failure in patients with inflammatory polyarthritis: Results from a UK prospective cohort study. Arthritis Res. Ther. 20, 1–6 (2018).
2. Ridker PM, Everett BM, Pradhan A *et al*. Low-Dose Methotrexate for the Prevention of Atherosclerotic Events. N. Engl. J. Med. 380, 752–762 (2019).
3. Kamen BA, Nylen PA, Camitta BM, Bertino JR. Methotrexate Accumulation and Folate Depletion in Cells as a Possible Mechanism of Chronic Toxicity to the Drug. Br. J. Haematol. 49, 355–360 (1981).
4. O'Dell JR. Therapeutic strategies for rheumatoid arthritis. N. Engl. J. Med. 350, 2591–2602 (2004).
5. Navarro VJ, Senior JR. Drug-Related Hepatotoxicity. N. Engl. J. Med. 354, 731–739 (2006).
6. Aithal GP. Hepatotoxicity related to methotrexate. Drug-Induced Liver Dis. 7, 593–604 (2013).
7. García Rodríguez LA, Williams R, Derby LE, Dean AD, Jick H. Acute liver injury associated with nonsteroidal anti-inflammatory drugs and the role of risk factors. Arch. Intern. Med. 154, 311–316 (1994).
8. Dávila-Fajardo CL, Swen JJ, Cabeza Barrera J, Guchelaar H-J. Genetic risk factors for drug-induced liver injury in rheumatoid arthritis patients using low-dose methotrexate. Pharmacogenomics 14, 63–73 (2013).
9. Sotoudehmanesh R, Anvari B, Akhlaghi M, Shahraeeni S, Kolahdoozan S. Methotrexate hepatotoxicity in patients with rheumatoid arthritis. Middle East J. Dig. Dis. 2, 104–9 (2010).
10. Amital H, Arnson Y, Chodick G, Shalev V. Hepatotoxicity rates do not differ in patients with rheumatoid arthritis and psoriasis treated with methotrexate. Rheumatology 48, 1107–1110 (2009).
11. Singh JA, Saag KG, Bridges Jr SL *et al*. 2015 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis. Arthritis Care Res. (Hoboken). 68, 1–25 (2016).
12. Owen SA, Lunt M, Bowes J *et al*. MTHFR gene polymorphisms and outcome of methotrexate treatment in patients with rheumatoid arthritis: analysis of key polymorphisms and meta-analysis of C677T and A1298C polymorphisms. Pharmacogenomics J. 13, 137–147 (2013).
13. Pestana A, Halprin KM, Taylor JR *et al*. Predictive value of HLA antigen for methotrexate-induced liver damage in patients with psoriasis. J. Am. Acad. Dermatol. 12, 26–29 (1985).
14. Li Y-J, Phillips EJ, Dellinger A *et al*. HLA-B*14:01 and HLA-B*35:01 are associated with trimethoprim-sulfamethoxazole induced liver injury. Hepatology 73, 268–281 (2021).
15. Teixeira M, Macedo S, Batista T *et al*. Flucloxacillin-induced hepatotoxicity - Association with HLA-B*5701. Rev. Assoc. Med. Bras. 66, 12–17 (2020).
16. Hope HF, Hyrich KL, Anderson J *et al*. The predictors of and reasons for non-adherence in an observational cohort of patients with rheumatoid arthritis commencing methotrexate. Rheumatol. (United Kingdom) 59, 213–223 (2020).
17. Goekoop-Ruiterman YPM, de Vries-Bouwstra JK, Allaart CF *et al*. Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the best study): A randomized, controlled trial. Arthritis Rheum. 52, 3381–3390 (2005).

18. de Jong PH, Hazes JM, Han HK *et al*. Randomised comparison of initial triple DMARD therapy with methotrexate monotherapy in combination with low-dose glucocorticoid bridging therapy; 1-year data of the tREACH trial. Ann. Rheum. Dis. 73, 1331–1339 (2014).

19. Steunebrink LMM, Versteeg LGA, Vonkeman HE *et al*. Radiographic progression in early rheumatoid arthritis patients following initial combination versus step-up treat-to-target therapy in daily clinical practice: Results from the DREAM registry. BMC Rheumatol. 2, 1 (2018).

20. Purcell S, Neale B, Todd-Brown K *et al*. PLINK: A tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81, 559–575 (2007).

21. Marchini J, Howie B, Myers S, McVean G, Donnelly P. A new multipoint method for genome-wide association studies by imputation of genotypes. Nat. Genet. 39, 906–913 (2007).

22. Pe'er I, Yelensky R, Altshuler D, Daly MJ. Estimation of the multiple testing burden for genomewide association studies of nearly all common variants. Genet. Epidemiol. 32, 381–385 (2008).

23. Devlin B, Roeder K. Genomic control for association studies. Biometrics 55, 997–1004 (1999).

24. Thul PJ, Akesson L, Wiking M *et al*. A subcellular map of the human proteome. Science 356, eaal3321 (2017) doi:10.1126/science.aal3321.

25. Lu HF, Hung K-S, Hsu Y-W *et al*. Association study between the FTCDNL1 (FONG) and susceptibility to osteoporosis. PLoS One 10, e0140549 (2015).

26. Astolfi A, Fiore M, Melchionda F *et al*. BCOR involvement in cancer. Epigenomics 11, 835–855 (2019).

27. Beenken A, Mohammadi M. The FGF family: Biology, pathophysiology and therapy. Nature Reviews Drug Discovery 8, 235–253 (2009).

28. Coebergh JA, Fransen van de Putte DE, Snoeck IN *et al*. A new variable phenotype in spinocerebellar ataxia 27 (SCA 27) caused by a deletion in the FGF14 gene. Eur. J. Paediatr. Neurol. 18, 413–415 (2014).

29. Van Swieten JC, Brusse E, de Graaf BM *et al*. A mutation in the fibroblast growth factor 14 gene is associated with autosomal dominant cerebral ataxia. Am. J. Hum. Genet. 72, 191–199 (2003).

30. Nicoletti P, Cartsos VM, Palaska PK *et al*. Genomewide Pharmacogenetics of Bisphosphonate-Induced Osteonecrosis of the Jaw: The Role of RBMS3. Oncologist 17, 279–287 (2012) doi:10.1634/theoncologist.2011-0202.

31. Tyler AL, Mahoney JM, Carter GW. Genetic interactions affect lung function in patients with systemic sclerosis. G3 (Bethesda) 10, 151–163 (2020).

32. Li Y, Chen L, Nie C *et al*. Downregulation of RBMS3 is associated with poor prognosis in esophageal squamous cell carcinoma. Cancer Res. 71, 6106–6115 (2011).

33. Collins C, Volik S, Kowbel D *et al*. Comprehensive genome sequence analysis of a breast cancer amplicon. Genome Res. 11, 1034–1042 (2001).

34. Miyoshi N, Ishii H, Mimori K *et al*. Abnormal expression of PFDN4 in colorectal cancer: A novel marker for prognosis. Ann. Surg. Oncol. 17, 3030–3036 (2010).

35. Sadeghi H, Nazemalhosseini-Mojarad E, Sahebi U *et al*. Novel long noncoding RNAs upregulation may have synergistic effects on the CYP24A1 and PFDN4 biomarker role in human colorectal cancer. J. Cell. Physiol. 236, 2051–2057 (2021).

36. Wang D, Zhu Z-Z, Jiang H *et al*. Multiple genes identified as targets for 20q13.12–13.33 gain contributing to unfavorable clinical outcomes in patients with hepatocellular carcinoma. Hepatol. Int. 9, 438–446 (2015).

37. Tabassum R, Mahajan A, Chauhan G *et al*. Evaluation of DOK5 as a susceptibility gene for type 2 diabetes and obesity in North Indian population. BMC Med. Genet. 11, 35 (2010).

38. Yasuoka H, Yamaguchi Y, Feghali-Bostwick CA. The membrane-associated adaptor protein DOK5 is upregulated in systemic sclerosis and associated with IGFBP-5-induced fibrosis. PLoS One 9, e87754 (2014).

39. Zhang L, Li R, Hu K *et al*. Prognostic role of DOK family adapters in acute myeloid leukemia. Cancer Gene Ther. 26, 305–312 (2019).

40. Liew SC, Gupta ED. Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism: Epidemiology, metabolism and the associated diseases. Eur. J. Med. Genet. 58, 1–10 (2015).

41. Kim TY, Kim JY, Sohn JH *et al*. Assessment of Substantial Liver Fibrosis by Real-time Shear Wave Elastography in Methotrexate-Treated Patients With Rheumatoid Arthritis. J. Ultrasound Med. 34, 1621–1630 (2015).

42. Osuga T, Ikura Y, Kadota C *et al*. Significance of liver biopsy for the evaluation of methotrexate-induced liver damage in patients with rheumatoid arthritis. 8, 1961–1966 (2015).

43. Hoffmeister RT. Methotrexate therapy in rheumatoid arthritis: 15 years experience. Am. J. Med. 75, 69–73 (1983).

44. Oh RC, Hustead TR, Ali SM, Pantsari MW. Mildly Elevated Liver Transaminase Levels: Causes and Evaluation. Am. Fam. Physician 96, 709–715 (2017).

45. Kent PD, Luthra HS, Michet C. Risk Factors for Methotrexate-Induced Abnormal Laboratory Monitoring Results in Patients with Rheumatoid Arthritis. 31, 1727–1731 (2004).

46. Shetty A, Cho WK, Alazawi W, Syn WK. Methotrexate Hepatotoxicity and the Impact of Nonalcoholic Fatty Liver Disease. Am. J. Med. Sci. 354, 172–181 (2017).

47. Luster MI, Simeonova PP, Gallucci RM *et al*. Role of inflammation in chemical-induced hepatotoxicity. Toxicol. Lett. 120, 317–321 (2001).

48. Hoekstra M, van Ede AE, Haagsma CJ *et al*. Factors associated with toxicity, final dose, and efficacy of methotrexate in patients with rheumatoid arthritis. Ann. Rheum. Dis. 62, 423–426 (2003).

49. Quintin E, Scoazec J-Y, Marotte H, Miossec P. Rare incidence of methotrexate-specific lesions in liver biopsy of patients with arthritis and elevated liver enzymes. Arthritis Res. Ther. 12, R143 (2010).

50. Eektimmerman F, Swen JJ, Madhar MB, Allaart CF, Guchelaar HJ. Predictive genetic biomarkers for the efficacy of methotrexate in rheumatoid arthritis: a systematic review. Pharmacogenomics J. 20, 159–168 (2020).

Pathway analysis to identify genetic variants associated with efficacy of adalimumab in rheumatoid arthritis

Frank Eektimmerman
Jesse J. Swen
Stefan Böhringer
Tom W.J. Huizinga
Wouter M. Kooloos
Cornelia F. Allaart
Henk-Jan Guchelaar

Pharmacogenomics 18(10), 945–953 (2017)

Abstract

Aim: About 30% of rheumatoid arthritis patients have no clinical benefit from TNF inhibitors. Genome-wide association (GWA) and candidate gene studies tested several putative genetic variants for TNF inhibitor efficacy with inconclusive results. Therefore, this study applied a systematic pathway analysis.

Patients & methods: A total of 325 rheumatoid arthritis patients treated with adalimumab were genotyped for 223 SNPs. We tested the association between SNPs and European League Against Rheumatism response and remission at 14 weeks under the additive genetic model using logistic regression.

Results: A total of three SNPs located in *CD40LG* (rs1126535), *TANK* (rs1267067) and *VEGFA* (rs25648) showed association with both end points. *TNFAIP3* (rs2230926) had the strongest effect related to European League Against Rheumatism response.

Conclusion: This exploratory study suggests that *TNFAIP3*, *CD40LG*, *TANK* and *VEGFA* play a role in the response to adalimumab treatment.

INTRODUCTION

TNF inhibitors are effective as second-line drug in rheumatoid arthritis (RA) patients after failure of (mono) therapy with disease modifying antirheumatic drugs.[1–3] However, about 30% of patients with (severe) RA have no clinical benefit from treatment with TNF inhibitors while they are at risk for side effects such as (serious) infections and malignancies.[4,5] Although several clinical and laboratory markers, such as C-reactive protein level, rheumatoid factor positive and anticyclic citrullinated peptide antibodies, are recognized as predictors for the response to TNF inhibitors, multiple studies showed that these biomarkers alone are weak predictors.[6] Genetics may play an additional role in the efficacy of TNF inhibitors and therefore studies have been performed to identify predictive genetic biomarkers for the response to TNF inhibitors for RA.[7–24] Despite significant efforts, clinical application of these candidate genetic biomarkers is not reached due to underpowered cohorts, conflicting results, lack of replication studies or questionable causality.

As seen in other diseases, it seems plausible that pharmacogenetic response in RA depends on the interaction of genes involved in antirheumatic drug mode of action and genes associated with RA pathogenesis.[25,26] Therefore, we selected genes related to seven different pathways where TNF plays a pivotal role. In addition, we included previously associated genes to explore the presence of putative biomarkers for the efficacy of adalimumab in RA. Characteristically, with this study, the use of a pathway approach combines the advantages of both candidate gene methods and GWA studies.[15]

The goal of this exploratory study was to determine genetic variants that are responsible for the efficacy of adalimumab.

MATERIALS & METHODS

Cohort ascertainment

Previously, regulation for reimbursement of treatment with a TNF inhibitor in The Netherlands required prescribers to provide documentation that the following criteria were met: a diagnosis of RA,[27] active disease with a disease activity score (DAS) 28 of 3.2 or higher[28] and previous insufficient response on at least two synthetic disease-modifying antirheumatic drugs, one of which was methotrexate (MTX). Accordingly, for patients prescribed adalimumab (ADA), data were collected by the central ADA distributor in The Netherlands (ApotheekZorg, Sittard, The Netherlands). No database was available, but instead, we approached every patient who met the inclusion criteria and requested a saliva sample for genotyping. Available patient data was retrieved from ApotheekZorg.

Between 2005 and 2007, 325 patients were selected who started with ADA; were 18 years or older; had an erythrocyte sedimentation rate of at least 28 mm/h; patient's assessment of disease activity (pain rating on a 100-mm visual analog scale) and were followed prospectively using ADA of minimal 14 weeks. The ethics committee of the Leiden University Medical Center approved the study protocol. All patients provided written informed consent.

Single nucleotide polymorphism selection

We investigated different genes related to the mechanism of action of TNF inhibitors; the inflammatory process of RA; and single nucleotide polymorphisms (SNPs) previously associated with genetic susceptibility to RA or anti-TNF treatment outcome. Briefly, a systematic approach was used to select candidate SNPs related to the mechanism of action of adalimumab, as described in detail by Kooloos *et al*.[29] From the pathway approach, 186 candidate SNPs in 111 genes divided into seven different pathways were selected. Additionally, 37 other significantly associated SNPs previously reported were selected.[30–35] Finally, 223 SNPs (minor allele frequency >0.05 and NCBI reported validation states >2) in 124 genes were selected (Table 6-1).

The pathway approach considers the variability in the entire pathway without restricting the analysis to a single candidate gene.[29] In theory, this would affect the stringency of multiple testing correction. Therefore, we calculated a global p-value, which corrects for each gene region included in the study.

DNA collection & genotyping

DNA was collected from 2 ml saliva using the Oragene™ DNA self-collection kit (DNA Genotek, Inc., Ontario, Canada). DNA samples were extracted according to instructions provided by the manufacturer. Genotyping was performed using a custom designed array with Veracode GoldenGate GT assays on the Illumina BeadXpress platform (Illumina, Inc., CA, USA).

Quality control

Prior to association analysis, quality control procedures were performed. First, low-quality DNA (defined as DNA yield lower than 10 µg and/or nucleic acid purity [260/280 nm ratio] lower than 1.6) was excluded from further analysis. Second, SNP genotyping plots for each assay were visually checked for the degree of clustering. SNPs that showed an unexpected number of clusters or poorly defined or separated clusters were removed. Additionally, monoallelic SNPs with a call rate of less than 0.98 and SNPs with a call rate of less than 90%

Table 6-1. Selected genes related to the mechanism of action of adalimumab

Mechanism of action	Genes	n	Ref.
Neutralization and blockage interaction	*ADAM17, IL1A, IL1B, IL1R1, IL1R2, IL1RAP, IL1RN, LTA, TNF, TNFRSF1A, TNFRSF1B*	11	[36,37]
Interaction with Fc receptor	*FCGR2A, FCGR2B, FCGR3A, FCGR3B*	4	[38]
Initiation of reverse signaling, leading to blockage or increased apoptosis or growth arrest	*BAK1, BAX, BCL2L1, BIRC2, BIRC3, CASP3, CASP7, CASP8, CFLAR, CHUK, FADD, IKBKB, IKBKG, MAP3K7, MAP3K7IP1, MAP3K7IP2, MAPK14, MAPK8, NFKB1, NFKB2, NFKB3, RIPK1, TANK, TNFAIP3, TP53, TRADD, TRAF2, XIAP*	28	[39,40]
Reduction of inflammatory cytokine production and angiogenic factor expression	*APOA1, CD11, CD28, CD40, CD40L, CD69, CSF1, CSF1R, CSF2, CSF2RA, CSF2RB, CSF3, CSF3R, FIGF, FLT1, FLT4, ICAM1, IFNA1, IFNB, IFNG, IFNGR1, IFNGR2, IL10, IL10RA, IL10RB, IL11, IL11RA, IL12A, IL12B, IL12RB1, IL12RB2, IL13, IL13RA1, IL13RA2, IL15, IL15RA, IL18, IL18R1, IL2, IL2RA, IL3, IL3R, IL4, IL4R, IL6, IL6R, IL7, IL7R, IL8, IL8RA, IL8RB, IL9, IL9R, KDR, LIF, LIFR, OSM, OSMR, PECAM1, SELE, TGFB1, VCAM1, VEGFA, VEGFB, VEGFC, VWF*	66	[36,41–43]
Restoration of immune regulation	*FOXP3*	1	[44]
Mediation of complement-dependent cytotoxicity and antibody-dependent cytotoxicity	*C2, C3, C4A, C4B, C5, C5AR1, C1QA, C1QB, C1QC, CR1*	10	[37,40]
Downregulation or discontinuation of bone and cartilage destruction	*TNFRSF11A, TNFSF11, TNFRSF11B, TRAF6*	4	[45,46]
Previously reported genes associated with efficacy of TNF inhibitors or susceptibility of RA	*ANAPC4, FCN1, FCRL3, HLA-DRB1, HMGN2, IRF5, ITGAV, LOC100133618, MMEL1, PADI4, PTPN22, RSBN1, RUNX1, SLC22A4, STAT4, TNF, TNFAIP3, TNFRSF1B, TRAF1*	37	[30–35]

Abbreviations: RA: Rheumatoid arthritis.

were removed. The reliability of the SNP detection based on the shape of the clusters and their relative distance to each other (distribution) was calculated with the GenTrain score. Patient samples with a GenTrain score of less than 0.6 were removed. Finally, genotype frequencies were tested for deviations from Hardy–Weinberg equilibrium. Under Hardy–Weinberg assumptions, deviant SNPs ($p<0.001$) were removed from further analysis.

Definition of effect

End points were the achievement of good response and remission at 14 weeks according to European League Against Rheumatism (EULAR) criteria.[47] The EULAR good response was defined as an improvement to the baseline of DAS28>1.2 and DAS28≤3.2 at 14 weeks. EULAR remission was defined as a DAS28≤2.6 at 14 weeks.

Statistical analysis

Statistical analyses were performed using SPSS 23.0 (SPSS, Inc., IL, USA) and Plink (version 1.07).[48] Associations between SNPs and EULAR good response or EULAR remission at 14 weeks were tested with multiple logistic regressions. Before multivariate analysis, gender, concomitant use of MTX, age and baseline DAS28 were univariately tested for association between the end points EULAR good response and EULAR remission at 14 weeks. Variables with a p-value of <0.05 were selected for multivariate analysis.

A global p-value was calculated using the p-min, tail strength and Sequence Kernel Association Test (SKAT) statistics.[49] Global p-values summarize the statistical significance of the values of all SNPs within each gene region resulting in a single p-value that is corrected for multiple testing. To account for nonindependence of SNPs, p-values from empirical distributions using permutations were computed for the tail strength and p-min statistics. SNPs were permuted as a block, keeping intact the relationship between covariates and outcome. For p-min and tail strength, individual tests were based on a logistic model with an additive genetic model and 1 × 104 permutations were used to obtain empirical p-values. The SKAT statistics does not assume independence of SNPs and was computed using bioconductor package globaltest without permutations.[50] Covariates were standardized for the SKAT statistic. Computations were parallelized using package parallelize.dynamic.[51]

Global p-values were computed using R version 3.2. Characteristics of the global tests can be summarized as follows: p-min performs well when there is a single SNP (or a single set of highly correlated SNPs) among all SNPs that are associated with the outcome, tail strength performs well when many SNPs have a small effect and SKAT performs well when effect sizes follow a normal distribution, in other words, a situation between p-min and tail strength.

RESULTS

In this exploratory study, 34 patients and 28 SNPs were removed for further analysis during quality control procedure, resulting in a total of 291 patients and 195 different SNPs for the analysis (Figure 6-1). In our cohort, RA patients with a mean age of 58.5 years and with

a mean DAS28 at baseline of 5.8 were studied (Table 6-2). The majority of the patients (82.1%) received concomitant MTX with an average dose of 22.2 mg/week. Nongenetic factors associated with adalimumab response were age, baseline DAS28, gender and the use of concomitant MTX. Baseline DAS28 and age showed a significant association with EULAR good response ($p=2.56\times10^{-9}$ and 2.34×10^{-2}, respectively) and EULAR remission ($p=3.24\times10^{-12}$ and 9.48×10^{-4}, respectively). Gender and concomitant use of MTX were not associated with either primary end points ($p>0.05$) and were not included as covariates.

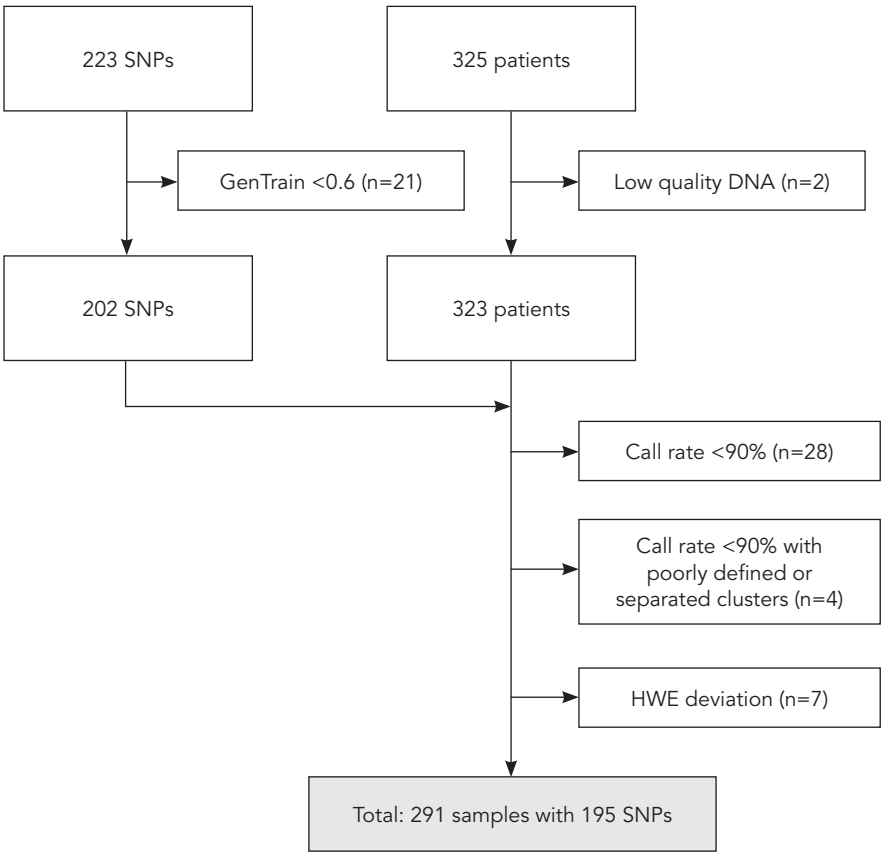


Figure 6-1. Study flow chart of sample and single nucleotide polymorphism selection for the analysis of single nucleotide polymorphisms associated with the efficacy of adalimumab in rheumatoid arthritis.
The calculated GenTrain score represents the reliability of the SNP detection based on the shape of the clusters and their relative distance to each other (distribution). The call rate for each sample is the number of SNPs successfully genotyped divided by the total number of SNPs.

Table 6-2. General characteristics of the study population (n=291)

Characteristic	Frequency
Female, n (%)	206 (70.8)
Age, years	58.5±11.7
Concurrent methotrexate	239 (82.1)
Methotrexate dose, mg/week	22.2±5.7
Previous use of biological agent, n (%)	14 (4.8)
DAS28, baseline	5.8±1.0
DAS28, 14 weeks	3.1±1.1
Relative change in DAS28 (%)	46.2±16.8
Response according to EULAR criteria:	
Good response, n (%)	157 (54.0)
Moderate response, n (%)	126 (43.3)
No response, n (%)	8 (2.7)
Remission according to EULAR criteria, n (%)	93 (32.0)

Abbreviations: DAS28: 28 joint disease activity score, EULAR: European League Against Rheumatism.

EULAR response at 14 weeks was dichotomized in good response (157/291 patients, 54%) or no adequate response (134/291 patients, 46%) with the latter including EULAR moderate and non-responders. We identified 12 genetic variants (odds ratios [OR] between 1.464 and 5.395) that were associated with adalimumab efficacy for EULAR good response at the 0.05 level (Table 6-3).

The global p-value for the outcome EULAR good response and p-min statistics was 0.029. We also computed global p-values for the tail strength and SKAT statistics which were 0.4 and 0.2, respectively. Seven SNPs (Table 6-3) demonstrated a significant association with EULAR good response ($p<0.029$), coding for the genes *TNFAIP3*, *VEGFA*, *CD40LG*, *TANK*, *FCGR2A*, *PADI4* and *VWF*. Five significant SNPs had an OR between 1.5 and 2.0, with p-values between 2.4×10^{-2} and 6.7×10^{-3}. The SNP coding for TNFAIP3 (rs2230926) had the strongest association with a p-value of 3.8×10^{-3} and OR of 5.4.

After 14 weeks, 93/291 (32%) patients achieved EULAR remission. We found eight SNPs, who were associated and showed a trend with EULAR remission at the 0.05 level (Table 6-4). Noteworthy, three SNPs encoding the *VEGFA*, *CD40LG* and *TANK* genes were associated with both end points, EULAR good response and remission. For the outcome EULAR remission the global p-values for p-min, tail strength and SKAT were 0.97, 0.89 and 0.89, respectively, which were not significant at the level of 0.05.

Table 6-3. Association between SNPs and EULAR good response at 14 weeks according to the multivariate additive genetic model (p<0.05, adjusted for age and DAS28 at baseline)

Gene	SNP	Chr:position	n	A1/A2 (+)	p-value	OR	(95% CI)	MAF
TNFAIP3	rs2230926†	6:138196066	291	T	0.003733	5.395	(1.727–16.86)	0.03952
VEGFA	rs25648†	6:43738977	202	T‡	0.003902	2.571	(1.354–4.883)	0.1658
CD40LG	rs1126535†	X:135730555	291	T	0.006627	1.871	(1.190–2.942)	0.1804
TANK	rs1267067†	6:138006504	284	C‡	0.008951	1.719	(1.145–2.581)	0.3204
FCGR2A	rs1801274†	1:161479745	291	T	0.01070	1.575	(1.111–2.231)	0.4605
PADI4	rs2240340†	1:17662639	291	G	0.01744	1.547	(1.080–2.218)	0.4227
VWF	rs216897†	12:6102607	291	C	0.02367	1.516	(1.057–2.175)	0.4450
IL1RAP	rs13321840	3:190275521	291	T	0.03049	1.530	(1.041–2.249)	0.2852
TNFRSF11B	rs1485286	8:119950668	291	C‡	0.03676	1.520	(1.026–2.252)	0.2955
IL10RA	rs2229114	11:117869878	150	C	0.04011	2.571	(1.044–6.334)	0.08667
IL1R1	rs3917243	2:102774988	291	A‡	0.04041	1.464	(1.017–2.107)	0.4381
nearBACE2	rs2837960	21:42511918	291	G‡	0.04716	1.633	(1.006–2.649)	0.1632
TNFAIP3	rs2230926†	6:138196066	291	T	0.003733	5.395	(1.727–16.86)	0.03952
VEGFA	rs25648†	6:43738977	202	T‡	0.003902	2.571	(1.354–4.883)	0.1658
CD40LG	rs1126535†	X:135730555	291	T	0.006627	1.871	(1.190–2.942)	0.1804

† SNPs have a significant association with EULAR good response (p<0.029). ‡ Minor allele.
Abbreviations: Chr: Chromosome; MAF: Minor allele frequency; OR: Odds ratio.

Table 6-4. Association between single nucleotide polymorphisms and European League Against Rheumatism remission (DAS28≤2.6) at 14 weeks according to the multivariate additive genetic model (p<0.05, adjusted for age and DAS28 at baseline)

Gene	SNP	Chr:position	n	A1/A2 (+)	p-value	OR	(95% CI)	MAF
TANK	rs1267067	6:138006504	284	C†	0.01748	1.708	1.098–2.656	0.3204
VEGFA	rs25648	6:43738977	202	T†	0.02275	2.164	1.114–4.204	0.1658
CD69	rs10844706	12:9910132	291	A†	0.02589	1.600	1.058–2.419	0.3677
LOC105378654	rs17534243	1:38650917	291	G†	0.02595	1.675	1.064–2.638	0.2423
TNF	rs1800629	6:31543031	282	G	0.03651	1.798	1.037–3.116	0.1844
CD40LG	rs1126535	X:135730555	291	C	0.03813	1.754	1.031–2.983	0.1804
TNFRSF11B	rs2073617	8:119964283	239	T†	0.04734	1.558	1.005–2.415	0.4937
CSF1R	rs11749913	5:149483427	291	C†	0.04759	1.501	1.004–2.243	0.3247
TANK	rs1267067	6:138006504	284	C†	0.01748	1.708	1.098–2.656	0.3204
VEGFA	rs25648	6:43738977	202	T†	0.02275	2.164	1.114–4.204	0.1658

† Minor allele.
Abbreviations: Chr: Chromosome; MAF: Minor allele frequency; OR: Odds ratio.

DISCUSSION

In an effort to elucidate pharmacogenetic biomarkers in TNF pathways and RA susceptibility genes related to the efficacy of adalimumab, we performed a cohort study in 291 RA patients treated with adalimumab. We found an association of three genetic loci coding for *VEGFA*, *CD40LG* and *TANK* with efficacy following adalimumab treatment in both EULAR response and remission. Overall, seven SNPs (ORs between 1.516 and 5.395) coding for *TNFAIP3*, *VEGFA*, *CD40LG*, *TANK*, *FCGR2A*, *PADI4* and *VWF* were significantly associated with EULAR response, of which the SNP coding for *TNFAIP3*, rs2230926, had the strongest effect and is, therefore, the most promising gene.

In the literature, data from several TNF inhibitor pharmacogenetic studies using the candidate gene approach are known. Usually, these studies focus on a single or a few loci or genes while for complex diseases such as RA, it seems more likely that drug response is the result of multiple genes. GWA study, in contrary, investigates multiple genes, but has the disadvantage that most associations identify genes of unknown causality, require a substantial sample size and require stringent correction for multiple testing to limit the risk of false-positive results. Therefore, in the current study, we used a pathway selection method using RA susceptibility genes related to TNF pathway.

A significant strength of our study is that only one TNF inhibitor, adalimumab, was investigated. Routine clinical practice shows that the response to TNF blockers is generally not a class phenomenon. Indeed, patients who do not respond to one type of TNF blocker may respond to another. Therefore, each TNF inhibitor could be associated with different genetic components responsible for the efficacy in RA, and combining multiple TNF inhibitors could lead to incorrect or missed associations.

We are aware that our research may have limitations. First, patients started adalimumab several years ago and this may have led to an increased number of patients with advanced RA since currently TNF inhibitors are prescribed at an earlier stage of the disease. This was also evident in the baseline DAS of our cohort, where most patients were categorized as severe RA (mean DAS>5.1).[28] Second, only eight patients (2.7%) were non-responders according to the EULAR criteria. This number was too low to allow comparison of non versus moderate versus good responders. Therefore, we combined non-responders and moderate responders. Unfortunately, this decreases the contrast between responder groups. Third, nongenetic factors such as serology (e.g., anticyclic citrullinated peptide antibodies or rheumatoid factor) and disease duration could influence the efficacy of TNF inhibitors. Unfortunately, our study was not designed as a prospective study and these factors were not retrievable. Fourth, the disadvantage of the pathway selection method is that no new (unknown) genes can be found.

In our study, *TNFAIP3* rs2230926 had the largest effect size with EULAR response, with an OR of 5.4. The wild-type allele (T) of this SNP is related to a better response of adalimumab as compared with the G-allele. TNFAIP3 is critical for limiting inflammation by terminating the TNF-induced NF-κB transcription factors as well as TNF-mediated apoptosis. Consequently, defects in the TNFAIP3 expression could lead to chronic inflammation and tissue damage.[52] This implies that the T-allele leads to less inflammation and apoptosis, and hence less tissue damage occurs. Multiple SNPs in the TNFAIP3 gene have been associated, including rs5029937 and rs6920220, with an increased risk of developing RA by 20 to 40%, respectively previously. The study of Zhu *et al*. found rs2230926 heterozygote related to poor RA outcome, which is in line with our findings.[53,54]

The minor allele of rs25648, coding for *VEGF*, is associated with a better outcome in both EULAR good response as EULAR remission compared with the wild-type allele. VEGF is an important protein involved in angiogenesis and is an important aspect of new tissue development, growth and tissue repair.[55] Since it is demonstrated that expression of VEGF is reduced in RA patients treated with TNF inhibitors, it is thought that TNF inhibitors may interfere with the angiogenesis in the inflammatory process of RA.[36] Our findings would seem to suggest that the minor allele (T) leads to increased VEGF expression and subsequently leads to a better prognosis of RA.

CD40LG is a member of the TNF superfamily, which is primarily expressed on the surface of activated T cells and stimulates B-cell proliferation and secretion of pro-inflammatory cytokines and chemokines after CD40-CD40LG ligation.[56] CD40LG is an extracellular target for TNF inhibitors. This was demonstrated in a study of Danese *et al*.,[57] where patients using infliximab had reduced levels of CD40LG. Anti-CD40L stimulated human $CD4^+$ T cells produced less IL-2, which results in less T-cell maturation and directly prevents the onset of autoimmune diseases, like RA.[58] Correspondingly, adalimumab may limit the inflammatory process involved in RA by inhibiting CD40LG. Likewise, the observed interindividual differences in response in our study may be the result of the SNP rs1126535 in the gene encoding of the CD40LG protein. Concerning our results, we hypothesize that patients carrying the *CD40LG* (rs1126535) C-allele may have less binding capacity to TNF inhibitors compared with patients genotyped carrying a *CD40LG* (rs1126535) T-allele.

TANK, a protein coding for TRAF family member associated NF-κB activator, is part of the TRAF family which mediates signals from cytokine signals through cell surface receptors and, thereby, activating downstream intracellular signaling cascades. TANK binds to TRAF1, TRAF2 and TRAF3. It is observed that the mechanism of action of TNF inhibitors includes the initiation of reverse intracellular signaling cascade by binding of antibodies to trans-membrane TNF. This may lead to a decreased production of pro-inflammatory cytokines

(like TNF-α), increased production of anti-inflammatory cytokines (like IL10) and induction of apoptosis in cells [36–37,59]. In our results, patients with a homozygous mutant genotype (rs1267067-CC) were more likely to achieve clinical response than patients carrying a wild-type allele (rs1267067-TT or rs1267067- CT). Hypothetically, this polymorphism may support the reverse signaling by less binding of TRAF1-TRAF3 to TANK.

In conclusion, with the use of a systematic pathway method, we identified four different SNPs in *CD40LG*, *TNFAIP3*, *TANK* and *VEGFA* as putative candidates associated with the efficacy of adalimumab. Of these *TNFAIP3* is the most promising candidate.

Acknowledgement

The authors are grateful to Tahar van der Straaten and Yavuz Ariyurek for helping with Illumina BeadStudio Data Analysis Software Modules (Illumina, Inc., CA, USA). Also, we want to thank all participants who agreed to participate in this study and the staff and pharmacists of ApotheekZorg (Sittard, The Netherlands) who helped in the acquisition of clinical data.

REFERENCES

1. Keystone EC, Kavanaugh AF, Sharp JT *et al*. Radiographic, clinical, and functional outcomes of treatment with adalimumab (a human anti-tumor necrosis factor monoclonal antibody) in patients with active rheumatoid arthritis receiving concomitant methotrexate therapy: a randomized, placebo-controlled, 52-week trial. Arthritis Rheum. 50(5), 1400–1411 (2004).
2. Maini RN, Breedveld FC, Kalden JR *et al*. Sustained improvement over two years in physical function, structural damage, and signs and symptoms among patients with rheumatoid arthritis treated with and methotrexate. Arthritis Rheum. 50(4), 1051–1065 (2004).
3. van Der Heijde D, Klareskog L, Rodriguez-Valverde V *et al*. Comparison of etanercept and methotrexate, alone and combined, in the treatment of rheumatoid arthritis: two-year clinical and radiographic results from the TEMPO study, a double-blind, randomized trial. Arthritis Rheum. 54(4), 1063–1074 (2006).
4. Poddubnyy D, Rudwaleit M. Efficacy and safety of adalimumab treatment in patients with rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis. Expert Opin. Drug Saf. 10(4), 655–673 (2011).
5. Furst DE, Kavanaugh A, Florentinus S, Kupper H, Karunaratne M, Birbara CA. Final 10-year effectiveness and safety results from study DE020: adalimumab treatment in patients with rheumatoid arthritis and an inadequate response to standard therapy. Rheumatology (Oxford) 54(12), 2188–2197 (2015).
6. Marotte H, Miossec P. Biomarkers for prediction of TNFalpha blockers response in rheumatoid arthritis. Joint Bone Spine 77(4), 297–305 (2010).
7. Krintel SB, Palermo G, Johansen JS *et al*. Investigation of single nucleotide polymorphisms and biological pathways associated with response to TNFα inhibitors in patients with rheumatoid arthritis. Pharmacogenet. Genomics 22(8), 577–589 (2012).
8. Plant D, Bowes J, Potter C *et al*. Genome-wide association study of genetic predictors of anti-tumor necrosis factor treatment efficacy in rheumatoid arthritis identifies associations with polymorphisms at seven loci. Arthritis Rheum. 63(3), 645–653 (2011).
9. Cui J, Saevarsdottir S, Thomson B *et al*. Rheumatoid arthritis risk allele PTPRC is also associated with response to anti-tumor necrosis factor alpha therapy. Arthritis Rheum. 62(7), 1849–1861 (2010).
10. Cui J, Stahl EA, Saevarsdottir S *et al*. Genome-wide association study and gene expression analysis identifies CD84 as a predictor of response to etanercept therapy in rheumatoid arthritis. PLoS Genet. 9(3), e1003394 (2013).
11. Swierkot J, Bogunia-Kubik K, Nowak B *et al*. Analysis of associations between polymorphisms within genes coding for tumour necrosis factor (TNF)-alpha and TNF receptors and responsiveness to TNF-alpha blockers in patients with rheumatoid arthritis. Joint Bone Spine 82(2), 94–99 (2015).
12. Avila-Pedretti G, Tornero J, Fernández-Nebro A *et al*. Variation at FCGR2A and functionally related genes is associated with the response to anti-TNF therapy in rheumatoid arthritis. PLoS ONE 10(4), e0122088 (2015).
13. Tan RJL, Gibbons LJ, Potter C *et al*. Investigation of rheumatoid arthritis susceptibility genes identifies association of AFF3 and CD226 variants with response to antitumour necrosis factor treatment. Ann. Rheum. Dis. 69(6), 1029–1035 (2010).

14. Sieberts SK, Zhu F, García-García J *et al*. Crowdsourced assessment of common genetic contribution to predicting anti-TNF treatment response in rheumatoid arthritis. Nat. Commun. 7, 12460 (2016).

15. Umicevic Mirkov M, Cui J, Vermeulen SH *et al*. Genomewide association analysis of anti-TNF drug response in patients with rheumatoid arthritis. Ann. Rheum. Dis. 72(8), 1375–1381 (2013).

16. Liu C, Batliwalla F, Li W *et al*. Genome-wide association scan identifies candidate polymorphisms associated with differential response to anti-TNF treatment in rheumatoid arthritis. Mol. Med. 14(9–10), 575–581 (2008).

17. Batalla A, Coto E, Gómez J *et al*. IL17RA gene variants and anti-TNF response among psoriasis patients. Pharmacogenomics J. 35(8), 2101– 2107 (2016)

18. Mewar D, Wilson AG. Treatment of rheumatoid arthritis with tumour necrosis factor inhibitors. Br. J. Pharmacol. 162(4), 785–791 (2011).

19. Lee YH, Bae SC. Associations between PTPRC rs10919563 A/G and FCGR2A R131H polymorphisms and responsiveness to TNF blockers in rheumatoid arthritis: a meta-analysis. Rheumatol. Int. 36(6), 837–844 (2016).

20. Dávila-Fajardo CL, van der Straaten T, Baak-Pablo R *et al*. FcGR genetic polymorphisms and the response to adalimumab in patients with rheumatoid arthritis. Pharmacogenomics 16(4), 373–381 (2015).

21. Ranganathan P. Pharmacogenomics of tumor necrosis factor antagonists in rheumatoid arthritis. Pharmacogenomics 6(5), 481–490 (2005).

22. Nishimoto T, Seta N, Anan R *et al*. A single nucleotide polymorphism of TRAF1 predicts the clinical response to anti-TNF treatment in Japanese patients with rheumatoid arthritis. Clin. Exp. Rheumatol. 32(2), 211–217 (2014).

23. Ceccarelli F, D'Alfonso S, Perricone C *et al*. The role of eight polymorphisms in three candidate genes in determining the susceptibility, phenotype, and response to anti-TNF therapy in patients with rheumatoid arthritis. Clin. Exp. Rheumatol. 30(6), 939–942 (2012).

24. Conigliaro P, Ciccacci C, Politi C *et al*. Polymorphisms in STAT4, PTPN2, PSORS1C1 and TRAF3IP2 genes are associated with the response to TNF inhibitors in patients with rheumatoid arthritis. PLoS ONE 12(1), e0169956 (2017).

25. Dornbrook-Lavender KA, Pieper JA. Genetic polymorphisms in emerging cardiovascular risk factors and response to statin therapy. Cardiovasc. Drugs Ther. 17(919), 75–82 (2003).

26. Mascheretti S, Schreiber S. Genetic testing in Crohn disease: utility in individualizing patient management. Am. J. Pharmacogenomics 5(4), 213–222 (2005).

27. Matsumoto Y, Uemura M, Hibino N, Yamamoto M. [Clinical usefulness of the 1987 revised criteria for rheumatoid arthritis by American Rheumatism Association]. Nihon Naika Gakkai Zasshi 77(5), 742–743 (1988).

28. Aletaha D, Landewe R, Karonitsch T *et al*. Reporting disease activity in clinical trials of patients with rheumatoid arthritis: EULAR/ACR collaborative recommendations. Ann. Rheum. Dis. 67(10), 1360–1364 (2008).

29. Kooloos WM, Wessels JAM, van der Straaten T, Huizinga TWJ, Guchelaar HJ. Criteria for the selection of single nucleotide polymorphisms in pathway pharmacogenetics: TNF inhibitors as a case study. Drug Discov. Today 14(17–18), 837–844 (2009).

30. Criswell LA, Lum RF, Turner KN *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. 50(9), 2750–2756 (2004).

31. Choy EH, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. N. Engl. J. Med. 344(12), 907–916 (2001).

32. Wood AJJ. Therapeutic strategies for rheumatoid arthritis. N. Engl. J. Med. 350(25), 2591–2602 (2009).

33. Kang CP, Lee KW, Yoo DH, Kang C, Bae SC. The influence of a polymorphism at position -857 of the tumour necrosis factor α gene on clinical response to etanercept therapy in rheumatoid arthritis. Rheumatology 44(4), 547–552 (2005).

34. Olsen NJ, Stein CM. New drugs for rheumatoid arthritis. Drugs 350, 2167–2179 (2004).

35. Tutuncu Z, Kavanaugh A, Zvaifler N, Corr M, Deutsch R, Boyle D. Fcgamma receptor type IIIA polymorphisms influence treatment outcomes in patients with inflammatory arthritis treated with tumor necrosis factor alpha-blocking agents. Arthritis Rheum. 52(9), 2693–2696 (2005).

36. Paleolog EW, Young S, Stark AC, McCloskey RV, Feldma M, Main RN. Modulation of angiogenic vascular endothelial growth factor by tumor necrosis factor alpha and interleukin-1 in rheumatoid arthritis. Arthritis Rheum. 41(7), 1258–1265 (1998).

37. Nesbitt A, Fossati G, Bergin M *et al*. Mechanism of action of certolizumab pegol (CDP870): in vitro comparison with other anti-tumor necrosis factor α agents. Inflamm. Bowel Dis. 13(11), 1323–1332 (2007).

38. Kohno T, Tam LTT, Stevens SR, Louie JS. Binding characteristics of tumor necrosis factor receptor-fc fusion proteins vs anti-tumor necrosis factor mAbs. J. Investig. Dermatology Symp. Proc. 12(1), 5–8 (2007).

39. Mitoma H, Horiuchi T, Tsukamoto H *et al*. Binding activities of infliximab and etanercept to transmembrane tumor necrosis factor-alpha. Gastroenterology 126(3), 934–936 (2004).

40. van den Brande JMH, Braat H, van den Brink GR *et al*. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. Gastroenterology 124(7), 1774–1785 (2003).

41. Charles P, Elliott MJ, Davis D *et al*. Regulation of cytokines, cytokine inhibitors, and acute-phase proteins following anti-TNF-alpha therapy in rheumatoid arthritis. J. Immunol. 163(3), 1521–1528 (1999).

42. Klimiuk PA, Sierakowski S, Domysławska I, Fiedorczyk M, Chwiecko J. Reduction of soluble adhesion molecules (sICAM-1, sVCAM-1, and sE-selectin) and vascular endothelial growth factor levels in serum of rheumatoid arthritis patients following multiple intravenous infusions of infliximab. Arch. Immunol. Ther. Exp. (Warsz) 52(1), 36–42 (2004).

43. Ulfgren AK, Andersson U, Engström M, Klareskog L, Maini RN, Taylor PC. Systemic anti-tumor necrosis factor alpha therapy in rheumatoid arthritis down-regulates synovial tumor necrosis factor alpha synthesis. Arthritis Rheum. 43(11), 2391–2396 (2000).

44. Goldstein I, Ben-horin S, Koltakov A *et al*. alpha1beta1 Integrin+ and regulatory Foxp3+ T cells constitute two functionally distinct human CD4+ T cell subsets oppositely modulated by TNFalpha blockade. J. Immunol. 178(1), 201–210 (2007).

45. Kubota A, Hasegawa K, Suguro T, Koshihara Y. Tumor necrosis factor-alpha promotes the expression of osteoprotegerin in rheumatoid synovial fibroblasts. J. Rheumatol. 31(3), 426–435 (2004).

46. Lee C-K, Lee EY, Chung SM, Mun SH, Yoo B, Moon H-B. Effects of disease-modifying antirheumatic drugs and antiinflammatory cytokines on human osteoclastogenesis through interaction with receptor activator of nuclear factor kappaB, osteoprotegerin, and receptor activator of nuclear factor kappaB ligand. Arthritis Rheum. 50(12), 3831–3843 (2004).

47. Prevoo MLL, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LBA, van Riel PLCM. 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. 38(1), 44–48 (1995).

48. Purcell S, Neale B, Todd-Brown K *et al*. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81(3), 559–575 (2007).

49. Wu MC, Lee S, Cai T, Li Y, Boehnke M, Lin X. Rare-variant association testing for sequencing data with the sequence kernel association test. Am. J. Hum. Genet. 89(1), 82–93 (2011).

50. Goeman JJ, van de Geer SA, van Houwelingen HC. Testing against a high dimensional alternative. J. R. Stat. Soc. Ser. B-Statistical Methodol. 68, 477–493 (2006).

51. Böhringer S. Dynamic parallelization of R functions. R. J. 5(2), 88–96 (2013).

52. Vereecke L, Beyaert R, van Loo G. The ubiquitinediting enzyme A20 (TNFAIP3) is a central regulator of immunopathology. Trends Immunol. 30(8), 383–391 (2009).

53. Zhu L, Wang L, Wang X *et al*. Characteristics of A20 gene polymorphisms and clinical significance in patients with rheumatoid arthritis. J. Transl. Med. 13(1), 215 (2015).

54. O'Rielly DD, Rahman P. Pharmacogenetics of rheumatoid arthritis: potential targets from susceptibility genes and present therapies. Pharmgenomics Pers. Med. 3(1), 15–31 (2010).

55. Marrelli A, Cipriani P, Liakouli V *et al*. Angiogenesis in rheumatoid arthritis: a disease specific process or a common response to chronic inflammation? Autoimmun. Rev. 10(10), 595–598 (2011).

56. Laman JD, Claassen E, Noelle RJ. Functions of CD40 and its ligand, gp39 (CD40L). Crit. Rev. Immunol. 16(1), 59–108 (1996).

57. Danese S, Sans M, Scaldaferri F *et al*. TNF-α blockade down-regulates the CD40/CD40L pathway in the mucosal microcirculation: a novel anti-inflammatory mechanism of infliximab in Crohn's disease. J. Immunol. 176(4), 2617–2624 (2006).

58. Blair BPJ, Riley JL, Harlan DM *et al*. CD40 ligand (CD154) triggers a short-term CD4(+) T cell activation response that results in secretion of immunomodulatory cytokines and apoptosis. J. Exp. Med. 191(4), 651–660 (2000).

59. Shen C, van Assche G, Rutgeerts P *et al*. Caspase activation and apoptosis induction by adalimumab: demonstration in vitro and in vivo in a chimeric mouse model. Inflamm. Bowel Dis. 12(1), 22–28 (2006).

General discussion

Rheumatoid arthritis (RA) is an inflammatory disease of the joints that affects circa 1% of the Western population.[1] It is a progressive disease that results in joint damage and disability unless the inflammation is slowed or stopped by appropriate drug treatment. The treatment principle is to suppress the inflammation at an early stage of the disease with aggressive drug treatment to reach specified and sequentially measured goals, such as remission or low disease activity (so-called treat-to-target approach).[2] This approach is not only in RA a common concept but also in the field of other chronic diseases, including diabetes,[3] hypertension,[4] and hyperlipidemia.[5] Thus, the treatment of RA has a clear target and could combination therapies when monotherapy fails to achieve the goal.

Despite using such clear clinical endpoints in the treat-to-target approach, still, not all RA patients obtain an adequate effect to reduce the disease activity. Conventional drugs used in RA – the "disease-modifying antirheumatic drugs" (DMARDs) – reach their optimal effect after two to three months of treatment. Subsequently, it remains a hurdle for rheumatologists to identify the responders and non-responders beforehand, while patients can be treated with a DMARD that had an insufficient clinical response for several months. A variety of reasons could be conceived for the efficacy of DMARDs, but we hypothesize that pharmacogenetics (PGx) plays a substantial part in it. In essence, the patient's PGx could be determined prior to prescribing DMARDs, making that the drug treatment could be adjusted earlier instead of attempting multiple months of treatment without potential success.

Precision medicine – also known as personalized treatment – with the use of PGx is an evolving field in which the treatment is tailored to the individual patient. Precision medicine is already widely recognized and is for instance embedded in the daily practice of oncology and cardiology. One example of PGx testing in daily clinical practice is on CYP2C9/VKORC1 for the change of the maintenance dose of warfarin, a vitamin K antagonist that is used to inhibit the formation of coagulation factors II, VII, IX, and X, and protein C and S.[6] Normally, due to the narrow therapeutic index and the wide variability in individual dosing, frequent monitoring of the international normalized ratio over weeks is needed to determine the right dose. Guidelines from the Dutch Pharmacogenetics Working Group (DPWG) and the Clinical Pharmacogenetics Implementation Consortium (CPIC) can aid physicians to set patients earlier on the right dose, with determining PGx variants in multiple, such as *CYP2C9*, *CYP4F2*, and *VKORC1* combined with non-pharmacogenetic variants.[7–10]

Although a large number of studies investigates PGx in RA, still, not a single genetic marker has been implemented in daily clinical practice. Thus, the field of RA is still in the proof of principle phase, whereas numerous challenges must first be addressed before the implementation of PGx. Contradictory or non-convincing study results hamper the implementation, among others caused by lack of power (small sample size) or the use

of multi-ethnicities or different evaluation times. This general discussion highlights the obstacles to the implementation and the future directions in the field of PGx testing in RA.

Methodological and statistical aspects

Most PGx studies in RA were retrospective analyses, that were limited to patients with available outcomes data and sufficient material (blood, saliva) for genotyping. As a result, retrospective studies have some disadvantages over prospective studies. First, data from retrospective studies are frequently old, and therefore the study can lack important data which cannot be supplemented or has potential confounding factors. Second, it can be difficult to identify an appropriate exposed comparison group (for example controls in a case/control study) within the same study, and as a consequence, result in a small sample size. Third, differential losses to follow-up retrospective studies can introduce selection bias, thereby it may ensure that the included patients are not representative of the studied population. In the field of RA, in Chapter 2 we concluded that most PGx studies associated with methotrexate (MTX) efficacy had a small study group (<100 patients) with a small effect size (OR between 1.0 and 1.5) or lack the proper multivariate analysis that prevents association by confounders.

The second issue in pharmacogenetic testing is multiple testing, which refers to simultaneously investigate more than one hypothesis on the same study group of study subjects. The risk of multiple testing is that a significant difference is more often found based on coincidence when doing multiple tests. A correction for multiple testing maintains a stricter level of significance, but there is no firm rule whether you should correct it or not. In our thesis, we correct mostly for multiple testing by the simplest and most conservative approach using the Bonferroni correction. In Chapter 6 we performed a pathway analysis, whereas the SNPs within gene regions overlap each other. Consequently, Bonferroni correction is too stringent, because it is based on independent tests, and therefore a global p-value must be calculated using p-min, tail strength, and sequence kernel association test statistics. In most PGx rheumatology studies, no correction for multiple testing was applied.

Yet it is not justified to claim that studies that do not correct for multiple testing or consist of small sample sizes are wrong or even useless in science. Exploratory studies are essential, have a low threshold, and show a basis for new (pharmacogenetic) findings with more knowledge about the mechanism of action (efficacy or toxicity) of drugs. However, associations with good causality must be validated to confirm the result. After proper validation, the studies must be prospectively tested for both confirming the pharmacogenetic association as feasible in the clinical practice.

Hurdles of the translation into clinical practice

PGx has several hurdles to overcome for translation into clinical practice in the field of RA. The main problem is the paucity of available data that PGx testing demonstrates clinical improvement. Furthermore, a common assumption is that the findings of clinical improvement must be replicated in independent cohorts. Problems could be that there is no equivalent replication cohort or mostly the findings could not be replicated. For example, therapeutic drug monitoring (TDM) has been researched for a long time before it was embedded in clinical daily practice to adjust the drug dose according to the explored therapeutic window. Just like TDM, research must be done elaborately, and eventually – when there is sufficient evidence – PGx will be implemented in daily practice. One example that PGx is used in daily clinical practice is for the chemotherapeutic agent 5-fluorouracil (5-FU). Hereby, multiple studies showed that there is a relationship between different allelic variants in the DPYD gene (the gene that encodes DPD) and a deficiency in DPD activity. A deficiency of DPD activity leads to an increased risk for 5-FU toxicity, and therefore pre-emptive PGx testing for DPYD variants is performed.

After validation, there must be carrying capacity among clinicians to implement it into clinical practice. Different organizations associated with PGx – such as CPIC, Dutch Pharmacogenetics Working Group (DPWG), Pharmacogenomics Research Network, and Ubiquitous Pharmacogenomics – contribute to the implementation of PGx in clinical practice by establishing pharmacogenomic information, developing implementation tools, and also release public guidelines to implement PGx in clinical practice.

Phenotype definition and evaluation time

In RA, numerous criteria are used to determine the efficacy of drug therapy. For instance, we described in **Chapter 2** that the endpoints DAS(28), remission (DAS<1.6 or DAS28<2.6), low disease activity (DAS<2.4 or DAS28<3.2), EULAR response criteria, and ACR20, 50, 70 response criteria are often used to determine the efficacy of MTX. Granted that the majority of recent studies increasingly inclined towards the EULAR response criteria, still a few studies used different endpoints. For this reason, it is difficult to combine or compare the results directly. Attention is not only needed for the efficacy endpoint, but also the time of evaluation of those endpoints. In the case of MTX, most studies used the evaluation time points after three or six months of therapy, which properly reflects the effect of low dose MTX in RA.

Different studies investigate the effect of pharmacogenetics on the side effects or efficacy of MTX. A systematic review and meta-analysis showed that *RFC-1* 80G>A (*SLC19A*) rs1051266 is associated with the toxicity of MTX.[11] Our systematic review (Chapter 2) showed that this

SNP (rs1051266) among five other SNPs was associated with MTX efficacy, but still needs further validation.

For future research, it is preferable to take a uniform approach; with consistent criteria and time of evaluation. For example, as an efficacy endpoint the EULAR response criteria is suitable since it also corrects for DAS at baseline. Three or six months after the start of the treatment are appropriate choices of evaluation, whereas DMARDs are effective. For pharmacogenetics related to efficacy, simultaneously testing multiple SNPs seems more obvious than testing single SNPs, because DMARDs act on different pathways and combining SNPs can probably impact the response. For example, the associated six SNPs in Chapter 2 could be used to test if they together form a better prediction and associations on the efficacy of MTX.

Functional SNPs

To better understand if pharmacogenetic variants are associated with the efficacy or adverse events it is essential to know if those variants are functional SNPs that alter the function of a gene. However, there are at least 3.1 million SNP in the human genome, and most of them are not defined as (non-)functional and pragmatically these are extrapolated to assign an effect to a gene. Most common polymorphisms (MAF>5%) are potential regulatory polymorphisms located in 1) noncoding regions, including promoter/upstream, downstream and intron regions, that may affect transcription; 2) in intron and untranslated regions transcribed as RNA that may affect transcription, RNA splicing, stability or translation; or 3) in intergenic regions of unknown function.

Even if a SNP is functional it can have minimal impact on the alteration of a protein and lead to clinically unimportant changes.[12] In our studies, we tested individual SNPs that may have a (minimal) effect or no effect on the gene, but it could also be possible that a set of SNPs that form a haplotype could have a functional association of the efficacy or toxicity of drugs. However, to detect haplotype associations, other genotypic methods with sequencing data are needed.

Prediction models: trend or necessity?

Prediction models for DMARD treatment are developed with the purpose to support drug decision-making for rheumatologists. In recent years the number of publications on statistical models and decision models increased, but yet, these models are not clinically applied. One of the obstacles is that validation is a necessity before a model can be applied. In the developing phase, good models have internal validation. Further to internal validation,

prediction models must also be externally validated in another cohort in the same type of patients, preferably by other investigators. Unfortunately, most multivariate prediction models fail external validation due to poor study design, missing data, or weak-mediocre key performance. Also, not all important issues are reported, and therefore the TRIPOD statements have been introduced. The TRIPOD statements consist of the minimal details to report when developing or validating a multivariate diagnostic or prognostic prediction model.[13]

As mentioned earlier, (in)efficacy of drug response is probably multi genetic, and therefore a combination of different pharmacogenetic biomarkers could play a role and needs further investigation. This is also embedded in our prediction model, which consists of four different genes. Even though the prediction model, tested in **Chapter 5**, compromises four SNPs in four genes, those genes were included a decade ago, while there were only a limited potential known SNPs. Nowadays, there are more investigated SNPs known and it seems that other SNPs have more potential to link them between MTX efficacy and PGx.

Future perspectives on genetic testing

Both the candidate gene studies and GWAS are subject to the same artifacts of spurious association findings. GWAS relies on the indirect association to locate a pharmacogenetic-causing variant but only identifies putative candidate genes that still need a functional assay to determine the functioning of the active substance rather than just its PGx part. The direct candidate gene analysis relies on *a priori* hypothesis to identify a pharmacogenetic-causing variant by direct sequencing.

A novel method, next-generation sequencing (NGS) could be the future that will unravel complex disease genetics, like RA. NGS performs sequencing of millions of small DNA fragments in parallel. These fragments are mapped together with the individual reads to the human genome. Each of the three billion bases in the human genome is sequenced multiple times, providing accurate data and more insight into unexpected DNA variation. The advantage of NGS is that it will capture a broader spectrum of mutations than Sanger sequencing, is unselective, and is used to interrogate full genomes or exomes to discover entirely novel mutations and disease-causing genes, and could detect mosaic mutations.[14]

However, sequencing has the property that it results in huge data and being that, could lead to more spurious findings than GWAS or candidate gene studies. A better method seems to select genes from significant associations in a GWAS and sequence those genes and filter potential associations. This not only leads to a narrow, and more objective result, but is also more affordable.

Prospects towards personalized treatment in rheumatoid arthritis

In the last decade, great progress has been made in the clinical management of RA to achieve low disease activity or remission (so-called treat-to-target principle).[2] Thanks to the treat-to-target approach patients are earlier onset on an effective DMARD and ultimately had less joint damage. Additionally, the introduction of the new drugs (TNF-and JAK-inhibitors) ensures that there is an ample choice in the treatment of RA and offers a solution when the conventional DMARDs had an insufficient clinical effect. Despite those developments, it remains the question of the field of rheumatology has still engrossment about genetic testing.

In this thesis, four SNPs were associated with the efficacy of adalimumab (**Chapter 6**) but need additional replication to validate those findings. In **Chapter 1** we found in the literature six potential SNPs associated with the efficacy of MTX, but five of them did not have any replication studies that could confirm the results. Also, the prediction model for MTX monotherapy (Chapter 4) seems useful, but still, nowadays most of the included non-genetic variants are taken into consideration for drug decision making and the four pharmacogenetic variants in the prediction model showed a small contribution to its total effect.

Up to now, no genetic variants have yet been robustly and consistently associated with response to DMARD use in RA. Also, the results of candidate gene studies, including ours, had led to conflicting results with margin effect sizes. Given the fact that MTX acts on various biological pathways, it is more likely that multiple genes are related to the efficacy and therefore a combination of multiple genes seems more logical. Genetic studies, that tested single SNPs, are not sufficient to unravel complex immunological diseases like RA or multi-target drug treatment as MTX. Therefore, future studies must focus more on a combination of multiple SNPs (haplotype), eventually in combination with other non-genetic factors. One method to take this into account is by using a polygenetic risk score (PRS). A PRS summarises the estimated effect of multiple genetic variants on an individual's phenotype, calculated as a weighted sum of trait-associated alleles. A practical example of the application of PRS is in the prediction of subtype-specified breast cancer, which was based on a large GWAS dataset.[15]

Future research should consider the potential effects of combining results from GWA studies with sequencing data, so the discovery of genetic variants will be accelerated and could ultimately lead to the implementation of pharmacogenetics in RA patients. Also, PRS could be used to improve the predictive value of the efficacy or toxicology of DMARDs and thus help to improve stratification for the screening of suitable DMARDs.

REFERENCES

1. Scott DL, Wolfe F, Huizinga TWJ. Rheumatoid arthritis. Lancet 376, 1094–1108 (2010).
2. Solomon DH, Bitton A, Katz JN *et al*. Review: Treat to target in rheumatoid arthritis: Fact, fiction, or hypothesis? Arthritis Rheumatol. 66, 775–782 (2014).
3. Garber AJ. Treat-to-target trials: Uses, interpretation and review of concepts. Diabetes Obes. Metab. 16, 193–205 (2014).
4. Staessen JA, Wang J, Thijs L. Cardiovascular protection and blood pressure reduction: a meta-analysis. Lancet 358, 1305–1315 (2001).
5. Graham I, Atar D, Borch-Johnsen K *et al*. European guidelines on cardiovascular disease prevention in clinical practice: Executive summary - Fourth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (Constituted by Eur. Heart J. 28, 2375–2414 (2007).
6. Hirsh J, Dalen JE, Deykin D *et al*. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest 119, 8S–21S (2001).
7. Johnson JA, Gong L, Whirl-Carrillo M *et al*. Clinical pharmacogenetics implementation consortium guidelines for CYP2C9 and VKORC1 genotypes and warfarin dosing. Clin. Pharmacol. Ther. 90, 625–629 (2011).
8. Swen JJ, Huizinga TW, Gelderblom H *et al*. Translating pharmacogenomics: Challenges on the road to the clinic. PLoS Med. 4, 1317–1324 (2007).
9. Swen JJ, Nijenhuis M, de Boer A *et al*. Pharmacogenetics: From bench to byte an update of guidelines. Clin. Pharmacol. Ther. 89, 662–673 (2011).
10. Swen JJ, Wilting I, de Goede AL *et al*. Pharmacogenetics: From bench to byte. Clin. Pharmacol. Ther. 83, 781–787 (2008).
11. Qiu Q, Huang J, Shu X *et al*. Polymorphisms and Pharmacogenomics for the Clinical Efficacy of Methotrexate in Patients with Rheumatoid Arthritis: A Systematic Review and Meta-analysis. Sci. Rep. 7, 44015 (2017).
12. Albert PR. What is a functional genetic polymorphism? Defining classes of functionality. J. Psychiatry Neurosci. 36, 363–365 (2011).
13. Collins GS, Reitsma JB, Altman DG, Moons KGM. Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD): the TRIPOD statement. BMJ 350, g7594 (2015).
14. Behjati S, Tarpey PS. What is next generation sequencing? Arch. Dis. Child. Educ. Pract. Ed. 98, 236–238 (2013).
15. Mavaddat N, Michailidou K, Dennis J *et al*. Polygenic Risk Scores for Prediction of Breast Cancer and Breast Cancer Subtypes. Am. J. Hum. Genet. 104, 21–34 (2019).

English summary
Nederlandse samenvatting

ENGLISH SUMMARY

Rheumatoid arthritis (RA) is a chronic, symmetrical, autoimmune disease characterized by inflammation of the mucous membranes in the joints. RA occurs in approximately 1% of the world population and often develops between the ages of 40 and 60 years, especially in women. Left untreated, the disease leads to inflammation and destruction of joints, leading to loss of physical function and inability to perform daily tasks. The exact cause of RA is unknown, but certain risk factors can increase the onset of the disease, such as age, smoking, genetics (particularly the HLA-DRB1 gene), and hormonal changes (pregnancy and breastfeeding).

For the treatment of RA, it is essential to intervene both early and aggressively with medication to prevent (potential) damage with a more favourable course of the disease. Treatment is usually started with the drug methotrexate (MTX), possibly in combination with other disease-modifying antirheumatic drugs (DMARDs) or with prednis(ol)one to quickly suppress the inflammation. Although the treatment of RA has improved considerably in recent decades, drug treatment does not work well for everyone. As a result, it is suspected that genetics plays a central role in the efficacy of current (RA) medications. The aim of this thesis is therefore to investigate which genetic factors influence the toxicity or effectiveness of the drugs used in RA.

Chapter 1 is a general introduction to RA. Here, the classification criteria for RA and the different ways of recording disease activity are described. The drug treatments with, among other things, MTX and biologicals are discussed here, but also the principle of genetics and the different methods of applying genetics. As MTX is a cornerstone in the treatment of RA, this thesis is mainly focused on this drug.

The exact mode of action of MTX is unknown, but it is known to act through at least eight different mechanisms in the cell. Different proteins are involved in each mechanism, which in turn can be influenced by genetics. In recent decades, much research has been done on the role of genetics in the treatment of MTX in RA. However, none of these studies tested with valid endpoints and have been adjusted for multiple testing. Therefore, in chapter 2 we performed a systematic review, applying both valid endpoints only and Bonferroni correction. Ultimately, it turns out that six associations were significant. These associations are promising for predicting the efficacy of MTX, but only one association has been confirmed in an independent cohort.

MTX is transported from the blood into the cell by a transport protein across the cell membrane. Once inside the cell, MTX is polyglutamated and becomes active in the cell. An important transport protein is the solute carrier (SLC), encoded by the *SLC* gene. Because

the coding for RA patients can be different, it is thought that SLC influences the toxicity and effectiveness of MTX. In collaboration with a group from Alabama, Birmingham, USA, we looked at five different SLC genetic variants to see whether this affects efficacy (**chapter 3**). We were able to conclude that this is not the case, both separately and in combination.

Because MTX does not appear to be effective in all patients, a predictive model has been developed. This model contains four genetic and three non-genetic factors (smoking, gender, and rheumatoid factor). The model was developed in 2006 for patients with monotherapy MTX. Today, however, patients are increasingly treated with combination therapy. In this study, described in **chapter 4**, three different Dutch cohorts had been examined whether the model is also suitable in contemporary practice. However, this turned out not to be the case.

As standard, both before and during the treatment of MTX, blood is drawn regularly to check liver function and other processes. This is done regularly because a side effect of MTX is liver damage. If these liver function values are elevated, MTX should be tapered or discontinued. Not only immediately after starting MTX, the liver enzyme values may be increased, even after years of treatment of MTX, though. Only a number of patients develop liver damage, so it is suspected that the cause is genetic. In **chapter 5** we performed a genome-wide association study (GWAS). A GWAS looks at a large part of the genome, without having to determine beforehand which individual SNPs are tested.

A GWAS is performed to provide important insights into the underlying mechanisms of liver damage caused by MTX. It can also help in the development of biomarkers to predict in advance patients who have a higher chance of developing drug side effects. Because not all patients develop liver damage, many different hospitals have been approached. In the end, seven different centres from Poland, Slovenia, England and the Netherlands participated in the study. No genetic variants were found that were a "hit". Nevertheless, some promising associations were found. This offers a perspective, and further research is needed to understand their content and implications.

If MTX and other conventional DMARDs do not exert sufficient clinical effects, the biologicals, or tumor necrosis factor-alpha (TNF-α) inhibitors, can be used. TNF-α is a cytokine that activates the immune system after the binding to the TNF receptors. Inhibitors ensure that the activation is prevented so that the inflammation is inhibited or stopped. Since 2004, several TNF-alpha inhibitors have been marketed, of which adalimumab is the most commonly used drug. The advantage of TNF-alpha inhibitors is that RA patients respond more quickly to the medication, but due to the high cost, they are only used at a later stage, after the failure of several DMARDs.

Adalimumab does not work equally well for all patients, so it has been investigated whether this difference has a genetic cause. We did this with pathway analysis. In this analysis, a series of SNPs are preselected based on the action and pharmacokinetics of adalimumab. This method was used in **chapter 6**, where we investigated which genes influence the efficacy of the biologic adalimumab in RA. For this, 186 SNPs in 111 genes were selected that occur in seven different pathways. We also added 37 significant SNPs from the literature, representing a total of 223 SNPs in 124 genes. The four genetic variants *CD40LG*, *TNFAIP3*, *TANK*, and *VEGFA* seems to play a crucial role in the efficacy of adalimumab.

Chapter 7 includes the general discussion, covering all issues in the design and implementation of genetics research. Importantly, much research remains to explore the genetic aspects in the treatment of RA.

NEDERLANDSE SAMENVATTING

Reumatoïde artritis (RA) is een chronische, symmetrische, auto-immuunziekte die zich kenmerkt door ontstekingen van het slijmvlies in de gewrichten. RA komt bij circa 1% van de wereldbevolking voor en ontstaat vaak op een leeftijd tussen de 40 en 60 jaar, met name bij vrouwen. Onbehandeld leidt de ziekte tot ontstekingen en ernstige schade aan gewrichten, wat weer leidt tot verlies van de fysieke functie en onvermogen om dagelijkse taken uit te voeren. De exacte oorzaak van RA is onbekend, maar er zijn bepaalde factoren die het risico op het ontstaan van de ziekte kunnen verhogen, zoals leeftijd, roken, genetica (in het bijzonder het HLA-DRB1 gen) en verandering van de hormonale spiegel (zwangerschap en borstvoeding).

Voor de behandeling van RA is het essentieel om zowel bijtijds als agressief medicamenteus in te grijpen om zo (potentiële) schade te voorkomen en een gunstiger verloop van de ziekte te bewerkstelligen. Doorgaans wordt de behandeling gestart met het geneesmiddel methotrexaat (MTX), eventueel in combinatie met een andere 'disease-modifying antirheumatic drugs' (DMARD's) of met prednis(ol)on om de ontsteking snel te onderdrukken. Alhoewel de behandeling van RA de laatste decennia sterk is verbeterd, slaat de medicamenteuze behandeling niet bij iedereen voldoende aan. Hierdoor bestaat het vermoeden dat genetische factoren een centrale rol spelen met betrekking tot de werkzaamheid van de huidige medicatie. Het doel van dit proefschrift is dan ook te onderzoeken welke genetische factoren van invloed zijn op de toxiciteit of effectiviteit van de geneesmiddelen die worden gebruikt bij RA.

Hoofdstuk 1 is een algemene inleiding over RA. Hier worden de classificatiecriteria voor RA en de verschillende manieren om de ziekteactiviteit vast te leggen beschreven. Ook de medicamenteuze behandelingen met o.a. MTX en biologicals komen hier aan bod, maar ook het principe van genetica en de verschillende toepassingen van nieuwe inzichten op dit gebied. Doordat MTX een hoeksteen is in de huidige behandeling van RA, is deze thesis voornamelijk gericht op dit geneesmiddel.

De exacte werking van MTX is onbekend, maar aangetoond is dat het tenminste via acht verschillende mechanismen in de cel zijn werking uitoefent. Bij elk mechanisme zijn verschillende eiwitten betrokken die weer op hun beurt door genetica kunnen worden beïnvloed. De laatste decennia is er veel onderzoek gedaan naar de rol van genetica in de behandeling van MTX in RA. Geen van deze onderzoeken is getest met valide eindpunten en gecorrigeerd voor het 'multiple testen'. Hierom hebben we in **hoofdstuk 2** een systematische review uitgevoerd, waarbij we zowel alleen valide eindpunten als Bonferroni-correctie hebben toegepast. Uiteindelijk blijkt dat er zes associaties zijn die significant waren. Deze

associaties lijken veelbelovend voor het voorspellen van de werkzaamheid van MTX, maar slechts één associatie is bevestigd in een onafhankelijk cohort.

MTX wordt vanuit het bloed door een transporteiwit over het celmembraan in de cel getransporteerd. Eenmaal in de cel wordt MTX gepolyglutameerd en wordt het werkzaam in de cel. Een belangrijk transporteiwit is solute carrier (SLC), gecodeerd door het *SLC* gen. Doordat de codering voor RA-patiënten verschillend kan zijn, wordt gedacht dat *SLC* invloed heeft op zowel de toxiciteit als de effectiviteit van MTX. In samenwerking met een groep uit Alabama, Birmingham, USA hebben we gekeken naar vijf verschillende genetische SLC-varianten en het daadwerkelijke effect op de werkzaamheid (**hoofdstuk 3**). We konden zowel separaat als gecombineerd concluderen dat dit niet het geval is.

Doordat MTX lang niet bij alle patiënten effectief blijkt, is er een voorspellend model ontwikkeld. Dit model bevat vier genetische en drie niet-genetische factoren (roken, geslacht en reumafactor). Het model is in 2006 ontwikkeld voor patiënten met monotherapie MTX. Tegenwoordig worden patiënten echter in toenemende mate behandeld met een combinatietherapie. In deze studie, beschreven in **hoofdstuk 4**, is er in drie verschillende Nederlandse cohorten naar gekeken of het model ook geschikt is in de hedendaagse praktijk. Dit bleek echter niet zo te zijn.

Er wordt standaard, zowel voor als tijdens de behandeling van MTX, regelmatig bloed geprikt om de leverfunctie en andere lichamelijke processen te controleren. Dit wordt gedaan omdat een bijwerking van MTX leverschade is. Indien deze leverfunctiewaarden zijn verhoogd, moet MTX worden afgebouwd of worden gestopt. Niet alleen direct na het starten van MTX kan het voorkomen dat de leverenzymwaarden zijn verhoogd, maar zelfs ook na jarenlange behandeling met MTX. Doordat slechts een klein gedeelte van de patiënten leverschade krijgt, bestaat het vermoeden dat de oorzaak hiervan genetisch is. In **hoofdstuk 5** hebben we een genoombrede associatiestudie ('genome-wide association studies', GWAS) uitgevoerd. Een GWAS kijkt naar een groot gedeelte van het genoom, zonder dat daarvoor vooraf moet worden vastgesteld welke individuele SNP's getest worden.

Een GWAS wordt uitgevoerd om belangrijke inzichten te geven in de onderliggende mechanismen van het ontstaan van leverschade door MTX. Tevens kan het helpen bij de ontwikkeling van biomarkers om vooraf patiënten te identificeren die een grotere kans hebben op het ontwikkelen van geneesmiddelbijwerkingen. Doordat lang niet alle patiënten leverschade krijgen, zijn veel verschillende ziekenhuizen benaderd. Uiteindelijk hebben er aan de studie zeven verschillende centra uit Polen, Slovenië, Engeland en Nederland deelgenomen. Er bleken geen genetische varianten gevonden te zijn die een "hit" waren. Desondanks werden er een aantal veelbelovende associaties gevonden. Dit

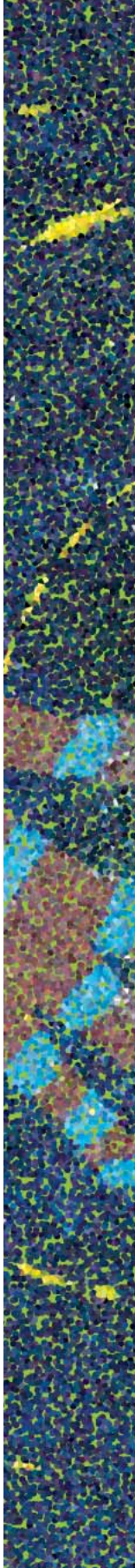
biedt perspectief, en er moet dan ook verder worden uitgezocht welke implicaties deze bevindingen hebben.

Als MTX en andere conventionele DMARD's onvoldoende klinische effecten uitoefenen, kunnen de biologicals, oftewel tumornecrosefactor alfa (TNF-α)-remmers, worden ingezet. TNF-α is een cytokine, die na binding aan de TNF-receptoren o.a. het immuunsysteem activeert. Hierbij zorgen remmers ervoor dat de activatie wordt voorkomen waardoor de ontsteking wordt geremd of gestopt. Sinds 2004 zijn er meerdere TNF-alfa remmers op de markt, waarvan adalimumab het meest gebruikte geneesmiddel is. Het voordeel van TNF-alfa remmers is dat RA-patiënten sneller reageren op de medicatie, maar door de hoge kosten worden deze pas in een later stadium, na het falen van meerdere DMARD's, gebruikt.

Adalimumab werkt niet voor alle patiënten even goed en daarom is onderzocht of dit een genetische oorzaak heeft. Dit hebben we met een pathway-analyse gedaan. Bij deze analyse wordt een reeks SNP's op basis van de werking en farmacokinetiek van adalimumab vooraf geselecteerd. Deze methode is gebruikt in **hoofdstuk 6**, waarbij we onderzochten welke genen invloed hebben op de effectiviteit van de biological adalimumab in RA. Hiervoor zijn 186 SNP's in 111 genen geselecteerd die in zeven verschillende pathways voorkomen. Ook hebben we uit de literatuur 37 significante SNP's toegevoegd, wat neerkomt op een totaal van 223 SNP's in 124 genen. De vier genetische varianten *CD40LG*, *TNFAIP3*, *TANK* en *VEGFA* blijken een rol te spelen bij de werking van adalimumab.

In **hoofdstuk 7** is de algemene discussie opgenomen, waarbij alle problematiek bij het ontwerpen en uitvoeren van genetica-onderzoek aan bod komt. Er ligt nog veel terrein braak om de genetische aspecten bij de behandeling van RA te onderzoeken.

Curriculum vitae



Frank Eektimmerman was born September 14th, 1987 in Almelo, The Netherlands. After completing his secondary school at the Pius-X-college Almelo, he started to study Pharmacy at the University of Groningen. His bachelor thesis was about spray-freeze-drying of lipophilic (fat-soluble) drugs, like diazepam, under the supervision of dr. Hans de Waard. During his Master's study, he followed interdisciplinary honours courses as part of the honours program "Leadership: make a difference!" and met lecturers and students from all degree programmes at the University of Groningen. Also during his master's studies, he completed several extracurricular courses, like medical cell biology, pharmacy and informatics, caput molecular pharmacology, and advanced pharmacokinetics. His master's thesis was on the pharmacokinetics of the local anesthetic ropivacaine in the treatment of total knee arthroplasty, under the direct supervision of drs. Pim Langendijk and the late Prof. Dr. Bob Wilffert, carried out in the Reinier de Graaf Gasthuis, Delft.

In 2013 he obtained his Pharm.D. and started as a pharmacist in the Martini hospital, with a special area of focus of the operation complex and the opiate registration. In 2014 he was a pharmacist in Diaconessenhuis Meppel, Bethesda Hoogeveen, and Isala Zwolle for the electronic prescription of medication of all medical specialties and the forwarding of the electronic recipes to the community pharmacies. In October 2015 he started his Ph.D. under the supervision of Prof. Dr. H.J. Guchelaar, Dr. J.J. Swen and C.F. Allaart. In 2018 Frank started his specialization to hospital pharmacist in the Amphia hospital, Breda, in combination with the Albert Schweitzer hospital, Dordrecht. Frank lives together with Paulien Lipman in Leiden.

Dankwoord

Graag bedank ik iedereen die heeft bijgedragen aan de totstandkoming van dit proefschrift.

Bijzonder erkentelijk ben ik mijn promotor en copromotoren. Henk-Jan, met jouw eruditie, enthousiasme, optimisme en snelle respons gaf je bezielend leiding aan mijn promotie-onderzoek. De plezierige samenwerking en communicatie resulteerden in verschillende waardevolle en motiverende onderzoeksideeën. Jesse, je kritische en initiatiefrijke houding hielden me scherp. René, jouw klinische blik met oog voor de toepasbaarheid en relevantie gaf het onderzoek het nodige gewicht. Zoals vaker in het leven verliep niet alles zonder tegenslagen. Met volharding werden uiteindelijk nieuwe wegen gevonden.

Gedurende het promotieonderzoek heb ik hulp gekregen van de studenten Denise, Jip en Moenira. Bedankt voor jullie inzet! Ook dank ik Tahar, Renée, Daniëlle, Rowena, Yavuz en Fina voor de hulp bij de DNA-extractie, het genotyperen en het meedenken bij de vele bepalingen. Voor de statische uitdagingen ben ik veel dank verschuldigd aan Stefan Böhringer. Zonder zijn hulp zou dit onderzoek onmogelijk zijn geweest. Daarnaast dank ik Wendy, Kyra, Mieke en Marion die mij veel werk uit handen hebben genomen op organisatorisch gebied.

Mijn dank gaat ook uit naar mijn (oud-)kamergenoten: Meta, Sofieke, Paul, Xiaoyan, Carin, Cathelijne, Maaike, Anabel, Michel, Anyue, Tom en Sylvia. Aan het einde van je PhD-project realiseer je je pas echt hoe een promotieproces verloopt. Het is af en toe wel even doorzetten. Verder dank ik mijn collega-promovendi van de reumatologie voor het wekelijkse uurtje met altijd enerverende interacties en waardevolle feedback. Dank jullie: Sytske Anne, Joy, Miranda, Emma en Féline.

Goede samenwerking is een voorwaarde voor goed onderzoek. Tijdens mijn onderzoek heb ik veelal op locatie hulp gekregen in Nijmegen (Johanne, Els, Thea, Marieke, Jaap en Alfons), Leiden (Gerrie en Cedric), Amsterdam (Renske, Ingrid en Mike) en Rotterdam (Mieke en Jard). Ook verdienen mijn buitenlandse collega's veel lof: Engeland (Suzanne en Nisha), Polen (Andrzej), Slovenië (Vita en Barbara) en de Verenigde Staten (Stella). Bedankt dat jullie mijn verzoeken om hulp altijd inwilligden en de moeite namen om met mij mee te denken.

Dank gaat voorts uit naar mijn familie en schoonfamilie, zeker voor de welkome ontspannende momenten zoals die in Porto, op Texel of aan het Idromeer. Naast mijn vader daarboven, blijft mijn moeder mijn grootste supporter. Haar trots, interesse en betrokkenheid waren hartverwarmend, evenals de steun die ik voelde van mijn broer en zus. Bedankt, Jaap en Linda dat jullie mijn paranimf willen zijn tijdens de plechtigheid.

Tot slot wil ik mijn liefste Paulien bedanken, die er altijd voor mij was. Die voor mij naar Leiden is verhuisd. Bedankt dat je mijn steun en toeverlaat bent geweest, niet alleen in de afgelopen jaren, maar altijd.