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

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

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Chapter 1:

General Introduction and Outline

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a prevalent autoimmune disease, which affects approximately one percent of all types of human populations (1;2). Generally, this immune-mediated disease is associated with symmetrically inflammation, destruction of the joints leading to overall functional impairment and (serious) comorbidity, like cardiovascular events (3-6).
Generally, the etiology of this inflammatory disease remains unclear due to the complexity of interacting factors representing a multifactor process. Still, important risk- and protective factors have been elucidated which are associated with development or severity of RA (7-11). These factors can be divided in two groups: environmental- and genetic factors. It has been demonstrated that these factors act synergistically in causing RA. Specifically, this phenomenon was highlighted by an interaction between smoking and HLA-DR risk alleles in RA patients (12;13). Likewise, it was observed that an environmental factor, like smoking, could increase the genetic risk course for RA. These interactions provide additional difficulties in clear understanding RA's etiology.
Similarly to etiology, the RA's pathophysiology is not fully understood. Hypothetically, after the stimulation of an environmental trigger, T-cells of the CD4+ type stimulate monocytes, macrophages and synovial fibroblasts to secrete three important pro-inflammatory mediators: Tumor Necrosis Factor alpha (TNFα), interleukin 1 (IL-1) and interleukin 6 (IL-6). It is thought that TNFα has a central place in the inflammatory cascade of RA leading to progression of inflammation and eventually erosion of bone and cartilage. Also, TNFα is recognized to be involved in stimulation of cytokine production (including its own), enhancing expression of adhesion molecules, neutrophil activation and it is also a co-stimulator for T-cell activation and antibody production by B-cells (14;15) (figure 1).

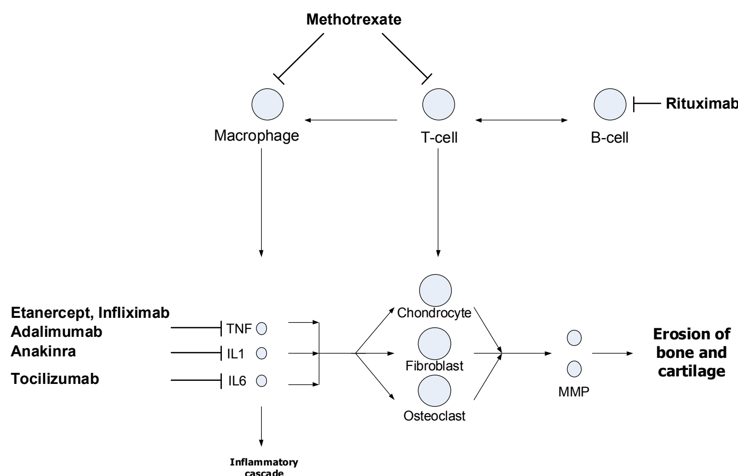

Figure 1. Pathophysiology and accessory therapeutic agents in rheumatoid arthritis

Abbreviation(s): IL1= interleukin 1, IL6= interleukin 6, MMP= matrix metalloproteinases, TNF= tumor necrosis factor.

Treatment of rheumatoid arthritis

In the last decades treatment outcome of RA has been successfully improved due to:
-1- the expanding knowledge of the disease's pathophysiology, which elucidated important key players in the inflammatory process as potential targets for therapy (14-16); -2- development of new

agents based on newly discovered targets leading to improved response percentages and facilitating a range of equivalent treatment modalities in drug therapy (e.g. for effectively switching to infliximab after failure of etanercept) (17;18); -3- the development of easy-to-use diagnostic tools to measure efficacy of therapy (19-22); -4- the recognition and acknowledgement that disability and joint damage occurs in an early state (6;23-25); -5- therapeutic strategy for RA patients that is focused on strictly- and early management of RA's disease course (26-28).
Despite an increasing knowledge on RA's etiology and pathogenesis, a therapy resulting in remedy of the disease is not achieved to date. Alternatively, treatment is aimed at remission of disease, by suppressing pro-inflammatory particles, like cytokines and lymphocytes (16;29). Notably, a widening arsenal of therapeutics has been developed, which have a general focus on modifying RA's disease course to alleviate pain, to suppress inflammation, to prevent joint damage and loss of function in order to postpone disability (Figure 1) (30). Hereby, an important role in modification of treatment outcome is being played by disease modifying antirheumatic drugs (DMARDs). Two types of DMARDs take a central place in the rheumatology clinics: methotrexate (MTX) and TNF inhibitors. Despite the fact that the mechanism of action of these drugs remains partially unclear, DMARDs have proven to be effective treatment modalities according to several disease activity measurements in clinical trials (26;31-36). For this reason, rheumatologists have increasingly prescribed both types of drugs. However, highly differential response rates in overall clinical efficacy and/or toxicity have been demonstrated in clinical trials with MTX and TNF inhibitors. Specifically, 40-70% and/or 15-30% in RA patients treated with MTX and TNF inhibitors fail to achieve a satisfactory response and/or develop adverse drug events, respectively (26;31-36). Because of the substantial differences in individual responses and also the knowledge that reduction of disease activity leads to less progression of RA, it is beneficial to predict which patients have a increased chance for responding to the different treatment modalities. Consequently, several studies have been performed considering an influence of demographic, clinical and immunological variables on treatment outcome to DMARDs (37-41). Similarly, genetic influences on response to DMARDs have also been explored (42-47). Generally, genetic factors are estimated to account for 15-30% of interindividual differences in drug metabolism and response. In this way, pharmacogenetics has the potential to increase drug efficacy and to ameliorate adverse drug events by applying genetic determinants of therapeutic response and is able to aid in predicting efficacy and toxicity of drug therapy in RA (48;49).

Pharmacogenetics

Pharmacogenetics is defined as the study of variability in drug responses attributed to genetic factors in different populations (48;49). In this context, the pharmacogenetics of most drugs is likely to be comparable to the genetics of complex diseases, like RA. In both cases numerous proteins are involved in complex pathways, and in this way one clear genetic explanation is not available (50).
The complete DNA sequence across the human genome, which consists of approximately 3.1 billion base pairs, has been determined. Approximately 99.9% of base pairs in the human genome are identical among individuals, whereas the remaining 0.1% reflects the individual differences in variants which may lead to differences in susceptibility to specific diseases and response to specific drugs. Single nucleotide polymorphisms (SNP) have been recognized as useful markers of genetic polymorphism. SNPs comprise genetic variation with a single-base difference between individuals resulting in due to alteration, deletion or insertion of the base (e.g. replacement of guanine by adenine). SNPs are widely distributed at a frequency of about one SNP in every 300–500 bases, which is approximately 1.5 million of this type of variants across the human genome. Hereby, SNPs are estimated to account for 90% of all genetic mutations (51;52).

Generally, in pharmacogenetics SNPs are involved in differences in drug response by affecting the expression of genes or by altering the types of amino acids and affecting their activities (49;53). In addition, as multiple SNPs exist within a single gene, several combinations of these polymorphisms (expressed by linkage disequilibrium- e.g. haplotypes) are important to consider in order to explain genetic variation as a whole in pharmacogenetic research (51).

Outline of this Thesis

The primary objective of this thesis is to assess the role of pharmacogenetics in the variation of treatment outcome in patients diagnosed with rheumatoid arthritis and treated with disease modifying antirheumatic drugs. Hereby, this thesis is divided in two parts: pharmacogenetics of methotrexate and of adalimumab in RA patients.

Part 1: Pharmacogenetics of methotrexate

In **Chapter 2** an overview is presented of the previously performed studies concerning genetic variability contributing to differences in response to MTX in RA treatment.

As it is generally accepted that MTX may act in RA through inhibition of folate pathway enzymes, other reports indicate that efficacy may also be related to the release of endogenous antiinflammatory adenosine. With this hypothesis, the relationship between SNPs in genes related to adenosine release and MTX treatment outcome in patients with recent-onset RA is explored in **chapter 3**.

So far, most genetic variants are selected for analysis based upon their hypothetical relation to the mechanism of MTX or inflammatory process in RA (**chapters 2**), such as genetic variants in the adenosine pathway (**chapter 3**). Ideally, functional genetic variants are chosen because the alteration in protein function is thought to influence drug action and thus may explain interindividual differences in drug response. **Chapter 4** assesses the role of SNPs with proven functional consequences. These SNPs are located in genes, which are thought to be related with the mechanism of action of MTX and/or immunopathogenesis of RA. In addition, replication analyses are performed in **chapter 4**, since previously applied endpoints for efficacy from other research reports are available. These replication analyses are important, since pharmacogenetic studies have the potential to result in reporting false positive findings.

Previously, a clinical pharmacogenetic predictive model was developed for predicting the efficacy of MTX monotherapy in patients with recent-onset RA comprising the Dutch BeSt Cohort. The model consists of non-genetic factors sex, rheumatoid factor and smoking status, Disease Activity Score (DAS) before starting MTX and 4 genetic polymorphisms (*MTHFD1* 1958G>A, *AMPD1* 34C>T, *ITPA* 94A>C and *ATIC* 347C>G). The performance of this model is validated in a second Dutch cohort (**chapter 5**) and in a Swedish cohort (**chapter 6**).

Chapter 7 evaluates the role of the haplotypes comprising the SNPs *MTHFR* 1298A>C and *MTHFR* 677C>T in treatment outcome to MTX in RA. Specifically, in this chapter optimalization of a previously designed pharmacogenetic model is aimed with addition of the number of haplotypes comprising *MTHFR* 1298A-677C alleles as additional criterion. Furthermore, the predictive value of the haplotype is compared with other genetic polymorphisms in predicting MTX efficacy.

Part 2: Pharmacogenetics of adalimumab

In **Chapter 8** an overview is given of the previously performed studies concerning genetic variability contributing to differences in response to TNF inhibitors in RA treatment.

In the next chapter, SNP selection for pharmacogenetic association studies is discussed. Additionally, a pharmacogenetic pathway approach is presented together with proposed criteria for systematic selection of SNPs. This method is applied for the selection of potential interesting SNPs within genes related involved in the mechanism of action of adalimumab and/or inflammatory process of RA (**chapter 9**).

Chapter 10 puts the presented systematically selection of SNPs in **chapter 9** into practice: efficacy of treatment with adalimumab is associated with genetic variants selected by a pharmacogenetic pathway approach using a custom made antiTNFα SNP array.
Furthermore, SNPs, which were previously associated with genetic susceptibility to RA and/or treatment outcome to TNF inhibitors, were examined for association with treatment outcome in **chapter 10**.

Chapters 11 and 12 provide a summary of this thesis (chapter 11) and present a general discussion including a perspective on future research (chapter 12).

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Part 1:

Methotrexate

Chapter 2:

Pharmacogenetics of methotrexate in rheumatoid arthritis

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Abstract

Over the last decades important progress is being made regarding disease modifying anti-rheumatic drugs (DMARDs), like methotrexate (MTX), in the treatment of rheumatoid arthritis (RA). Nevertheless, a substantial part of the patients fail to achieve a good response and/or experience toxicity, which limits further treatment leading to progression of inflammation and destruction of joints. These high interindividual differences in drug response gave rise to the need for prognostic markers in order to individualize and optimize therapy with these anti-rheumatic agents. Besides demographic and clinical factors, studies in the research field of pharmacogenetics have reported potential markers associated with clinical response on treatment with MTX. However, publicized conflicting results and underlying interpretation difficulties inhibit drawing definitive conclusions. Presently, clinical implementation of pharmacogenetics as an important step for individualizing drug therapy in RA is not feasible yet. Replication and prospective validation in large patient cohorts are required before pharmacogenetics can be used in clinical practice. This review provides the current state of art in genotyping RA patients as a potential guide for clinical decision making.

Introduction

Rheumatoid arthritis has a prevalence of ~1% in the Western population (1). This autoimmune disease is characterized by a chronic inflammatory process within the synovial joints, progressive (radiological) joint damage and significant functional impairment (2). In the last decades patients have been treated with traditional disease modifying anti-rheumatic drugs (DMARDs) including methotrexate (MTX), sulphasalazine and leflunomide, or a combination of DMARDs. More recently, growing evidence for the central role of tumour-necrosis factor alpha (TNFα) in the pathogenesis of RA has led to the introduction of TNF inhibitors, such as etanercept, infliximab and adalimumab (3). These biological DMARDs have proven to play an important role in the treatment of persistent RA in patients, who achieve an incomplete response or develop adverse drug events to traditional DMARDs (4-6). In addition, biologicals with alternate mechanisms of actions such as rituximab, abatacept and tocilizumab have recently been developed, (7-9). To date, the place in RA therapy of these new agents is less established.
Ideally, RA therapy is based on strict monitoring of disease activity and tight control treatment in order to prevent progression of joint damage and functional disability (10). Namely, it is established that high and variable disease activity is related to increasing joint damage and that effective intervention stops this progression (11;12). In current clinical practice, newly diagnosed RA patients are treated with traditional DMARDs, in which methotrexate (MTX) is the drug of first choice (13;14). In case of unfavourable response, side effects and/or drug toxicity, alteration of dose regimen or drug therapy towards a combination of DMARDs and/or biologicals is recommended.(4;15;16).
Still, different response rates are seen in RA patients treated with MTX. Substantial percentages of 30-40% of RA patients fail to achieve a satisfactory response. Moreover, 15-30% of the patients develop adverse drug events (16-18). These different responses lead to studies identifying influence of demographic, clinical and immunological variables on treatment outcome with MTX (19). Next to these factors, genetic influences have also been explored in the last decade. Generally, pharmacogenetics has the potential to increase drug efficacy and to ameliorate adverse events (20;21). Therefore, its application might be of great clinical benefit for individuals affected with RA. Studies have reported associations between single nucleotide polymorphisms (SNPs) in genes encoding enzymes related to the pharmacokinetics and pharmacodynamics of MTX and treatment outcome (22-25). The ultimate aim of using pharmacogenetic markers is to predict the probability of a wanted or unwanted drug response in individual patients (20;21).
This review presents an overview of genetic variability contributing to differences in response to MTX in RA treatment.

Pharmacogenetics of methotrexate

Although the exact mechanism of action of MTX is unclear, numerous enzymes have demonstrated to be important for its anti-proliferative and immunosuppressive effects (26;27). Before MTX is being metabolized inside the cell, MTX enters the cell e.g. by the transporter-enzyme reduced folate carrier (RFC). Efflux from the cell is facilitated by ATP-binding cassette (ABC) transporters, e.g. ABCC1 to 5 and ABCG2 and (less proven by) ABCB1 (28;29). If MTX enters the cell, the drug is polyglutamated, meaning that groups of glutamic acid are added to MTX. This process is catalyzed by the enzyme folylpolyglutamate synthetase (FPGS) and reversed by gammaglutamyl hydrolase (GGH), respectively.

Polyglutamated MTX (MTXPGs) inhibits several enzymes directly such as thymidylate synthase (TYMS), dehydrofolate reductase (DHFR), whereas indirect inhibition occurs on methylenetetrahydrofolate reductase (MTHFR), a key enzyme in the folate pathway (26). MTXPGs also inhibit the conversion of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) to formyl-AICAR, which is facilitated by the enzyme AICAR transformylase (ATIC). Accumulation of AICAR has a direct inhibitory effect on other enzymes, like adenosine monophosphate deaminase (AMPD1). This accumulation may lead to the release of adenosine, a potential anti-inflammatory agent (30;31).
To date, SNP selection for pharmacogenetic association studies concerning MTX is done within genes encoding enzymes in these hypothetical pathways regarding MTX's mechanism of action. However, the association of polymorphisms in these pathway genes have yielded mixed results. Table 1 presents the pharmacogenetic data of RA patients treated with MTX monotherapy.
Regarding transport enzymes, association studies of MTX treatment outcome to genetic polymorphisms in the genes *ABCB1*, *RFC* and *ABCC2* have been performed (25;32-40). It has been found that SNPs in the transporters ABCB1 and RFC associate with MTX efficacy and toxicity. However, conflicting data were seen. For example, studies with the *ABCB1* 3435 C>T have reported that the genotype TT was associated with efficacy (34) and inefficacy (36). In addition, one study detected an association of the TT genotype with toxicity (40). For *ABCC2*, no associations of SNPs with toxicity were found (25).
The best-studied SNPs concerning MTX treatment outcome are at the positions 677C>T and 1298A>C within the gene coding for the folate key-enzyme MTHFR (24;25;37;39-47). This enzyme catalyzes the conversion of homocysteine to methionine for a variety of metabolic reactions (30). Functional studies have elucidated that these two polymorphisms are associated with diminished enzyme activity of MTHFR leading to homocysteinemia (48). In fact, it is demonstrated that a decrease in activity could lead to homocysteinemia and eventually could be related to toxicity, e.g. influencing the gastrointestinal tract in RA patients on MTX therapy (48). As a consequence, several reports studied the association of *MTHFR* 677C>T and 1298A>C with toxicity. Regarding *MTHFR* 677C>T, seven studies found no association with overall MTX-induced toxicity (37;41-43;47-49), whereas other studies found associations with GI toxicity for the CT genotype (48), increased MTX discontinuation due to increased liver enzyme levels for 677 T-allele carriers (24), alopecia in Afro-Americans (25), and overall toxicity (24;45;46). In other studies, *MTHFR* 1298 A-allele carriers were related to side effects in two reports (41;42), whereas two groups found no association (43;45) and two groups detected an association between 1298 C-allele carriers with overall toxicity and gastrointestinal toxicity (37;49).
Additionally, associations with MTX efficacy were assessed in most of the studies, involving *MTHFR* genetics. Of the seven studies performed, only three studies found that patients genotyped for *MTHFR* 677CC were more likely to achieve a good response, defined as a decrease or an obtained absolute value of disease activity score (DAS) (37;44;49). Also, reports on *MTHFR* 1298A>C provide inconclusive results. One report found associations with efficacy of the 1298AA genotype and a decrease in DAS (37). In contrast, three studies reported an association between C-allele carriers and disease improvement as defined as the likelihood to be treated with a higher dose, a tendency for remission or decrease in ESR and/or CRP level (44-46). Still, five reports did not find associations of *MTHFR* 1298A>C with efficacy (40-43;49). The enzymes methylenetetrahydrofolate dehydrogenase (MTHFD1), methylenetetrahydrofolate (SHMT1), and thymidylate synthase enhancer region (TSER) are indirectly influenced by MTXPGs (26). Regarding efficacy, one SNP in the *MTHFD1* gene could be related with inefficacy to MTX treatment (39). Yet, for *SHMT1* and *TSER* an association with a single genetic polymorphism within each gene and developing side effects and alopecia in specific was demonstrated (47) (table 1).

Two direct targets of MTX are the enzymes DHFR and TYMS (26;30). Regarding *DHFR*, one study was performed in which no associations with efficacy or toxicity were found (37). For *TYMS*, four studies were performed (36;39;40;43). Only, one study reported an association of a polymorphism, 6 basepair (bp) deletion, within the 3'UTR region of the gene and achieving good response, defined as likelihood to be treated with a higher dose or decrease in CRP level (43).

Recently an association between MTX and HLA-G antigens, defined as nonclassical major histocompatibility complex class Ib molecules important for maintaining anti-inflammatory conditions, was found in an in vitro study (50). The *HLA-G* 14 bp deletion is thought to increase *HLA-G* mRNA and protein stability, possibly leading to prolonged anti-inflammatory actions. Therefore, MTX may act synergistic with this deletion. It was shown that MTX induces soluble HLA-G, whereas a homozygous deletion of 14bp in this *HLA*-gene was more frequently detected in patients with response to MTX. However, the role of the *HLA-G* 14 bp polymorphism in vivo in clinical response to MTX remains conflicting (50-52).

Generally, regarding MTX-induced toxicity, no associations between polymorphisms in the pathway enzymes including, *TYMS*, *DHFR*, *AMPD1*, *ITPA* genes and the occurrence of side effects in RA patients exist (36;37;40;53) (Table 1).

Direct involved in MTX's polyglutamation are the enzymes FPGS and GGH. Two SNPs, 114G>A and 1994A>G, in the *FPGS* gene were not reported to be related to efficacy or toxicity in RA patients (54). Concerning *GGH*, in three studies no significant effect of three SNPs, -401C>T, 452C>T and 16C>T, with efficacy was demonstrated (39;49;54). However, an association of *GGH* -401C>T with toxicity was seen in one study (49).

Gene	Function	Genetic polymorphism(s)	Clinical effect on:	
			Toxicity	**Efficacy**
MTHFR	Catalyzes methylene THF to methyl-THF; indirect target MTX	677C>T	- Effect on GI toxicity (48); T-allele associated with toxicity and increased liver enzyme levels (24;45;46); No association with toxicity (37;48;40-43;47;49)	-No association with efficacy (24;25;40;42;43); Association with efficacy (37;44;49)
		1298 A>C	A-allele associated with toxicity (41;42); C-allele associated with toxicity and GI toxicity (37;40;49); No association with toxicity (43;45)	No association with efficacy (40;42;43;45;49); Association with efficacy (37;46); May affect efficacy (44)
ATIC	Conversion of AICAR to 10-formyl-AICAR; target of polyglutamated MTX	347C>G	GG associated with toxicity and GI toxicity (47;49;53); No effect on toxicity (36)	Association with efficacy (39;53;47); No association with efficacy (36;49)
DHFR	Reduction of DHF to THF; target of MTX	-473G>A, 35389G>A	No effect on efficacy or toxicity (37)	
MTHFD1	catalyzes interconversion of 1-carbon derivatives of THF; indirect target MTX	1958G>A	*	AA associated with inefficacy (39)
SHMT1	catalyzes conversion of serine and THF to glycine and methylene-THF: indirect target MTX	1420C>T	No association with toxicity (47;49); CC associated with alopecia and CNS side effects (47)	No association with efficacy (39); CC associated with efficacy (49);
TSER	Enhancer region of TYMS; indirect target of MTX	‘5 UTR 28bp repeat	No association with toxicity (43;49); Association with toxicity and alopecia (47)	No association with efficacy (39;43;49)
TYMS	Conversion of dUMP to dTMP; target of MTX	‘3 UTR 6bp deletion	No effect on toxicity (36;40)	May affect MTX efficacy (as defined by MTX dose and CRP level) (43); No effect on efficacy (as defined by MTX dose) (36;40)
AMPD1	Conversion of AMP to ADP and ATP; indirect target MTX	34C>T	No association with toxicity (53)	T-allele associated with efficacy (39;53)
MTR	Methylation of homocysteine to methionine; indirect target MTX	2756A>G	No association with toxicity (40;53); AA associated with toxicity (49)	No association with efficacy (40;49;53)
MTRR	Methylation of cofactors required for MTR action; indirect target MTX	66A>G	No association with toxicity (40;53); GG associated with toxicity (49)	No effect on efficacy (40;49;53)
ITPA	Conversion IMP to ITP; indirect target MTX	94C>A	No association with toxicity (53)	CC associated with efficacy (39;53)
ADORA2A	Adenosine A2a receptor	5 SNPs (4 in intron+ 1 in downstream)	All SNPs associated with Toxicity (55); Two SNPs with GI toxicity (55)	*
FPGS	Adding polyglutamates to MTX; prolonging cellular retention MTX	1994A>G, 114G>A	No effect on efficacy or toxicity (54)	
GGH	Conversion of long chain polyglutamated MTX into short chain by removing polyglutamates	452C>T, 16C>T	No effect on toxicity (54)	May affect efficacy (54); No association with efficacy (39)
		- 401C>T	CC associated with toxicity (49)	No association with efficacy (49);

ABCB1	Efflux transporter on cells; efflux of MTX	3435C>T	ABCB1 3435 TT associated with toxicity (40); No association with toxicity (36)	No association with efficacy (40); TT associated with efficacy (34); TT associated with inefficacy (36)
		+1236C>T, 2677G>T	No effect on efficacy or toxicity (25;40)	
RFC	Folate entry in the cell	-43T>C, 696C>T	*	No effect on efficacy (32)
		80G>A	RFC1 80GG associated with toxicity (40); No association with toxicity (36;49;53)	No effect on efficacy (32;36;37;40;49); RFC 80A-allele associated with efficacy (35)
ABCC2	Efflux transporter on cells of MTX	1249 G>A, 1058 G>A, IVS23 +56 T>C	No effect on toxicity (25)	*
HLA-G	Persistence of anti-inflammatory conditions	14bp deletion	*	-14/ -14 bp associated with efficacy (50;51) . No effect on efficacy (52)

Table 1. Pharmacogenetic association studies of methotrexate with treatment outcome in rheumatoid arthritis

* = No information on association(s) with specific efficacy or toxicity was present regarding this SNP under study
Abbreviations and accessory full names of formal genes can be relocated in the NCBI gene database

Because, it is thought that MTX has an influence on adenosine pathway, SNPs within genes coding for *AMPD1*, *ATIC*, *MTR*, *MTRR*, *ITPA* were correlated with treatment outcome in several studies (36;40;47;53). Our group identified significant associations with clinical response, defined as an absolute value of DAS of less than 2.4, and the SNPs *AMPD1* 34C>T, *ATIC* 347C>G, *ITPA* 94C>A. In the toxicity analysis, only *ATIC* GG was associated with toxicity (53).
In general, several studies demonstrated no effect of *MTR* and *MTRR* on MTX efficacy (40;49;53). Regarding toxicity, in only one study (49) a relation between *MTR* 2756A>G and *MTRR* 66A>G and toxicity in a small group of patients was seen. However in two previously performed studies this was not reported (40;53). Since, the anti-inflammatory effects of adenosine are mediated by adenosine receptors, one group studied polymorphisms in genes coding for the adenosine receptor (*ADORA2A*) in relation with MTX therapy outcome. Five SNPs, were reported to be associated with adverse events on MTX. Specifically, two SNPs were related with gastrointestinal side effects (55) (Table 1).
Several nongenetic factors have been reported to influence efficacy of MTX treatment over the last years. These factors include demographic, life-style and clinical determinants such as disease activity at baseline, gender and smoking. Still, associations of these factors have not been translated into clinical tools in order to guide MTX treatment in RA patients. However, recently, a pharmacogenetic model in combination with clinical factors to predict MTX efficacy in recent-onset RA was developed (39). In this study it was reported that the clinical factors gender, rheumatoid factor combined with smoking status and disease activity at baseline were predictive for MTX response. The included genetic factors were the SNPs *AMPD1* C>T, *ATIC* 347C>G, *ITPA* 94 A>C and *MTHFD* 1958G>A. The prediction resulted in the classification of 60% of the RA patients into MTX responders and nonresponders, with 95% and 86% as true positive and negative response rates, respectively. Evaluation of this predictive model in a second group of 38 RA patients supported our results (39). Still, this model needs further prospective validation before its implementation in clinical practice.

Conclusion

MTX has been demonstrated to be effective drugs in the treatment of RA. Still, various percentages in efficacy and toxicity are seen. Unfortunately, these interindividual differences cannot be predicted in individual patients and markers such as polymorphisms, are necessary to individualize and optimize drug treatment. Yet, most pharmacogenetic studies performed have an insufficient sample size (power) to detect true associations with treatment response. In addition, other factors, like non-genetic factors, ethnicity and clear endpoints, influence treatment outcome. Particularly, disease activity score (DAS) at baseline determines to a large extend the response of RA patients treated with DMARD therapy as was demonstrated from previous studies. Also regarding clear endpoints, various use of disease activity parameters and/or cutoff levels for the definition of response, e.g. elevated liver enzyme levels in the case of side effects and an absolute value of DAS in the case of efficacy, may contribute to different results. In order to optimally compare studies or perform meta-analyses, criteria regarding efficacy and toxicity should be standardized. Finally, opposite or alternative results found may be explained by differences in SNP allele frequencies between various ethnic populations, which makes these association studies unlikely to compare.
Therefore, definitive conclusions about the role of genetic prognostic factors in treatment outcome to MTX cannot be drawn. Large randomized prospective studies are required to effectively replicate and validate these findings, before a pharmacogenetic approach is applicable in daily clinical practice.

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Chapter 3:

Relationship between genetic variants in the adenosine pathway and outcome of methotrexate treatment in patients with recent-onset rheumatoid arthritis

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Abstract

Objective.

Among patients with rheumatoid arthritis (RA), there is a high degree of interindividual variability in the degree of response to methotrexate (MTX) treatment. This study was undertaken to explore polymorphisms in genes contributing to anti-inflammatory adenosine release as novel predictors of MTX treatment outcome.

Methods.

In 205 patients with newly diagnosed RA, 5 polymorphisms in 5 genes coding for enzymes related to the release of adenosine were analyzed. All patients received standardized MTX treatment (up to 25 mg per week orally), combined with folic acid. MTX efficacy was evaluated by the Disease Activity Score (DAS) and compared among genotypes. The association between MTX-related adverse events and genotype was also assessed. The following polymorphisms were determined: *AMPD1* 34C>T, *ATIC* 347C>G, *ITPA* 94C>A, *MTR* 2756A>G, and *MTRR* 66A>G. When significant differences were found by chi-square analysis, odds ratios (ORs) and 95% confidence intervals were calculated.

Results.

Patients carrying the *AMPD1* 34T allele, *ATIC* 347CC, or *ITPA* 94CC were more likely to have a good clinical response, as defined by a DAS of <2,4 (OR [95% confidence interval] 2,1 [1,0–4,5], 2,5 [1,3–4,7], and 2,7 [1,1–8.1], respectively). The likelihood of a good clinical response was increased if patients possessed all 3 favorable genotypes (OR 27.8 [95% confidence interval 3,2–250]). Regarding toxicity, only *ATIC* G allele carriers experienced a greater frequency of adverse events (OR 2,0 [95% confidence interval 1,1–3,7]).

Conclusion.

Polymorphisms in the *AMPD1, ATIC*, and *ITPA* genes are associated with good clinical response to MTX treatment. These findings indicate that genotyping may help in the identification of patients who will benefit most from MTX treatment and may assist clinicians in making treatment decisions regarding patients with recent-onset RA.

Introduction

Patients with rheumatoid arthritis (RA) show considerable variation in their clinical course and response to treatment (1,2). Despite the fact that most clinical study findings support the use of combination therapy to optimally suppress disease activity, most patients with newly diagnosed RA begin with monotherapy; methotrexate (MTX) is the preferred first-line disease-modifying anti-rheumatic drug (DMARD) (3–6).

Although results of randomized controlled clinical trials indicate that MTX alters the clinical course of RA, only ~40% of the patients exhibit a good clinical response (7–9). While achieving good response early in the disease process is key to minimizing the joint damage and functional decline characteristic of RA (6,10,11), it is not yet possible to predict which patients will respond to MTX. In most studies to date that have demonstrated MTX efficacy, predictors for response have not been specifically investigated. Clear predictors of response to MTX would be useful in directing treatment choices in the early phase of the disease.

In candidate gene–driven pharmacogenetic studies, polymorphisms in genes coding for proteins involved in pharmacokinetic or pharmacodynamic pathways related to the drug under study are selected, and possible associations with treatment outcome are investigated (12–14). Specific to MTX, several studies have shown that single-nucleotide polymorphisms (SNPs) in genes coding for the folate pathway enzymes are associated with treatment response (15–17). Although MTX may act in RA through inhibition of folate pathway enzymes, more recent reports indicate that its efficacy may be related to the release of endogenous anti-inflammatory adenosine (18–20) (Figure 1).

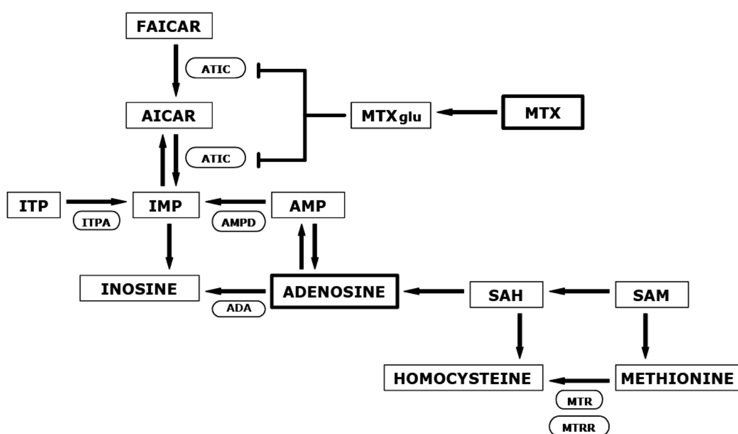

Figure 1. Simplified representation of the adenosine metabolism pathway[a,b,c]

a. Shown are enzymes and metabolites involved in the stepwise release of adenosine.
b. Abbreviation(s): FAICAR= formyl–5-aminoimidazole-4-carboxamide ribonucleotide, ITPA= inosine triphosphate pyrophosphatase, IMP= inosine monophosphate, ATIC= aminoimidazole carboxamide ribonucleotide transformylase, MTXglu= methotrexate polyglutamate, AMPD= adenosine monophosphate deaminase, ADA= adenosine deaminase, SAH= S-adenosylhomocysteine, SAM= S-adenosylmethionine, MTRR= methionine synthase reductase.
c. See ref. 18 for detailed information on the mechanism of action of MTX.

Studies on clinical outcome in patients with other complex conditions such as cardiovascular diseases have already alluded to the relevance of polymorphisms in genes coding for enzymes related to adenosine release (15,21–25). We hypothesized that genetic variants in these genes are associated with MTX treatment outcome. To investigate this, we assessed the relationship between SNPs in genes related to adenosine release and MTX treatment outcome in patients with recent-onset RA.

Patients and Methods

Role of the funding source

The rheumatologists participating in the Foundation for Applied Rheumatology Research were responsible for the study design and data collection in the BeSt study. The authors are responsible for the current subcohort data analysis, including genotyping, interpretation of data, preparing this manuscript, and the decision to publish. Centocor and Schering-Plough did not participate in any of these activities.

Patients

The 247 patients enrolled in this study comprised a subcohort of the 508 patients participating in the BeSt (Behandelstrategieën voor Reumatoide Artritis [Treatment Strategies for Rheumatoid Arthritis]) study (26). Inclusion criteria for the study included fulfillment of the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for RA (27), age of ≥18 years, and disease duration of <2 years. Patients also had to have active disease, defined as at least 6 swollen joints (of 66) and at least 6 tender joints (of 68), and either an erythrocyte sedimentation rate (ESR) of ≥28 mm/hour or a score of >20 mm on a 100-mm visual analog scale (VAS) for patient assessment of global health (0 mm = best; 100 mm = worst). Individuals were ineligible for the BeSt study if they had previously been treated with DMARDs other than antimalarial agents or were receiving concomitant treatment with an experimental drug. The local ethics committee at each participating hospital approved the study protocol, and all patients provided written informed consent before enrollment into the study.

Study design

The BeSt study was a randomized, multicenter, single-blind, clinical study comparing the clinical efficacy of 4 different treatment strategies in early RA: sequential monotherapy starting with MTX (n = 126), step-up from MTX to combination therapy with MTX and sulfasalazine (SSZ) (n = 121), initial combination therapy with MTX, SSZ, and high-dose (with tapering) prednisolone (n = 133), or initial biologic therapy with infliximab plus MTX (n = 128). Only patients who had been allocated to single use of MTX (n = 247) were included in the current analysis.

The primary goal of therapy in the BeSt study was clinical response as defined by a European League Against Rheumatism (EULAR) Disease Activity Score (DAS) of ≤2.4 (28,29). The DAS is a validated composite outcome measure consisting of the Ritchie Articular Index (RAI) (30), the number of swollen joints (of 44), general well-being as indicated by the patient on a VAS, and the ESR. A research nurse who was blinded with regard to the allocated treatment group assessed the DAS every 3 months.

All patients included in this analysis started on a regimen of oral MTX 7.5 mg weekly, increasing to 15 mg weekly after 4 weeks, in combination with folic acid (1 mg per day). In the event of insufficient clinical response (DAS ≤2.4) at the 3-month followup visit, the MTX dosage was increased stepwise to 25 mg weekly, given either orally or parenterally according to the rheumatologist's judgment. If the clinical response remained insufficient at the 6-month followup visit, patients were treated ac-

cording to the next step of the BeSt protocol, i.e., patients assigned to MTX sequential monotherapy were switched to SSZ 1,000 mg twice daily, and SSZ 1,000 mg twice daily was added to the MTX regimen for patients assigned to initial step-up combination therapy. Concomitant treatment with nonsteroidal antiinflammatory drugs and intraarticular injections of corticosteroids were allowed for all treatment groups. For the current analysis, clinical data from the first 6 months of followup were used to represent MTX treatment only. Responders were defined as patients with a DAS of ≤2.4 (good clinical response) based on the EULAR response criteria (28,29), and nonresponders as patients with a DAS of ≤2.4 at the 6-month followup visit.

Toxicity was evaluated by tabulating reported adverse drug events. Adverse drug events were spontaneously reported by the patients, were ascertained from nonspecific questioning by the investigator about the patient's well-being, or were found upon physical examination or determination of clinical laboratory parameters during the study. In cases of adverse drug events, MTX treatment was continued at the lowest tolerated dose or, if MTX was not tolerated at all, the DMARD therapy was changed. The following noninfectious adverse drug events were specifically evaluated: gastrointestinal adverse drug events (defined as general well-being, nausea, vomiting, diarrhea, or constipation); liver adverse drug events (defined as elevated liver enzyme levels resulting in MTX dosage adjustment or discontinuation), pneumonitis, and skin and mucosal disorders. Patients were also monitored for leukopenia (white blood cell count <4 x 10^9/liter) and for elevations in levels of alanine aminotransferase and alkaline phosphatase to >3 times the upper limit of normal (i.e., >135 units/liter and >360 units/liter, respectively).

Five SNPs in genes related to adenosine release (31) (Figure 1) were selected, taking into consideration the following criteria: validated SNP, SNP causes nonsynonymous amino acid change, indications for clinical relevance from previous publications (15,21–25,32,33), and a preferred minimal genotype frequency of ~10%. The 5 selected genes were those coding for adenosine monophosphate deaminase (*AMPD1*), aminoimidazole carboxamide ribonucleotide transformylase (*ATIC*), inosine triphosphate pyrophosphatase (*ITPA*), methionine synthase (*MTR*), and methionine synthase reductase (*MTRR*). The following SNPs were analyzed: *MTRR* 66A>G
(rs1801394), *MTR* 2756A>G (rs1805087), *AMPD1* 34C>T (rs17602729), *ITPA* 94C>A (rs1127354), and *ATIC* 347C>G (rs2372536).

DNA was isolated from peripheral white blood cells by a standard manual salting-out method. As a quality control, positive controls (Control DNA CEPH 347-02; Applied Biosystems, Foster City, CA) and negative controls (water) were used. In addition, 5–10% of samples were genotyped in duplicate, and no inconsistencies were observed.

Genotyping was performed using real-time polymerase chain reaction with TaqMan, according to the protocol provided by the manufacturer (Applied Biosystems). Genotype frequencies were in Hardy-Weinberg equilibrium, and the success rate was 99.5% for *MTRR* 66A>G, 100% for *MTR* 2756A>G, 99.5% for *AMPD1* 34C>T, 99.5% for *ITPA* 94C>A, and 100% for *ATIC* 347C>G. Genotype distributions were as follows: for *AMPD1* 34C>T, 74% CC, 25% CT, 1% TT; for *MTRR* 66A>G, 20% AA, 53% AG, 28% GG; for *MTR* 2756A>G, 70% AA, 27% AG, 2% GG; for *ITPA* 94C>A, 85% CC, 15% CA, 0% AA; and for *ATIC* 347C>G, 47% CC, 45% CG, 8%GG.

Statistical analysis.

Differences in baseline characteristics were analyzed by Student's *t*-test for continuous variables or chi-square test for dichotomous variables. For response and toxicity, differences in genotype distribution were tested by 3 x 2 cross-tabulations for each genotype, and by 2 x 2 cross-tabulations for carriers versus noncarriers, with analysis by 2-sided chi-square test. When genotype distributions differed, we used binary logistic analysis to calculate odds ratios

(ORs) for achieving good response or experiencing adverse drug events. Age and sex were identified as possible confounders and were used as covariates in all regression analyses. The primary efficacy end point was good clinical response (DAS ≤2.4) at 6 months. For classification as having good clinical response based on the DAS, patients had to be available for evaluation at a given time point; no values were carried forward. Secondary end points were good clinical improvement, defined as a change of >1.2 in the DAS, and moderate clinical improvement, defined as a change of >0.6 in the DAS. Additionally, for efficacy analyses, the following possible confounding factors were identified: DAS at baseline, duration of joint symptoms before enrollment, duration of RA before enrollment, rheumatoid factor (RF) positivity, modified Sharp/van der Heijde radiographic score (34) at baseline, ESR, RAI, and C-reactive protein level.

For safety analyses, all patients whose MTX regimen was altered prior to the 6-month followup visit were assessed for adverse drug events after the change in therapy and were included in the safety analyses. Analyses of laboratory measurements were performed for completers only. In the toxicity regression analysis, the following potential confounding factors were tested: body weight, creatinine clearance rate, MTX dosage group (15 mg/week or 25 mg/week), and alcohol use.

All statistical analyses were performed using SPSS 11.5 software (SPSS, Chicago, IL). Since 5 hypotheses were tested, Bonferroni adjustment was performed for multiple comparisons. Both adjusted and unadjusted *P* values were calculated. *P* values less than 0.05 were considered significant.

Results

Patient disposition and baseline characteristics.

DNA samples could be obtained from 205 of the 247 patients randomized to receive MTX monotherapy in the BeSt study. There were no statistically significant differences in baseline characteristics between patients with and those without available DNA samples (data not shown). Baseline demographic and disease characteristics of the 205 RA patients who were genotyped are presented in Table 1. The reported ethnicity distribution in the study population was 93% Caucasian (n = 191), 2.4% Asian (n = 5), 1.0% African (n = 2), and 3.4% other (n = 3 Hindustani, 3 Surinamese, 1 Israeli). All results remained similar when performed with and without inclusion of non-Caucasian patients.

Characteristics	Baseline value
Demographic	
Gender [female / male %]	68.8 / 31.2
Age [years] (sd)	54.6 (±13.3)
RF positivity [%]	67.3
Disease duration in weeks [median] (range)	2.0 (0-104.7)
Measures of disease activity	
Duration of joint complaints in weeks [median] (range)	25.0 (1.1-584.3)
DAS (sd)	4.5 (± 0.8)
ESR [median mm/hr] (range)	38 (2 - 143)
CRP [median mg/L] (range)	23 (0 - 238)
RAI [median] (range)	13 (2 - 47)

Swollen joints [median] (range)	13 (3 - 36)
Sharp van der Heijde score [median] (range)	4 (4 - 49.5)

Table 1. Baseline demographic and disease characteristics among the 205 patients with genotyping data

Abbreviation(s): DAS= Disease Activity Score in 44 joints, ESR= Erythrocyte Sedimentation Rate, RF= Rheumatoid factor, CRP= C-reactive protein, RAI=Ritchie Articular Index.

Association of *AMPD1* 34C>T, *ATIC* 347C>G, and *ITPA* 94C>A polymorphisms with good clinical response to MTX therapy.

At 6 months, 186 patients remained in the study, of whom 47% had a good clinical response (DAS ≤2.4) (n = 87) (Figure 2). Among these responders, 43% were receiving MTX 15 mg weekly and 57% were receiving MTX 25 mg weekly.
Three of the 5 selected genetic polymorphisms were associated with good clinical response at 6-month followup (Figure 3). Patients carrying the *AMPD1* T allele were 2)1 times more likely to achieve good clinical response when compared with patients possessing the *AMPD1* CC variant. For *ATIC* and *ITPA,* associations between the CC genotype and good clinical response were found (Figure 3). The numbers and percentages of responders by genotype are presented in Table 2).

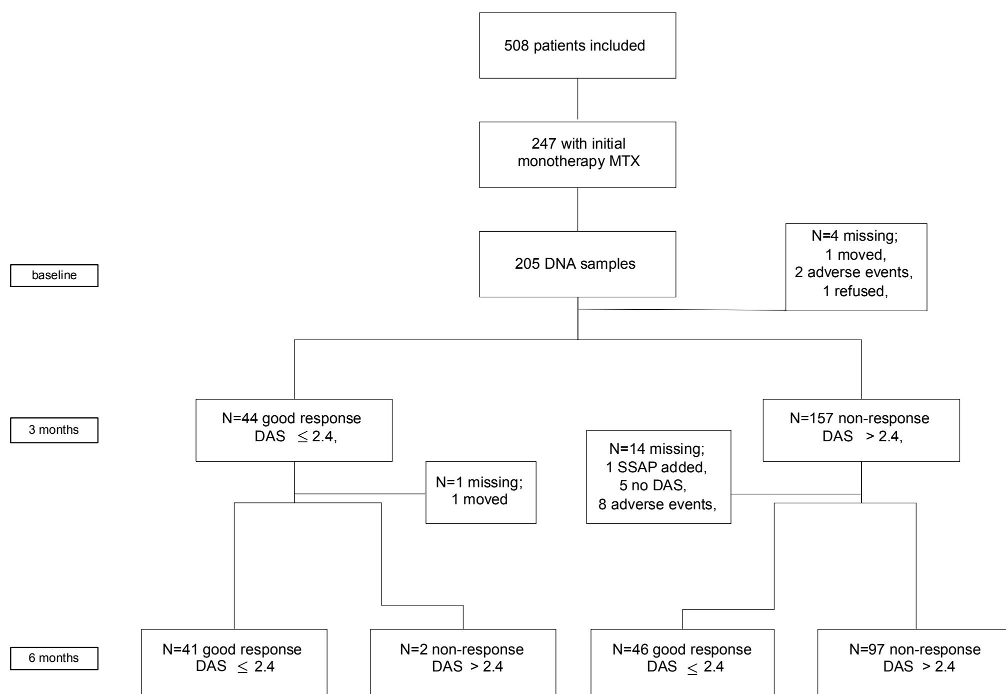

Figure 2. Disposition of the patients

Abbreviation(s): MTX= methotrexate; DAS= Disease Activity Score; SSAP= sulfasalazine.

To assess whether these 3 favorable polymorphisms showed an additive effect with regard to response to MTX therapy, additional analyses were performed for each combination of the *AMPD1*, *ATIC*, and *ITPA* genotypes. Among patients carrying the combinations *AMPD1* T allele and *ATIC* CC (n = 22), *AMPD1* T allele and *ITPA* CC (n = 41), and *ATIC* CC and *ITPA* CC (n = 82), the percentages with good clinical response at 6 months increased to 68%, 63%, and 56%, respectively. Among the 16 patients who carried all 3 favorable genotypes, 88% achieved a good clinical response. Logistic regression analyses revealed that the OR for achievement of good clinical response in this group was 27.8) The explained variance (R^2) of these combined favorable genotypes for MTX treatment response was 24.2% (Figure 3). In contrast, if patients carried all 3 unfavorable genotypes, i.e., the *AMPD1* CC and *ITPA* CA genotypes and the *ATIC* G allele (n = 10), the response rate at 6 months was only 10%.

	AMPD1			*ATIC*			*ITPA*	
	CC	**CT**	**TT**	**CC**	**CG**	**GG**	**CC**	**CA**
Population	151 (73%)	50 (24%)	3 (2%)	97 (47%)	92 (45%)	16 (8%)	174 (85%)	30 (15%)
Good clinical response at six months	57 (38%)	28 (56%)	1 (33%)	51 (53%)	30 (33%)	6 (38%)	79 (45%)	7 (23%)
Methotrexate 15 mg weekly	25/36 (69%)	15/15 (100%)	1/1 (100%)	22/25 (88%)	15/22 (68%)	4/5 (80%)	38/47 (81%)	3/5 (60%)
Methotrexate 25 mg weekly	30/98 (31%)	13/29 (45%)	0/2 (0%)	28/61 (46%)	14/60 (23%)	2/9 (22%)	39/107 (36%)	4/22 (18%)
Adverse drug events at six months	42/146 (29%)	16/50 (32%)	1/3 (33%)	21/94 (22%)	33/91 (36%)	6/15 (40%)	51/169 (30%)	8/30 (27%)

Table 2. Methotrexate response and adverse drug events at 6 months by AMPD1, ATIC and ITPA genotypes[a,b,c,d]

a. *MTR* and *MTRR* were not associated with methotrexate (MTX) efficacy or toxicity. Values are the number [%].
b. Genotype data missing on 1 of the 205 patients.
c. Data on MTX dosage missing on 2 of the 87 responders at 6 months.
d. Abbreviation(s): *AMPD1* = adenosine monophosphate deaminase, *ATIC* = aminoimidazole carboxamide ribonucleotide transformylase, *ITPA* = inosine triphosphate pyrophosphatase.

After adjustment for multiple comparisons, the association of the *ATIC* CC genotype with MTX response remained significant *(P* = 0.035), and the combination of favorable *AMPD1*, *ATIC*, and *ITPA* genotypes remained significantly associated with good clinical response (Figure 3). The regression analysis using the parameter good clinical improvement as opposed to good clinical response also revealed an association with the *ATIC* CC genotype in comparison with G allele carriers (OR 2.5 [95% confidence interval 1.3– 4.8], *P* = 0.007). No associations between the *MTRR* and *MTR* polymorphisms and good clinical response were found (data not shown).
In the regression analysis to predict good clinical response, only DAS at baseline and RF positivity appeared to be significant predictive factors (Figure 3). Patients who had a lower DAS at baseline and/or were RF negative were more likely to show good clinical response at 6 months. We also investigated whether the possible confounding factors were affected by genotype; no significant associations between the possible confounding factors examined and genotype variants were observed.

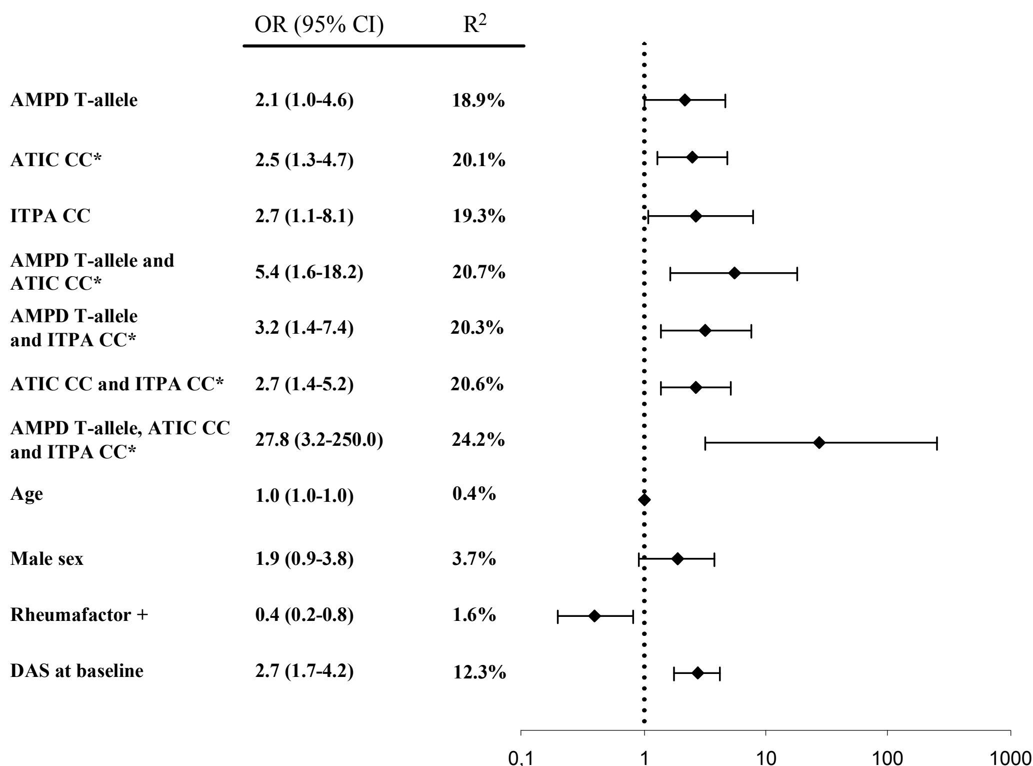

Figure 3. Associations between *AMPD1* 34C>T, *ATIC* 347C>G, and *ITPA* 94C>A polymorphism and good clinical response to methotrexate[a,b,c]

a. Data presented are odds ratios (ORs) (diamonds), 95% confidence intervals (95% CIs) (bars), and R^2 values with correction for the potential confounding factors of age, sex, rheumatoid factor (Rheumafactor) positivity, and Disease Activity Score (DAS) at baseline.
b. Odds ratios presented for age, sex, rheumatoid factor positivity, and DAS at baseline are results found without inclusion of genotypes as independent variables.
c. * $P< 0.05$ after Bonferroni adjustment.

Safety findings.

Safety data were available on 200 patients at 6 months; 4 patients did not return for the 6-month followup visit and 1 patient had moved away. Thirty percent of these patients (n = 60) experienced at least 1 adverse drug event during 6 months of treatment (Table 3). The percentage of patients experiencing an adverse drug event was similar in both dosage groups, although more patients receiving MTX 25 mg per week discontinued therapy due to adverse drug events.

During 6 months of treatment, patients carrying the *ATIC* G allele were twice as likely to experience any adverse drug event compared with patients without the allele (Figure 4). However, after adjustment for multiple comparisons, the association between the *ATIC* G allele and adverse drug events did not remain significant. No other associations with MTX-induced adverse events were identified. In the logistic regression analysis, none of the identified potential confounding factors was predictive of adverse drug events.

Adverse Drug Event	Frequency at 6 months
Skin and mucosa disorders	17 (8.5%)
Pneumonitis	0 (0%)
Hepatic	
elevated liverenzymes	16 (8%)
Gastrointestinal	
(general wellbeing, nausea, vomiting,diarrhoea, constipation)	26 (13.0%)
Overall adverse drug events in total population	60 (30%)

Table 3. Number of patients (percentage) with adverse drug events during six months of treatment

Values for overall adverse drug events are the number (%) of patients experiencing 1 event; values for the individual types of event are the number of events (% of patients).

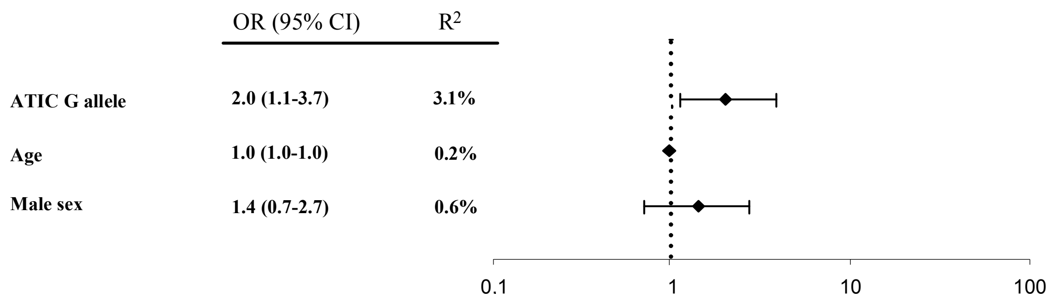

Figure 4. Association between *ATIC* 347C>G polymorphism and the occurrence of adverse drug events during 6 months of methotrexate therapy

Data presented are odds ratios (ORs) (diamonds), 95% confidence intervals (95% CIs) (bars), and R^2 values with correction for the potential confounding factors of age and sex.

We also examined the interaction between achievement of good clinical response (DAS ≤2.4) at 6 months, the *AMPD1*, *ATIC*, and *ITPA* genotypes, and the occurrence of adverse drug events. Responders at 6 months (n = 87) were selected, and regression analyses were performed. In general, patients with good clinical response at 6 months experienced fewer adverse drug events compared with nonresponders (OR 0.45, 95% confidence interval 0.22–0.91). This finding was also observed in nonresponders carrying the *ATIC* G allele; the OR of adverse drug events was increased from 2.0 to 2.8 (95% confidence interval 1.1–7.5) in this group.
For responders carrying the *AMPD1* T allele, the single *ATIC* CC or the single *ITPA* CC genotype, or combinations of these genotypes, no associations with the occurrence of adverse drug events were

found. The numbers and percentages of patients experiencing adverse drug events by genotype for *AMPD1*, *ATIC*, and *ITPA* are presented in Table 2)

Discussion

Results of this analysis show an association between allelic variants in the adenosine monophosphate deaminase (AMPD), 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, and inosine triphosphate pyrophosphatase (ITPA) genes and clinical response to MTX therapy in patients with recent-onset RA. Patients carrying the *AMPD1* T allele, the *ATIC* CC genotype, or the *ITPA* CC genotype are 2–3 times more likely to have a good clinical response, defined by a DAS of ≤2.4, following 6 months of MTX therapy. Additionally, the rate of good clinical response is increased substantially in patients carrying the 3 favorable genotypes.
With regard to the occurrence of adverse drug events, the only association found was with the *ATIC* G allele. This association was not significant after adjustment for multiple testing. No associations between methionine synthase or methionine synthase reductase and MTX efficacy or toxicity were found.
Previously, only the contribution of the *ATIC* 347C>G polymorphism has been studied in relation to the efficacy and safety ofMTX. In 2 articles, Dervieux et al report that RA patients with a higher mutation index respond better to MTX therapy (15,35). This composite mutation index was calculated for each patient by summing the scores for 3 SNPs in different genes, including *ATIC*. The patients with a higher mutation index showed a linear decline in the number of tender and swollen joints, the physician's global assessment of disease activity on a VAS, and the Health Assessment Questionnaire (36). Furthermore, it was suggested that patients with the *ATIC* 347GG genotype had an increased likelihood of response to MTX treatment. In addition, similar to our findings, Weisman et al showed that patients with the *ATIC* 347GG genotype more frequently experienced side effects overall, and specifically, gastrointestinal adverse drug events (37).
It is difficult to compare these findings with our results since study designs and data analysis differ. We chose to assess the contribution of genetic markers predictive of treatment outcome in the BeSt study because that study had clear and objective outcome measures and standardized treatment regimens in a well-described population of patients with recent-onset RA (26). Multivariate data analysis with Bonferroni adjustment for multiple comparisons was performed after 6 months of treatment, with controlling for identified confounders of response. Moreover, the selected patients were all treated with MTX monotherapy for an identical time period, and had not used any DMARDs prior to enrollment.
In contrast, other investigations have used crosssectional study designs with variable disease durations, MTX dosages, and treatment durations (15,35,37). In one study, combination DMARD therapy was allowed (37). Cross-sectional analyses reflect rheumatology practice, but population stratification may have occurred by selecting patients who are still being treated with MTX. With the design of the present study, the influence of sequential monotherapy and other possible confounders of treatment outcome is excluded.
The association of *ATIC* 347GG with side effects was established without controlling for confounders (37). The associations of clinical efficacy and overall toxicity with higher pharmacogenetic indexes were found in multivariate analysis in which other factors were included (15,35,37), but the composite mutation indexes used were calculated with grouping of different genotypes in 2 of the 3 studies. Moreover, the pharmacogenetic index calculation is based on the assumption that the contribution of every polymorphism is small, but that every polymorphism affects the response in the same direction with an equal, additive value. However, there are no data that support this assumption. In summary, different study designs and statistical methods should be taken into account in comparing results from different pharmacogenetic studies. We believe our results are more applicable to patients with recent-onset, non–DMARD-treated RA.

As with most genetic studies, the current study was not sufficiently powered to derive definitive conclusions. Further, while adjusting our results for multiple testing minimized false-positive associations, it also increased the chance of Type II error due to the conservative nature of the Bonferroni adjustment (38,39). Accordingly, we have presented both adjusted and unadjusted results.
Our primary efficacy parameter was good clinical response at 6 months of MTX treatment; in other reports, remission has been described as the primary goal of therapy (7,40,41). To examine whether the identified genotypes for good clinical response at 6 months were also predictive of remission at 1 year of followup, an additional analysis of patients carrying the *ATIC* CC genotype was performed. Results of this analysis showed that in 35% of the 97 patients carrying the *ATIC* CC genotype, disease was in remission, defined as a DAS <1.6, at 1 year; previous reports have indicated that remission has been achieved at 1 year in 10–25% of patients receiving MTX (8,42). This observation thus indicates that this variant may be associated with a prolonged and increased clinical response.
Our data showed that MTX therapy was less beneficial for *ATIC* G allele carriers, *ITPA* A allele carriers, and patients with the *AMPD1* CC genotype. While 47% of the overall population exhibited good clinical response at 6 months, comparison of good clinical response among allelic variants showed that the response percentages were 58% in patients with the *ATIC* CC genotype and 37% in *ATIC* G allele carriers. Also, good clinical response was achieved with 6 months of MTX therapy in 50% of the patients with the *ITPA* CC genotype compared with 26% of the *ITPA* A allele carriers, and by 60% of the *AMPD1* T allele carriers compared with 42% of the patients with the *AMPD1* CC genotype.
These findings suggest that pharmacogenetic testing before initiation of therapy may help to guide clinical treatment decisions, for example, in identifying patients with all 3 favorable genotypes, in whom MTX treatment is more likely to be efficacious. As another example of such clinical use, we analyzed the patients with all 3 unfavorable genotypes, i.e., the ATIC G allele, the ITPA A allele, and the AMPD1 CC genotype. In patients with these genotypes, other DMARD therapy may be chosen rather than MTX, because their response rate at 6 months was only 10%. Thus, such pharmacogenetic testing could avoid ineffective treatment and, at the same time, indicate high potential for effective therapy in 14% of the RA population.
Ideally, our findings regarding the effect of genetic variants in *AMPD1*, *ITPA*, and *ATIC* genes on MTX treatment outcome should be replicated and prospectively tested in a randomized controlled study comparing clinical response in 2 groups of patients (43,44). In such a study, patients in the first group would receive standard MTX treatment. In the second group, the pharmacogenetic test results would dictate whether patients receive standard MTX treatment (patients with the favorable genotypes) or other DMARDs (patients without the favorable genotypes).
The polymorphisms tested were selected based on the hypothesis that the mechanism of action of MTX is related to adenosine release (Figure 1). The enzymes whose genetic polymorphisms were studied relate to adenosine and were chosen because in vitro studies showed that polymorphisms altered their enzyme function or expression. Moreover, other reports have indicated the clinical relevance of these SNPs in different complex traits (15,21–25). Although the effect of variant alleles in relation to cellular adenosine homeostasis has not yet been explored, several in vitro effects have been shown (32,33,45–48).
Adenosine is thought to mediate the antirheumatic effects of MTX via adenosine receptor signaling (48–50). Binding of this compound to specific receptors enhances the antiinflammatory properties of MTX. The *AMPD1* 34C>T mutation generates an AMPD enzyme with lower activity (32). *AMPD1* catalyzes the conversion of AMP to inosine monophosphate (IMP). Alternatively, AMP is converted to adenosine. Thus, deficiency of *AMPD1* could enhance adenosine release. In addition, both *ITPA* and *ATIC* may lead to formation of adenosine. ITPA polymorphisms have been shown to lead to ITPA deficiency. ITPA catalyzes the conversion of ITP to IMP, whereas ITP is formed by phosphory-

lation of IMP. Deficiency of ITPA interrupts this cycle and possibly nfluences its balance with AMP and adenosine (33). Furthermore, MTX inhibits *ATIC*. This leads to cellular accumulation of AICAR, a nucleoside precursor (18,24). AICAR inhibits adenosine deaminase, which results in reduced conversion of adenosine to inosine.

Since understanding of these enzymes, their substrates, and interactions remains imprecise, no conclusions about the mechanism of action of MTX in relation to adenosine release can be drawn. Nevertheless, our results strongly indicate that MTX therapy works via the adenosine pathway. Moreover, we have confirmed that the genetic profile of RA patients is indeed a determinant of response to MTX treatment (15,16,45).

In summary, results of this analysis identify patients with adenosine genotypes who are most likely to achieve good clinical response with MTX. Findings of our pharmacogenetic analysis identified markers in the *ATIC*, *ITPA*, and *AMPD1* genes that may assist the rheumatologist in making clinical treatment decisions for patients with recent-onset RA.

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Chapter 4

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

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Abstract

Objective

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

Methods

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

Results

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

Conclusion

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

Introduction

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

Patients and methods

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

Study design and treatment.

Patients who were allocated to initial monotherapy with MTX (n=247) and for whom DNA samples were available (n= 205) were included in the current analysis. The primary goal, regarding efficacy of therapy in the BeSt study (16) groups was a clinical response as defined by the Disease Activity Score (DAS) of ≤2.4 at 6 months (17;18). The DAS is a validated composite outcome measure consisting of the Ritchie articular index (RAI) (19), the swollen joint count (SJC), general well-being as indicated by the patient on a VAS and the ESR. A research nurse who was blinded to the allocated treatment group assessed the DAS.

All patients included in this analysis began treatment with a regimen of oral MTX 7.5 mg weekly, with the dosage increasing to 15 mg weekly after 4 weeks, in combination with folic acid (1 mg daily). In the event of insufficient clinical response (DAS >2.4) at the 3-month follow up visit, the MTX dosage was increased stepwise from 5 mg every 2 weeks to 25 mg weekly. In case of intolerance, MTX could also be given by parenteral route of administration. In case of adverse drug events, MTX was continued at the highest tolerated dose. If MTX was not tolerated at all, the patient was treated with the next DMARD according to the BeSt protocol. Concomitant therapies with nonsteroidal anti-inflammatory drugs as well as intra-articular injections with corticosteroids were allowed for all treatment groups.

Evaluation of clinical efficacy and toxicity

Responders were defined as patients who were receiving MTX and had a DAS of ≤2.4 at 6 months (good clinical response). Nonresponders were defined as patients who were receiving MTX and had a DAS of >2.4. A total of 186 patients were available for the analysis of associations between selected SNPs and response to MTX at 6 months (16). Patients who experienced adverse events but continued to be treated with MTX at 6 months were included in the efficacy analysis. Baseline variables possibly influencing the patient's disease state and MTX response were previously selected on the basis of literature (20).

Toxicity was evaluated by counting each reported adverse drug event (ADE) and its consequences for the patient and treatment. Adverse drug events were spontaneously reported by the patients, or were reported as a result of non-specific questioning on patients' wellbeing by the investigator, by physical examination or laboratory measurements during follow up. In case of adverse drug events, MTX was continued at the lowest tolerated dose, or if MTX was not tolerated at all, the rheumatologists adjusted DMARD therapy according to the protocol. Of all reported ADEs, the following non-infectious adverse drug events were evaluated explicitly: gastrointestinal adverse drug events defined as patients' general wellbeing, nausea, vomiting, diarrhoea, and constipation; liver adverse drug events defined as all cases of elevated functional liverenzymes resulting in MTX dose adjustment or discontinuation; pneumonitis; skin and mucosal disorders. Moreover, patients were evaluated for leucopenia ($<4.10^9$/L), ALAT 3 times upper limit of normal (>135 U/L) and for alkaline phosphatase (AF) 3 times the upper limit of normal (>360 U/L). The analyses of laboratory measurements were performed for completers only.

Selection of SNPs

Seven nucleotide polymorphisms (SNPs) with proven functional consequences in seven candidate genes related to the mechanism of action of MTX and/or immunopathogenesis of RA were selected. Based on literature, the following criteria were taken into consideration: validated SNP, proven functional consequences of the variant (21-26) and indications for clinical relevance, as defined by previous associations with treatment outcome in RA (tables 1 and 3) (25;27-32).

The genetic polymorphisms included were in genes coding for dihydrofolate reductase (*DHFR*), multi-drug resistance-1 (*ABCB1*), inosine triphosphate pyrophosphatase (*ITPA*), human leukocyte

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

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

Gene	Role in relation with MTX and/or RA	Genetic variant	Hypothetical effect
ABCB1	Efflux transporter; (hypothetically) efflux of MTX	3435C>T	Decrease of mRNA stability and enzyme expression; May increase intracellular MTX levels (36-38)
DHFR	Conversion DHF to THF; target of MTX	-829C>T	Increase of enzyme expression leading to increased enzyme production and MTX resistance (21;22)
HLA-G	Downregulation of inflammatory process; involved in immune tolerance	->+14 bp	Decrease of HLA-G mRNA stability; Decrease of sHLA-G protein production (25;48)
IMPDH 2	Catalyzation of conversion IMP to XMP; involved in the purine metabolism	787C>T	Decrease of enzyme activity (24)
ITPA	Conversion ITP to IMP; involved in the purine metabolism; indirect target MTX	IVS2 +21A>C	Alteration of enzyme structure and Decrease of enzyme activity (26)
TGFB1	Involved in immunosuppression and inflammatory conditions	869T>C	May decrease or increase enzyme production (59-62)
TLR4	Mediation of secretion of inflammatory cytokines;	896A>G	Decrease of inflammatory cytokine; May associate with susceptibility to RA (53;55)

Table 1. Characteristics of functional genetic polymorphisms

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

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

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

Results

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

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

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

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

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

Functional polymorphisms in relation with toxicity

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

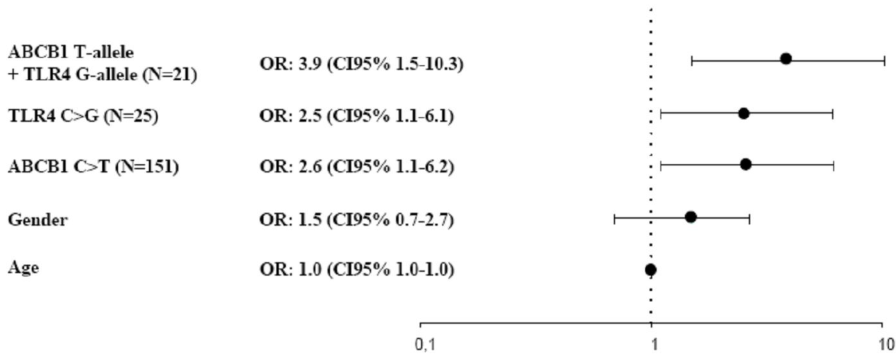

Figure 1. Genetic variants and occurrence of adverse drug events during 6 months of MTX therapy[a,b]

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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Chapter 5:

Cross-validation of a clinical pharmacogenetic model to predict the efficacy of MTX monotherapy in established RA

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Abstract

Objective
To cross-validate a clinical pharmacogenetic model to predict the efficacy of methotrexate (MTX) monotherapy in patients with established rheumatoid arthritis and previous failure of disease modifying antirheumatic drugs (DMARDs).

Methods
A clinical pharmacogenetic model developed in DMARD naive recent-onset RA patients, was applied in MTX naive RA patients and failure of other DMARDs. Patients from an RA inception cohort (N=593) were eligible if they fulfilled the ACR classification criteria for RA, received MTX monotherapy for at least 6 months and failed on at least one DMARD before MTX. Concomitant oral prednisone was allowed if the dose did not exceed 10 mg. Hundred-and-eighteen patients fulfilled these inclusion criteria, DNA and clinical data were available for 75 of those patients, and 71 patients were genotyped successfully. Risk scores for non-response as defined by DAS>2.4 at 6 months, were calculated using the pharmacogenetic model utilising eight baseline factors: gender, rheumatoid factor, smoking status, DAS, and four polymorphisms in the genes MTHFD1, AMPD1, ITPA and ATIC.

Results
The mean (SD) DAS at baseline was 3.55 (1.11). The time-averaged dose of MTX was 13.9 mg/week (range 5-25 mg/week); 48% of the patients used folic acid suppletion and 16% used prednisone. At 6 months, the DAS decreased with a mean (SD) of -0.72 (1.13) points ($p<0.0001$), resulting in 33% responders (n=25) and 67% non-responders (n=50). At baseline, risk scores ranged from 0 to 10.5. Seventy-five percent (56/75) of the patients could be categorized into predicted responders (risk score ≤ 3.5) and predicted non-responders (risk score ≥ 6) using the eight baseline variables. Comparison of the observed and predicted response at 6 months showed that the true positive response ratio was 47% (14/30) and the true negative response ratio was 81% (21/26).

Conclusion
The pharmacogenetic model predicts the efficacy of MTX monotherapy better in DMARD naive recent-onset RA patients than in patients with preceding DMARD failure. Updating the model is needed to improve its usefulness in patients with established RA and DMARD failure.

Introduction

Early treatment response in newly diagnosed rheumatoid arthritis (RA) patients is effective in decreasing functional disability and bone destruction (1,2). In addition, combination strategies of Disease Modifying Anti–Rheumatic Drugs (DMARDs), with or without anti-tumor necrosis factor (anti-TNF) agents, show higher response rates compared to monotherapy strategies in treating RA and considerably improve the prognosis (3,4).
Although combination strategies are more efficacious, such intensive approach is probably not necessary for all newly diagnosed RA patients since ~30 to 40% of the patients will experience a sustained good clinical response to methotrexate (MTX) monotherapy (5,6). To avoid unnecessary combination treatment, it is important to determine whether patients have a high probability of response to MTX monotherapy.
Recently a clinical pharmacogenetic model to predict response to MTX monotherapy has been developed in DMARD-naive patients with early onset RA (Table 1) (7). This model includes disease activity, gender, smoking and rheumatoid factor status next to four polymorphisms in genes related to the MTX mechanism of action and correctly predicts the response to MTX in early RA (7).
Next to MTX, other DMARDs such as sulphasalazine (SSZ) are still frequently used as a first DMARD for treating RA in daily clinical practice. Of note, the number of previous DMARDs and disease duration have been associated with reduced response to MTX (8,9). It is unclear whether the clinical pharmacogenetic model is still informative if MTX is intended after failure of another DMARD. In order to tailor treatment choices in RA, there is a need for prediction of response to MTX therapy in patients with established RA patients with previous DMARD failure.
The objective of this study was to cross-validate the clinical pharmacogenetic model with clinical and genetic factors to predict the efficacy of MTX monotherapy in established RA patients with previous DMARD failure.

Methods

Patients

Eligible patients were selected from the database of the inception cohort of early RA patients at the Department of Rheumatology of the Radboud University Nijmegen (10). Inclusion criteria of the cohort are: fulfillment of the ACR criteria for RA (ACR 1978), no use of DMARDs, a symptom duration < 1 year. Additional inclusion criteria for the present study were: use of MTX mono-therapy for at least 6 months, no use of oral prednisone > 10mg/day during the first 6 months of the MTX course, treatment with at least one other DMARD previously, availability of clinical data at start of MTX and after 6 months comprising the Disease Activity Score (DAS), and availability of a DNA sample.

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

Table 1. The original clinical pharmacogenetic model to predict response to MTX monotherapy

Abbreviation(s): RF = Rheumatoid Factor; DAS = Disease Activity Score; *AMPD1*= adenosine monophosphate deaminase; *ATIC*= aminoimidazole, carboxamide ribonucleotide transformylase; *ITPA*= inosine triphosphate pyrophosphatase; *MTHFD1*= methylenetetrahydrofolate dehydrogenase

RA cohort

Patients in the Nijmegen RA inception cohort were regularly assessed regarding disease activity and medication use (10). Medication and clinical information was registered in a computerized database. DNA had been collected in an unselected subsample of patients. At the time of patient selection for the present study in 2007, there were N=593 patients included in the database. N=352 patients had used MTX at any time point; MTX was the first DMARD used in n=36 patients, and a total of n=151 patients had switched to MTX monotherapy after one or more previous DMARDs, most often SSZ. Out of those 151 patients on MTX monotherapy, 118 had used MTX for at least 6 months. For 76 of them, DNA and DAS values were available. One patient used oral prednisone > 10mg/day and was excluded. Consequently, genotyping was performed in 75 patients, and was complete in 71 patients. The dates of start of MTX of the included patients ranged uniformly from 1988 to 2006. There were no significant differences between patients that were included (n=75) and patients that were not included (n=76) regarding age, gender, rheumatoid factor, smoking, DAS at diagnosis and MTX dose.

Variables

Age and disease duration at the time of MTX initiation (baseline) were calculated. As menopausal status was unavailable, age categories of ≤55 and 55< were used as proxy. For Rheumatoid Factor (RF) positivity, the most recent value before baseline was used. Smoking status was defined as current smoker and nonsmoker at the time of diagnosis. Joint counts were assessed by trained research nurses, Visual Analogue Scales (VAS, 0-100 mm.) for general health and pain were filled in by the patient. ESR was determined using the Westergren method. The DAS was calculated using the Ritchie Articular Index (RAI), the 44 swollen joint count (SJC), ESR and general health, according to the original formula (11).

Treatment

All treatment decisions were to the discretion of the treating rheumatologist and the patient. Concomitant use of methylprednisolone and Non-steroidal Anti-inflammatory Drugs (NSAIDs) was allowed. Patients receiving oral prednisone > 10mg/day during the first 6 months of the MTX course were not included.

MTX response evaluation

For the purpose of the present study, the same response definition as in the previous study form Wessels et al. was used (7). Responders were defined as patients who were treated with MTX for 6 months and had a DAS≤2.4 at 6 months, nonresponders were defined as patients who were treated with MTX for 6 months but had a DAS>2.4 at 6 months (12).

Prediction rule

The variables of the prediction rule for the probability of non-response (DAS>2.4) after 6 months of MTX use were: gender, menopausal status, RF status, smoking status, DAS at baseline, and 4 polymorphisms in AMPD1, ATIC, ITPA, MTHFD1 (7). To arrive at a clinical useful prediction rule, weighted scores had been assigned by rounding the regression coefficients in the final prediction model. In the final model, menopausal status did not contribute anymore to the clinical prediction rule (7).

Genotyping

Genomic DNA was extracted from peripheral venous blood (10 ml/sample) according to standard protocols. All included patients were genotyped for SNPs in genes coding for adenosine monophosphate deaminase (*AMPD1*; rs17602729), aminoimidazole carboxamide ribonucleotide transformylase (*ATIC*; rs2372536), inosine triphosphate pyrophosphatase (*ITPA*; rs1127354) methylenetetrahydrofolate dehydrogenase (*MTHFD1*; rs17850560). Genotype frequencies of *AMPD1* (p=0.068), *ATIC* (p=0.22), *ITPA* (p=0.23), and *MTHFD1* (p=0.065) were not significantly different to the distributions as expected based on the HapMap-CEU sample (http://www.ncbi.nlm.nih.gov/sites/entrez). Genotype distributions were as follows: for *AMPD1* 34C>T, 68% CC, 31% CT, 1% TT; for *ATIC* 347C>G, 49% CC, 41% CG, 9% GG; for *ITPA* 94C>A, 80% CC, 20% CA; and for *MTHFD* 1958G>A, 23% GG, 59% GA, 18% AA. Hardy-Weinberg equilibrium was not calculated because of the small sample. Genotyping was performed using real-time polymerase chain reaction with TaqMan®, according to the protocol provided by the manufacturer (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands).

Statistical analysis

Responders and non-responders were compared at baseline regarding the clinical and genetic variables in the prediction model and regarding former medication use, using Student's t-test, Wilcoxons' test, Chi-square test or Fisher-Exact test, as appropriate.

No variable selection procedure was attempted for model building. The variables of the existing prediction formula were applied in a logistic regression model with MTX response (yes/no) as dependent variable, and the clinical variables (nongenetic model) and the clinical and genetic variables combined (pharmacogenetic model) as independent variables. The exponential of the regression coefficient, e^{β} is an estimate of the adjusted Odds Ratio. In congruence with the original publication (7), the probability of a non-response was modeled. Of both the nongenetic model and the pharmacogenetic model, discrimination was evaluated using the area-under-the-receiver-operating-characteristic-curve (c-statistic) and the variance explained (Nagelkerke R^2). The clinical prediction rule and the cut-points for nonresponse, intermediate, and response were applied as delineated in the original publication (7). The resulting clinical prediction scores were compared between responders and nonresponders using Student's t-test. Using observed and predicted responses the true positive and true negative response ratios were calculated. It was attempted to update the nongenetic and pharmacogenetic models by addition of the predefined variables: number of previous DMARDs and disease duration (8), and SSZ as previous DMARD.

All analyses were performed using SAS 8.02 (SAS Institute Inc, Cary, NC, USA).

Results

Description of the cohort

The starting dose of MTX of the included patients was in median 15 mg/week, ranging from 2.5-22.5 mg/week. After 6 months the median MTX dose was also 15 mg/week, with a range of 5-25 mg/week. The median time-averaged dose of MTX was 13.9 mg/week (range 5-25 mg/week). Of the included patients 36/75 (48%) used folic acid suppletion and 12/75 (16%) used concomitant oral prednisone in the allowed dose of ≤10 mg/day. The mean (SD) DAS at baseline was 3.55 (1.11). After 6 months, the DAS had significantly decreased with mean (SD) -0.72 (1.13) points ($p<0.0001$), resulting in 33% responders (n=25) with a DAS≤2.4 and 67% non-responders (n=50) with a DAS>2.4.

In table 2, baseline values of responders and nonresponders are compared. The prespecified variables for the pharmacogenetic model were: gender, menopausal status, Rheumatoid Factor positivity, smoking status, DAS at baseline, and 4 polymorphisms in AMPD1, ATIC, ITPA, MTHFD1 genes. Of these variables, only baseline DAS was significant at a level of $p<0.05$. Notably, none of the 4 genetic factors were statistically significant at that p-level. Moreover, of all variables tested, only RF positivity, the baseline DAS, the tender joint counts, pain, ESR and the number of previous DMARDs would satisfy a liberal selection criterion of $p<0.20$ for model building.

Baseline variable	Responders (N=25)	Non-responders (n=50)	P-value
Age	55 (13)	58 (14)	0.33
Age > 55	12 (48%)	30 (60%)	0.32
Female gender	15 (60%)	35 (70%)	0.39
RF positivity	41 (82%)	16 (64%)	0.09
Disease duration (months)	19 (7-49)	19 (5-43)	0.99
Current smoker	8 (32%)	15 (30%)	0.86
DAS baseline	3.1 (1.3)	3.8 (0.9)	0.01
SJC44	12 (5-18)	14 (10-18)	0.31
TJC53	4 (2-9)	11 (7-19)	0.002
RAI	5 (2-7)	8 (5-12)	0.0005
Pain VAS	37 (25)	48 (22)	0.07
General Health VAS	46 (25)	50 (24)	0.47
ESR	14 (7-36)	24 (13-44)	0.14
Folic acid use	10 (40%)	26 (52%)	0.33
Oral prednisone use	3 (12%)	9 (18%)	0.74
Number of previous DMARDs	1 (1-3)*	1 (1-4)*	0.15
Previous DMARDs>1	6 (24%)	20 (40%)	0.17
Previous DMARD was SSZ	21 (84%)	36 (72%)	0.25
MTHFD1 1985 AA genotype	4 (17%)	9 (18%)	0.99
AMPD1 34 CC genotype	14 (58%)	35 (73%)	0.21
ITPA 94 A-allele carrier	4 (16%)	11 (22%)	0.54
ATIC 347 G-allele carrier	13 (52%)	25 (50%)	0.87

Table 2. Baseline variables of responders and nonresponders

Abbreviation(s): RF = Rheumatoid Factor; DAS = Disease Activity Score; SJC44 = 44 swollen joint count; TJC53 = 53 tender joint count; RAI = Ritchie Articular Index, SJC44 and TJC53 both are based on the joints assessed in the RAI; VAS = visual analogue scale; SSZ=Sulphasalazine; *AMPD1*= adenosine monophosphate deaminase; *ATIC*= aminoimidazole, carboxamide ribonucleotide transformylase; *ITPA*= inosine triphosphate pyrophosphatase; *MTHFD1*= methylenetetrahydrofolate dehydrogenase.

Cross-validation of the clinical pharmacogenetic model

The variables entered in the logistic regression model to predict non-response to MTX at 6 months according to a DAS≤2.4 were prespecified by the existing prediction model (Table 3).

First, the nongenetic variables were simultaneously entered in a logistic regression model; next the genetic variables were added. The sample was too small for including interaction terms. According to the odds ratio's it is seen that the contribution of gender was small (OR near 1) and that smoking was inversely associated with non-response (OR below 1). Age, RF positivity and DAS at baseline were all predictive for non-response.

The genetic factors were not statistically significant, three of the genetic factors had a sign ('-') reversed to the expectation, the sign for AMPD1 was in agreement with the expectation according to the publication of Wessels et al. (7).

When the original clinical prediction rule was applied on the baseline variables, the resulting risk scores ranged from 0 to 10.5. The mean (SD) risk score was 5.1 (2.3) and 3.8 (2.7) for non-responders and responders, respectively. The difference between these scores was significant (p=0.03). Accordingly, 75% (56/75) of the patients could be correctly categorized into predicted responders (risk score ≤ 3.5) and predicted non-responders (risk score ≥ 6) using the eight baseline

variables of the pharmacogenetic model (Table 4). Comparison of the observed and predicted response at 6 months showed that the true positive response ratio was 47% (14/30) and the true negative response ratio was 81% (21/26).

	Nongenetic model		Pharmacogenetic model	
Variable	**β**	**OR (95% CI)**	**β**	**OR (95% CI)**
Female gender	0.028	1.1 (0.3-3.3)	-0.12	0.8 (0.2-2.7)
Age ≥55	0.78	2.2 (0.7-6.7)	0.89	2.4 (0.7-8.0)
DAS at baseline	0.70	2.0 (1.2-3.5)	0.77	2.2 (1.2-4.0)
RF-positive	1.13	3.1 (0.8-11.5)	0.98	2.7 (0.7-11.0)
Smoking	-0.47	(0.6 (0.2-2.0)	-0.47	0.6 (0.2-2.2)
MTHFD1 1958 AA genotype	--	--	-0.31	0.7 (0.2-3.2)
AMPD1 34 CC genotype	--	--	0.34	1.4 (0.4-4.9)
ITPA 94 A-allele carrier	--	--	-0.21	0.8 (0.2-3.6)
ATIC 347G-allele carrier	--	--	-0.23	0.8 (0.3-2.4)

Table 3. Regression coefficients and odds ratio's of the logistic regression models to predict nonresponse to MTX

Abbreviation(s): RF = Rheumatoid Factor; DAS = Disease Activity Score; *AMPD1*= adenosine monophosphate deaminase; *ATIC*= aminoimidazole, carboxamide ribonucleotide transformylase; *ITPA*= inosine triphosphate pyrophosphatase; *MTHFD1*= methylenetetrahydrofolate dehydrogenase

	Predicted response		
Observed response	*Nonresponder*	*Intermediate*	*Responder*
Pharmacogenetic model			
Nonresponder	21	11	16
Responder	5	4	14
Nongenetic model			
Nonresponder	4	16	28
Responder	1	3	19

Table 4. Observed and predicted response after 6 months of MTX, using the original pharmacogenetic and nongenetic models.

Discriminatory performance of the nongenetic model and the pharmacogenetic model was studied using the area-under-the-ROC curve (Figure 1). The area-under-the-curve was 0.73 for the nongenetic model and 0.77 for the pharmacogenetic model. The variance explained (R^2) was 0.22 and 0.28 for the nongenetic and pharmacogenetics models, respectively. To assess the relative contribution of both models in predicting (DAS-) response in comparison to a very reduced model, a model including only baseline DAS was used. It showed an area-under-the-curve of 0.70 and an R^2 of 0.14.

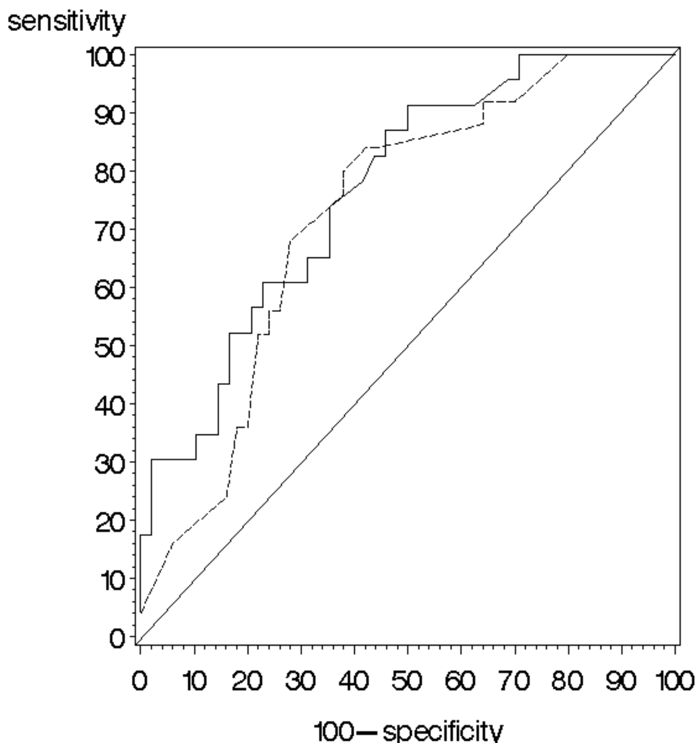

Figure 1. ROC curves for predicting the response to MTX using the original pharmacogenetic (solid line) and nongenetic (broken line) models.

Updating

Updating the clinical and pharmacogenetics models by removing the interactions of age and gender, and smoking and RF positivity, and adding the variables 'number of previous DMARDs' and 'SSZ as previous DMARD' did not improve the discriminatory performance (not shown). Disease duration was no candidate for uptake in an updated model, according to the results of the univariate analysis (Table 2).

Discussion

This study shows that the previously published clinical pharmacogenetic model to predict MTX mono-therapy efficacy in patients with established RA does not perform as good as in DMARD naïve patients with recent-onset RA. Previously it was found that the clinical pharmacogenetic model had a true positive and negative response rate of 95% and 86% respectively, whereas in the current cross-validation positive and negative response rates of only 47% and 81% were found. There are several possible explanations for these findings.

Clinical prediction rules developed in relatively small patient samples, which is not uncommon in medicine, generally perform less well in replication studies using independent samples. Therefore, cross-validation is necessary to assess the performance of clinical prediction rules in future patients (13). The original clinical pharmacogenetic model was developed in early RA patients who were DMARD naïve (7). In that same study, the model was validated in a small sample of early-onset DMARD-naïve RA patients from another hospital. In the current study, the model was applied in RA patients with established disease and a history of DMARD use, notably SSZ. By taking a different population, it was tested whether the model would be applicable for predicting MTX non-response in patients for whom MTX is not the first DMARD.

The MTX response in the present study was less in comparison with the MTX response in the study in which the clinical pharmacogenetic model was derived (7). Only one-third of the patients reached a response as defined by a DAS>2.4 at 6 months after start of MTX mono-therapy. These patients therefore appear to be more therapy-resistant, but also MTX dosages were lower than in the original study. Since MTX dosage was increased according to the discretion of the treating rheumatologist, the median MTX dosage of 15 mg/week at 6 months after initiation was less in comparison with recent tight control strategies in treating RA (2,14). Previously, it has been shown that tight disease control with increased MTX dosages, up to 30 mg/week, will lead to increased response rates even after re-employment after previous MTX failure (15-17). The clinical pharmacogenetic model was also based on a tight control strategy with increased dosages MTX, including folic acid suppletion (7). Therefore, it might be that MTX non-responders in our inception cohort would have been responders if treated with tight-control strategies instead of daily practice.

Yet, it also has been shown that RA patients failing previous DMARD monotherapy have an increased likelihood to fail on the next DMARD after switching (18,19). In this context, RA patients with longer disease duration do not respond as well to treatment compared with patients with early disease (9). Further, patients with DMARD failure are a subset of all patients. As a consequence, predictive factors for response may differ as well. In contrast with the original study, in the present study gender appeared to have no predictive value, and smoking appeared to be associated with response instead of non-response. The latter may have been caused by the fact that in the current study, smoking was only assessed at diagnosis, not at start of MTX which was in median 19 months later. Also, menopausal status was not assessed, but menopausal state is not of influence in calculating the risk score (7).

Interestingly, our results suggest that the clinical prediction model is capable to predict non-response to MTX also in patients with previous DMARD failure. This is despite the fact that the variables 'disease duration', 'number of previous DMARDs', and 'previous SSZ use' did not improve the clinical (nongenetic) model. The pharmacogenetic model showed no substantial improvement over the clinical model in this study. Failure on previous DMARDs, notably SSZ, could select patients into a subsample for which the proposed genetic factors are not discriminative. However, in our study there appeared to be no selection in direction of the less favorable genotypes, as the gene frequencies in this study were similar to the frequencies as expected based on the HapMap-CEU sample and similar to the frequencies as found by Wessels et al. earlier (7,20). Therefore, it remains

yet unclear whether the four selected genetic variants are true markers for MTX response (21,22). Fine mapping of these and other genes related to MTX treatment outcome is necessary in order to probably detect better pharmacogenetic factors for MTX response. Large collaborations between groups and continued collection of patients and data on MTX pharmacogenetics in RA are still needed in order to validate the pharmacogenetic model, in DMARD naïve early-onset RA treated with MTX according to a tight control strategy. In addition, the robustness of the pharmacogenetic model could also be tested by cross-validation in a larger population of patients again with established RA and previous DMARD failure, treated according to a tight control strategy.

In conclusion, the pharmacogenetic model predicts the efficacy of MTX monotherapy better in DMARD naive recent-onset RA patients than in patients with preceding DMARD failure. Updating the model is needed to improve its usefulness in patients with established RA and failure of previous DMARDs.

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Chapter 6

External Validation of the Clinical Pharmacogenetic Model for predicting MTX monotherapy efficacy using a Swedish Cohort of patients with recent-onset rheumatoid arthritis

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Abstract

Objective.

Previously, comparison of genetic, demographic and other clinical factors between methotrexate (MTX) responders and nonresponders has led to a clinical pharmacogenetic model including 4 genetic polymorphisms and 4 nongenetic factors to predict MTX therapy outcome. The aim of this study is to validate this predictive model in Swedish patients with recent-onset RA.

Methods

Genetic and nongenetic factors were collected in 355 patients participating in the Swefot clinical trial. All patients received up to 20 mg MTX monotherapy weekly for 3 to 4 months. After this treatment period, efficacy was evaluated by Disease Activity Score (DAS28). Based on individual scores calculated by factors compromising the prediction model, patients were classified as predicted responders, nonresponders and intermediate responders. Hereby, predicted response and observed response on MTX were compared by calculating accuracy, true negative and true positive predictive values (TNPV and TPPV) and by constructing receiver operating curves (ROC). Furthermore, predictive values of the original BeSt cohort, in which the predictive model was derived, and the validation cohort were compared.

Results

At baseline, no significant differences in frequencies of genetic and nongenetic factors compromising the predictive model were observed between this validation cohort and the BeSt cohort. With application of the model, the TNPV and TPPV observed in patients of the validation cohort, were significantly lower compared to the values observed in the original cohort (for TPPV 70% vs. 95%; for TNPV 68% vs. 86% respectively; all $p<0.05$). Regarding the number of classifiable patients, the level of accuracy of the model and the AUC, no significant differences were found between the two cohorts.

Conclusion

In this study, a pharmacogenetic model for predicting efficacy of MTX in patients with RA was validated. Our data imply that response prediction with this model is feasible in a substantial part of the patients. In order to implement this model in rheumatology clinical practice, additional replication and (ideally) performance of prospectively designed studies is eligible.

Introduction

Over the last two decades, several genetic risk factors for RA like *HLA-DRB1* (shared epitope) and *PTPN22* have been introduced (1;2). Besides the diagnostic ability of genetics, it has been demonstrated, although to a lesser extent, that genetics could also influence treatment outcome in RA patients (3-5). Intelligibly, a pharmacogenetic effect could be an explanation for the high variability of effective drug responses, up to 30-40%, between RA patients in large clinical trials (6;7).
Most genetic factors in relation to RA treatment outcome have been studied with MTX as the drug under study. Specifically, increasing knowledge about the hypothetical mechanism of MTX action and its role in the inflammatory pathway in RA has led to a substantial number of candidate genetic variants, mostly single nucleotide polymorphisms (SNPs), potentially influencing its efficacy in RA patients (8). However, genetics can not solely account for variability of effective drug response. Clinical and demographic factors, like disease activity- and rheumatoid factor (RF) status, have been shown to be related with treatment outcome (9-11). Still, only a part of the reported pharmacogenetic studies have also included nongenetic factors for the association with treatment outcome. However, in order to detect an individual or a synergystic effect of biomarkers on MTX response, genetic- and nongenetic factors should be analyzed collectively.
In a previous study of our group, comparison of genetic, demographic and other clinical factors between MTX responders and nonresponders led to a clinical pharmacogenetic model to predict MTX therapy outcome in a Dutch cohort of patients with early RA (12). This model demonstrated a true positive (predictive) rate of 95% and a true negative (predictive) rate of 86% and was able to categorize 60% of the patients into responders or non-responders defined as achieving DAS >2.4 and DAS ≤2.4 at 6 months, respectively. Despite the potential ability of the model to predict MTX efficacy, validation in larger cohorts is required before this pharmacogenetic model can assist rheumatologists in treatment-decision making. Therefore, the aim of this study is to validate the performance of this previously designed predictive model in an independent Swedish cohort of patients with recent-onset rheumatoid arthritis treated with MTX monotherapy (Swefot).

Patients and methods

RA patients

The 355 patients enrolled in this study are originated from a cohort participating in the “Swedish Pharmacotherapy” (Swefot) randomized clinical trial (13). In this trial, the addition of conventional DMARDs was compared with the addition of anti-TNFα drugs in patients with early rheumatoid arthritis (RA), who had failed initial treatment with MTX at 3 to 4 months.
Patients were recruited by rheumatologists in fifteen rheumatology units in Sweden, which collaborated in this study. Inclusion criteria contained a diagnosis of RA according to the revised ACR (formerly ARA) criteria; RA symptom duration of less than 1 year; no prior disease modifying anti-rheumatic drug (DMARD) therapy; no oral glucocorticoid therapy or stable glucocorticoid therapy for at least 4 weeks of at most 10 mg daily prednisolone (or equivalent); Disease Activity Score (DAS) based on 28-joint count of more than 3.2. Exclusion criteria were contraindications to any of the trial medications, and prior treatment with any DMARD. All patients gave written informed consent prior to inclusion.

Study design

In the Swefot trial, all patients (n=487) started with MTX monotherapy at an initial dose of 10 mg weekly. This dosage was increased biweekly by 5 mg increasing to a maximum of 20 mg weekly. Next to MTX, folic acid suppletion was prescribed in tablets of 5 mg to be taken 1-6 times weekly. In this study, monitoring of liver enzymes and blood counts was performed and abnormalities could lead to dose adjustments based on well-established clinical routines.

At a follow-up visit at least 3 and at most 4 months after the baseline visit, disease activity scores (DAS28) (14) were estimated. If patients were responders on MTX therapy (defined in the protocol as DAS28 <3.2), MTX was continued and patients were followed in usual care. If patients did not obtain response (defined as DAS28 >3.2), patients were randomized to either arm A (the addition of sulphasalazine plus hydroxychloroquine) or arm B (the addition of infliximab) according to the study protocol (REF Swefot study).

Patients who received MTX monotherapy and were evaluated at the follow-up visit at least 3 and at most 4 months after the baseline visit and for whom DNA samples were available (N=355) were included in the current analysis.

Clinical evaluation

The clinical pharmacogenetic predictive model was based on obtaining good clinical response as defined by DAS44 (12). Specifically, responders were defined as patients who were receiving MTX and had a DAS of ≤2.4 (good clinical response). Nonresponders were defined as patients who were receiving MTX and had a DAS of >2.4.

Notably, the original model is based on the DAS44 which includes a 44-joint count. In order to validate the predictive model in the SWEFOT data, main clinical endpoint for each patient in the Swefot cohort was converted: patient's DAS28 scores (based on 28 joints) were recalculated to DAS scores based on a 44-joint count (DAS44) using the transformation formula: DAS28= (1,072 X DAS44) + 0,938 (15).

Of the 355 patients included for the efficacy analysis, patients were unavailable for efficacy analysis due to lack of information about RF status (N=3), missing DAS at baseline and/or after 3-4 months (N=4) or due to incomplete genotype data (N=15). Consequently, remaining patients (N=333) were included for validation of the predictive model.

Genotyping

The standard method used for DNA extraction is modified salting-out method. SNPs for analysis in this study were selected according to the genetic polymorphisms previously included in the pharmacogenetic model (12). The selected SNPs were in genes coding for adenosine monophosphate deaminase (*AMPD1*), aminoimidazole, carboxamide ribonucleotide transformylase (*ATIC*), inosine triphosphate pyrophosphatase (*ITPA*) and methylenetetrahydrofolate dehydrogenase (*MTHFD1*). Specifically, these SNPs were analyzed: *AMPD1* 34C>T (rs17602729), *ITPA* 94A>C (rs1127354), *ATIC* 347C>G (rs2372536) and *MTHFD1* 1958G>A (rs17850560).

The method used for genotyping was TaqMan allelic Discrimination (Applied Biosystems, Foster City, U.S.A.). 5' Nuclease assay was performed according to a standard protocol in a 384-well format with 10 ng of DNA per sample. Detection of the final fluorescent signals from probes, which targeted alleles for each SNP, was performed at 7900 Sequence Detector (Applied Biosystems, Foster City, U.S.A.).

During the genotyping the following frequency distributions of these SNPs were observed: for *AMPD1* 34C>T, 74% CC, 24% CT, 2% TT; for *ITPA* 94A>C, 1% AA, 14% AC, 85% CC; for *ATIC* 347C>G, 47% CC, 42% CG, 11% GG and for *MTHFD1* 1958G>A, 27% GG, 49% GA, 24% AA. Genotype frequencies for all 4 SNPs were in Hardy-Weinberg equilibrium in this cohort ($p>0.05$). The

success rates for each SNP were as follows: for *AMPD1* 34C>T 99%; for *ITPA* 94A>C 98%, for *ATIC* 347C>G 99% and for *MTHFD1* 1958G>A 98%.

Statistical analysis

Swefot data were analysed for validation of the previously designed predictive model for MTX efficacy in the BeSt cohort (12). Briefly, reciprocal comparison in multivariate regression analyses of 17 polymorphisms and 24 nongenetic factors in the BeSt cohort led to a predictive model for MTX efficacy. This model consisted of gender, RF and smoking status, the DAS at baseline, and 4 SNPs in the *AMPD1, ATIC, ITPA,* and *MTHFD1* genes. Each patient was scored based on the regression coefficients of the independent variables and categorized in three groups: patient with scores of ≤3.5 (predicted response), patients with scores between 3.5 and 6 (predicted intermediate response) and patients with scores of ≥6 (predicted non-response). Additionally, a nongenetic model was developed based on gender, RF and smoking status, the DAS at baseline, which led to a subdivision of patients based on achieving a score of ≤2 (predicted responder), a score of >2 but <5.5 (intermediate predicted responder) and a score of ≥5.5 (predicted nonresponder).

For initial analysis in this study, baseline factors (included the prediction model) between patients in the BeSt cohort and the validation cohort were compared using chi-square test. Next, Swefot patients were individually scored based on the sum of points obtained by each baseline factor included in the original predictive model. Based on these calculated scores, patients were classified in one of the three groups of predicted response according to the original pharmacogenetic- and nongenetic model. Predicted response and observed response on were compared by calculating true negative and true positive predictive values (TNPV and TPPV) and levels of accuracy. Specifically, accuracy was calculated by the proportion of true results (the number of true positives en negatives) in the patient population.

Additionally, receiver operating characteristic (ROC) curves were constructed to evaluate the discriminative performance of the pharmacogenetic model in comparison with the nongenetic model in the SWEFOT cohort. Finally, levels of accuracy, ROC curves and TNPV and TPPV between the BeSt cohort and the validation cohort were compared using a chi-square test.

Notably, due to the absence of information on smoking status in the Swefot cohort, smoking status per individual could not be applied in the pharmacogenetic model. In this way, the maximum number of points which could be obtained was 10.5 (Table 1). Also, due to this absence the nongenetic model could not be optimally tested in the Swedish validation cohort and was therefore left out of the analysis.

All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics

The response of the included RA patients (N=333) at 3-4 months after start with treatment of MTX monotherapy was 41% according to DAS44 ≤2.4. This was not significantly different in comparison with obtained good clinical response of patients in the original BeSt cohort at 6 months (41% vs. 47%; p>0.05) (12). In addition, no significant differences in baseline factors included the prediction model between patients in the validation cohort and the BeSt population were observed (Table 1).

Variable	Score	Original cohort in % (N=186)	Validation cohort in % (N=333)[b]
Gender			
Male	0	30	28
Female	1	70	72
DAS at baseline			
≤3.8	0	25	26
>3.8 and ≤5.1			
2nd quartile	3	25	22
3rd quartile	3	26	30
>5.1	3.5	26	23
Rheumatoid factor			
Negativity	0	32	30
Positivity	1	68	70
MTHFD1 1958 AA	1	21	23
AMPD1 34 CC genotype	1	74	75
ITPA 94 A-allele carrier	2	15	15
ATIC 347 G-allele carrier	1	53	53

Table 1. Comparison of baseline factors between patients in the original (BeSt) cohort and patients in the validation (Swefot) cohort[a,b]

a. Abbreviations: AMPD1= adenosine monophosphate deaminase; ATIC= aminoimidazole, carboxamide ribonucleotide transformylase; ITPA= inosine triphosphate pyrophosphatase; MTHFD1= methylenetetrahydrofolate dehydrogenase; DAS= Disease activity score

b. No significant differences between the validation cohort and the population were observed for gender, DAS at baseline (based on quartiles), RF status and *MTHFD1*, *ATIC*, *AMPD1*, and *ITPA* genotype frequencies (p>0.05)

Validation of the pharmacogenetic model in the Swedish cohort

Assigned scores as defined by the pharmacogenetic model for the Swedish cohort ranged from 1 to 10.5. Application of the pharmacogenetic model cut off values for predicted (non)response to MTX monotherapy resulted in a TPPV rate of 70% (38 of 54 patients) and a TNPV rate of 68% (122 of 179 patients). In total, 233 patients (70%) in the Swefot cohort were classified as either predicted responder or nonresponder, whereas 100 patients (30%) were classified as predicted intermediate responders (Table 2). Hereby, the accuracy of the model in this cohort of patients was 48%, which represents the proportion of true results (TP+TN/All patients= 33+122/333).
In figure 1, a ROC of the pharmacogenetic model is demonstrated. Hereby, the discriminative ability of the model (AUC-area under the curve) was 75% (95%C.I. 70%-81%).

	Predicted response		
	Predicted response	Predicted intermediate response	Predicted non-response
Observed response			
Pharmacogenetic model			
Responder (DAS ≤2.4)	38	42	57
Nonresponder (DAS >2.4)	16	58	122
Total	54	100	179

Table 2. Comparison of the number of observed and predicted response to MTX therapy at 3-4 months for patients in the validation (Swefot) cohort[a,b]

a. Predicted response = DAS ≤3.5, Predicted intermediate response = DAS >3.5 & DAS <6.0, Predicted nonresponse = DAS ≥6.0
b. Abbreviations: DAS= Disease activity score.

Comparison performance original cohort and validation cohort

TPPV and TNPV demonstrated in this analysis, were significantly lower in the validation cohort compared to the predictive values observed in the original cohort (for TPPV 70% vs. 95%: $p<0.0001$ and for TNPV 68% vs. 86%: $p=0.004$, respectively) (12). Regarding the number of patients classified, level of accuracy of the model and AUC no significant differences were found between the Swefot cohort and BeSt cohort (for number of patients classified 70% vs 60%: $p=0.182$; for accuracy 48% vs. 53%: $p=0.572$ and for AUC 75% vs. 85%: $p=0.111$, respectively) (Table 3).

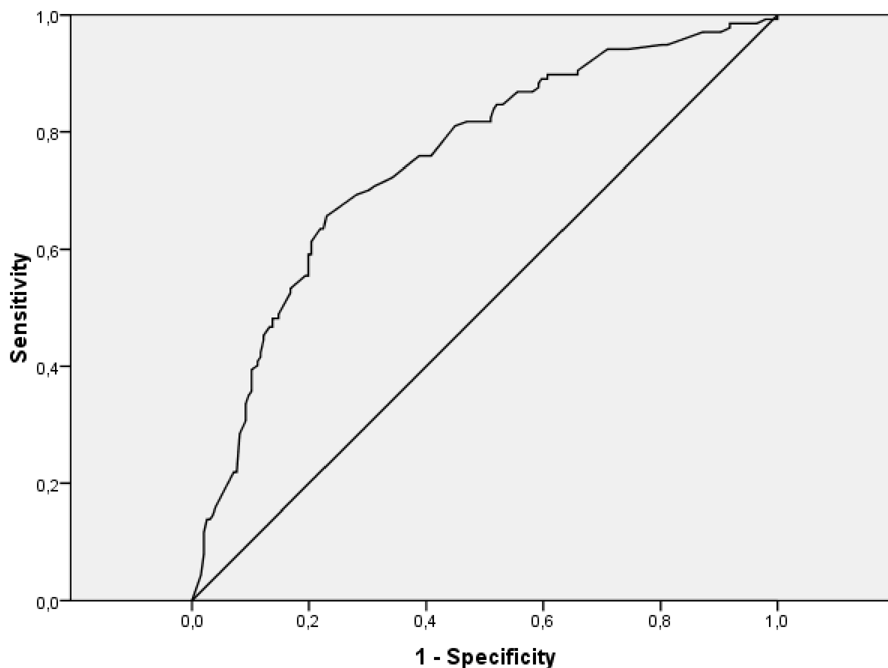

Figure 1. Receiver operating characteristic curve for predicting response to methotrexate in the validation (Swefot) cohort

	Original Cohort in % (N=186)	Validation cohort in % (N=333)[b]	P-value[b]
Predictive values			
N of patients classified (%)	60	70	0.182
TPPV (%)	95	70	0.0001
TNPV (%)	86	68	0.0040
Accuracy (%)	53	48	0.572
AUC (%) (95% C.I.)	86 (80-91)	75 (70-81)	0.111

Table 3. Comparison of predictive values for response to MTX therapy at 3-4 months for patients in the validation (Swefot) cohort[a] and patients in the original (BeSt) cohort[a,b]

a. Abbreviations: AUC= area under the curve; TPPV= true positive predictive values; TNPV= true negative predictive values
b. Tested by chi-square test

Discussion

In this study, a model for predicting the efficacy of MTX monotherapy was validated in a cohort of Swedish RA patients (Swefot). Importantly, no significant differences in frequencies of genetic and nongenetic factors compromising the predictive model were observed between this validation cohort and the BeSt cohort at baseline. Application of this pharmacogenetic model resulted in a TPPV rate of 70% and a TNPV rate of 68%. In total, 233 patients (70%) were classified as either predictive responder or nonresponder. With application of the model, the TPPV and TNPV observed in patients of the validation cohort were significantly lower compared to the values found in the original cohort. However, for the predictive values accuracy, number of patients classified and AUC the results were comparable. Overall, these validation data imply that efficacy of a substantial part of early RA patients treated with MTX could be predicted by this clinical pharmacogenetic model.
Interestingly, the performance of the pharmacogenetic model was already found to be comparable at initial validation in a separate, but much smaller, group of Dutch RA patients (N=38) in the original manuscript (12). In this Dutch group TPPV rate was 70 % and TNPV rate was 68%, whereas 68% of the patients could be categorized as either predictive responder or nonresponder. However, in comparison with the original BeSt cohort significant differences were observed concerning TPPV and TNPV. Several explanations are possible to declare these differences.
Firstly, differences in the performance of the model may partially be due to the lower response rate achieved (DAS ≤2.4) in the validation cohort with a smaller period of time on MTX therapy before evaluation of response compared with the original cohort (41% at 3 months vs. 47% at 6 months, respectively).
Next, no significant associations of the four individual SNPs included in this model and treatment response were found in the validation cohort ($p>0.05$). In the analyses of the original BeSt cohort, the four genetic variants increased the AUC of the pharmacogenetic model with 9% compared with the nongenetic model (85% vs. 76%, respectively) (12). However, in the validation cohort, a clear difference between AUCs of the pharmacogenetic- and nongenetic models could not be analyzed due to absence of smoking status. Conclusively, it remains unclear, if the four variants are true markers for MTX response, if other variants in the four genes are responsible for the effect on treatment outcome, ór whether the extent of genes or the effect of other genes involved in the mechanism of action of MTX is more important than the current genes. Since the MTX responsive phenotype may be considered polygenetic, selecting SNPs according to a candidate gene (mono- or oligogenetic) approach will repeatedly lead to only a limited explanation of variance in MTX response. Hereby, other genes could be more involved in MTX's mechanism of action than *MTHFD1*, *ATIC*, *AMPD1*, and *ITPA*. Future research on the mechanism of action of MTX is therefore required.
Finally, in this study the DAS28 of each patient was converted to the original DAS based on a 44 joint count. However, since cut-off criteria for response are applied to these scores, different response rates could be observed. Specifically, in the Swefot trial, patients were defined as responder if a DAS28 of less than or equal to 3.2 was achieved. Responders in the BeSt study were defined as achieving an original DAS of less than or equal to 2.4. Comparison of these response rates revealed that 18 patients were defined responder according to DAS, but nonresponder according to DAS28. These patients account for almost 13% of the patients in the DAS responder group. Hypothetically, due to a different observed response distribution in the validation cohort, different predictive values could be the result. Therefore additional analyses were performed considering responders according to DAS, but were nonresponder according to DAS28 (n=18), as nonresponders. Regarding the number of patients classified, TPPV, TNPV, accuracy and AUC, no significant differences compared with the results in table 3 were found (data not shown).

The additional recommendation for classification of patients in the intermediate group at baseline, as reported in our previously study (achieving a decrease of >1.2 or ≤1.2 in DAS at 3 months) (12), was left out of the validation analyses. Notably, no data was present due to alternative study design of the Swefot clinical trial.

In general, lower values for the Swefot cohort of patients found in this study (except for the number of patients classified) were anticipated. Namely, effect sizes found by association studies could appear to be smaller, but closer to a genuine effect size, than the first reported effect size (16). A well-designed meta-analysis of comparing the effect size (e.g. TPPV) with this predictive model in more cohorts of patients could provide the most optimal legitimate effect size.

Screening for markers in serum of patients with arthritis is a helpful tool and common practice in rheumatology diagnostics. For example, in a meta-analysis by Nishimura et al it was demonstrated that the sensitivity and specificity of testing for RF status within RA patients was 69% and 85% (17). Notably, this meta-analysis included studies that evaluated patients for the utility of RF for diagnosis or suspected RA. The sensitivity and specificity could be recalculated to a TPPV of 82% and a TNPV of 72% for the diagnosis of RA by testing for RF positivity. Interestingly, these predictive values, explained by RF-status, are comparable with the values regarding the predictive model's TPPV and TNPV found in our study. In this way, testing for response to MTX monotherapy in recent-onset RA patients prior to treatment appears to be qualitatively equivalent to testing for RF status to diagnose RA in clinical practice.

In this study, a pharmacogenetic model for predicting the efficacy of MTX in patients with RA was validated. Notably, the exact role as predictive markers of response for the four genetic polymorphisms included in this model remains to be determined by future studies. Still, our results demonstrate that predicting treatment response is feasible in RA. Additional replication and (ideally) performance of prospectively designed study with this model in large cohorts is warranted to demonstrate the legitimate predictive value in rheumatology practice.

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Chapter 7

The influence of the number of haplotypes of *MTHFR* 1298A-677C alleles on the predicted probability to respond to methotrexate in early RA patients

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Introduction

Two nonsynonymous genetic variants within the methylenetetrahydrofolate reductase (*MTHFR)* gene, *MTHFR* 677C>T (rs1801133) and *MTHFR* 1298A>C (rs1801131), have been extensively studied over the last decade. It has been demonstrated that these single nucleotide polymorphisms (SNPs) cause an amino acid replacement at codon 222 (Ala222Val) and at codon 429 (Glu429Ala), respectively (1;2). Additional haplotype analyses have revealed that these polymorphisms are in linkage disequilibrium (LD), meaning that a combination of C and A alleles of the two SNPs occur more frequently in a population than would be expected on a random basis (3).
MTHFR is a key enzyme in the folate pathway, which catalyzes the conversion of homocysteine to methionine for a variety of metabolic reactions (4). *In vitro* studies have demonstrated that *MTHFR* 677C>T and *MTHFR* 1298A>C, are associated with diminished enzyme activity of MTHFR leading to homocysteinemia and a disturbed folate balance (1;2;5). Moreover, a functional interaction between these two SNPs have been observed, which resulted in a synergistic effect on MTHFR enzyme activity (6).
Alteration of enzymatic function related to these SNPs might modulate disease susceptibility, but studies also have proposed *MTHFR* 677C>T and *MTHFR* 1298A>C as genetic determinants of clinical therapy outcome. These studies have mainly been performed with MTX (7-10). Notably, MTX functions as a folate antagonist, which inhibits indirectly MTHFR. Recently, a meta-analysis reported that the *MTHFR* 677C>T polymorphism was significantly associated with increased toxicity in rheumatoid arthritis (RA) patients on MTX treatment (11). Regarding efficacy, opposing results have been found for *MTHFR* 677C>T and *MTHFR* 1298A>C, whereas most of the performed studies did not find any association (12-17). Notably, a substantial part of the studies did not take linkage disequilibrium between these two *MTHFR* SNPs into account. Only a few studies have examined associations between haplotypes of *MTHFR* 1298A>C and *MTHFR* 677C>T and MTX efficacy in RA patients. In the reports of Urano et al and Taniguchi et al no significant associations of response defined as lower MTX dosages and the haplotype *MTHFR* 1298A-677C were found (16;18). In addition, in the study of Urano et al, carriers of the *MTHFR* 1298C-677C haplotype did show a relation with lower MTX dosage (18). Furthermore, Hughes et al reported no significant associations with MTX treatment outcome with any of the haplotypes comprising *MTHFR* 1298A>C and *MTHFR* 677C>T (14). In the study of Kurzawski et al a lower probability of remission was seen in patients genotyped for *MTHFR* 1298AA and 677CC in comparison with patients with 1 or 0 copies of 677C-1298A. Moreover, the presence of both 677T and 1298C alleles was associated with an increased frequency of remission (8).
We have reported that MTX treated RA patients genotyped for *MTHFR* 1298AA and 677CC were associated with efficacy (19). Additional analyses demonstrated that the number copies of the haplotype as determined by the 1298A-677C SNPs importantly strengthened the association with good clinical improvement (achieving an improvement of DAS >1.2) at 6 months.
Previously, we developed a pharmacogenetic model in combination with clinical factors to predict MTX efficacy in recent-onset RA. In this study it was reported that the clinical factors gender, rheumatoid factor combined with smoking status and disease activity at baseline together with genetic factors were predictive for MTX response. The included genetic factors were the SNPs *AMPD1* 34C>T, *ATIC* 347C>G, *ITPA* 94 A>C and *MTHFD1* 1958G>A. The prediction resulted in the classification of 60% of the RA patients into MTX responders and nonresponders (defined as achieving DAS >2.4 or DAS ≤2.4, respectively), with a 95% and 86% true positive and negative response rate, respectively. Thus 40% of the patients (n=74) could not be classified resulting in a group with a predicted intermediate probability of response to MTX (a score between 3.5 and 6 points). For these patients an evaluation was added using good clinical improvement at 3 months as an intermediate

endpoint. This would enable clinicians to decide on continuation or alteration of MTX therapy at an early stage of treatment and could possibly prevent treatment delay of three months (unnecessary MTX exposure due to inefficacy). Still, with the addition of this interim evaluation, categorization into responders and nonresponders at 6 months remained suboptimal. Regarding the influence of *MTHFR* 1298A>C and *MTHFR* 677C>T and particularly their haplotypes on achieving good clinical improvement, addition of these genotypes to the pharmacogenetic model to improve categorization of patients in the predicted intermediate response group could be beneficial.
In this paper, it is aimed to assess the discriminative performance of the pharmacogenetic predictive model by the addition of the number of copies of the *MTHFR* 1298A-677C haplotype. Also, it is aimed to increase the percentage of patients for which the pharmacogenetic model predicts response or nonresponse by incorporating the number of copies of this haplotype.

Methods

Patients

Characteristics of the patients enrolled in this study are similar to characteristics earlier described by our group (19;20). Briefly, the 205 patients enrolled in this study comprised a subcohort of the patients participating in the BeSt study. Main inclusion criteria were a diagnosis of early RA as defined by the American College of Rheumatology (ACR) 1987 criteria for RA (21), age ≥18 years, symptom duration of <2 years and active RA according to the BeSt study protocol (22). Main exclusion criteria were previous treatment with DMARDs other than antimalarials and concomitant treatment with an experimental drug. Further details have been published elsewhere (22). The local ethics committee at each participating hospital approved the study protocol. All patients gave informed consent before enrolment into the study.

Study design and evaluation of clinical efficacy

The discriminative performance of the pharmacogenetic model was defined as the difference in area under the curves (AUCs), obtained by plotting receiver operating curves (ROCs), between curves with and without inclusion of two copies of the haplotype into the model. Notably, *AMPD1* 34C>T, *ITPA* 94C>A, *ATIC* 347C>G and *MTHFD1* 1958G>A have been related with good response at 6 months (defined as obtaining a DAS ≤2.4) (23). Therefore, the discriminative performance of carrying 1 or 2 and 2 copies alone of the *MTHFR* haplotype in comparison with these four good response-related SNPs were presented according to the endpoint good response at 6 months. For this analysis 205 patients were included in the study. Of these, 74 patients were predicted having an intermediate probability of response to MTX (20) and this cohort was analysed for increasing the percentage of patients for which the pharmacogenetic model predicts response or nonresponse by incorporating the number of copies of the haplotype. Hereby, the primary goal was good clinical improvement (achieving an improvement of DAS >1.2) at 3 months.

Genotyping

DNA isolation, genotyping techniques and success rates of the SNPs *MTHFR* 1298A>C, *MTHFR* 677C>T, *AMPD1* 34C>T, *ATIC* 347C>G, *ITPA* 94 A>C and *MTHFD1* 1958G>A were as previously described (19;23).

Statistical analysis

Frequencies of the number of copies of the *MTHFR* haplotypes were calculated using chi-square tests. Additionally, the estimated probability for achieving good response was calculated for each

individual patient by adding the number of *MTHFR* haplotype to the prediction model. Next, receiver operating characteristic (ROCs) were derived to demonstrate the discriminative performance of the model with and without 1 or 2 and 2 copies alone of the *MTHFR* haplotype. In the predicted intermediate responders, associations of genotypes and haplotypes with treatment outcome were analyzed using chi-square tests and/or Fisher exact tests.
All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

Assessment of the discriminative performance of the pharmacogenetic predictive model by the addition of the number of copies of the *MTHFR* 1298A-677C haplotype.
In the cohort (N=205) the following haplotype distributions were present: for 2 copies 677C-1298A 13% (n=27); for 1 copy 677C-1298A 40% (n=82); for 0 copies 677C-1298A 46% (n=94) (Table 1).
No differences in predicted probability with the addition of number of copies of the haplotypes as predictors for good response were found (p>0.05). Specifically, similar AUCs were observed for all three ROC curves, as depicted in figure 1 (AUC=86% and 95% C.I.= 81-91%). Furthermore, comparison of ROC curves showed that the AUCs of the *MTHFR* 1 or 2 and 2 copies genotypes were smaller compared to the AUCs of other SNPs included in the model (table 2- ROCs not displayed). Specifically, the discriminative ability (AUC) of both number carriers of the *MTHFR* haplotype was 50% (95%C.I. 40-59%) in comparison with an AUC of 61% of the single SNP *ATIC* 347 C>G. Also, when the AUC of the *MTHFR* 2 copies genotype was compared with the AUC of *AMPD1* 34C>T, *ITPA* 94C>A, *ATIC* 347C>G and *MTHFD1* 1958G>A combined a significant difference was observed (50% vs. 69%, respectively: p=0.009- not displayed in table 2).

		***MTHFR* 1298 A>C**			
		AA	*AC*	*CC*	Total
***MTHFR* 677C>T**	*CC*	27	43	22	92
	CT	39	53	1	93
	TT	18	0	0	18
	Total	84	96	23	203

Table 1. *MTHFR* 1298A>C and *MTHFR* 677C>T genotype distribution to demonstrate the number of copies of the *MTHFR* 1298A-677C haplotype[a]

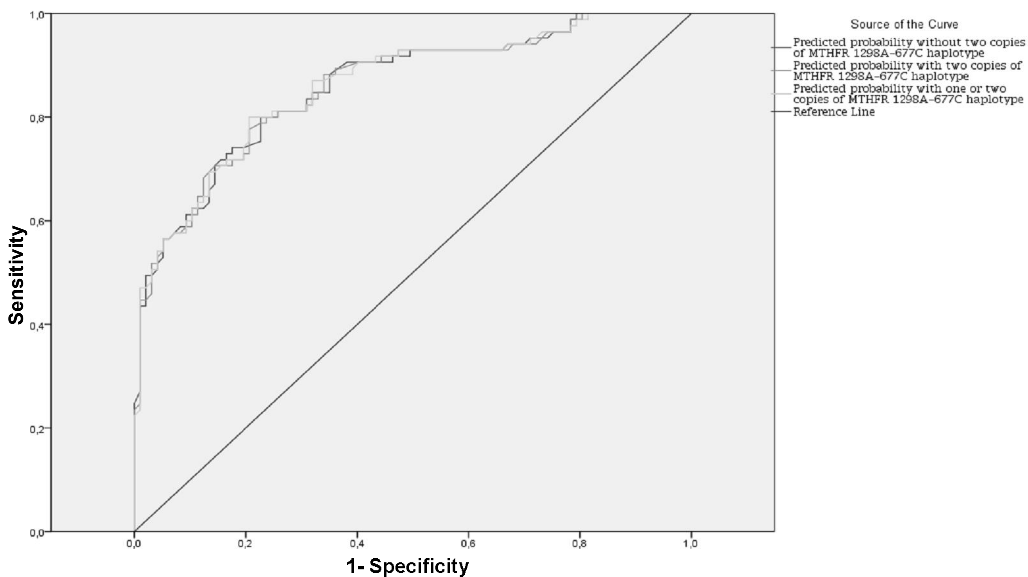

Figure 1. Receiver operating characteristic curve for predicting the response to methotrexate with and without the number of copies of the *MTHFR* 1298A-677C haplotype

Abbreviation(s): *MTHFR*= methylenetetrahydrofolate reductase.

SNPs	AUC in % (95% C.I.)
MTHFR 2 copies vs. 0 or 1	50% (42-59%)
MTHFR 1 or 2 copies vs. 0	50% (42-59%)
ITPA 94 CC vs. CA or AA	56% (47-64%)
MTHFD1 1958 GG or GA vs. AA	56% (47-64%)
AMPD1 34 CC vs. CT or TT	57% (49-66%)
ATIC 347 CC vs. CG or GG	61% (53-70%)

Table 2. AUCs (95%C.I.) for predicting the response to methotrexate of the number of copies of the haplotype *MTHFR* 1298A-677C, *ITPA* 94C>A, *MTHFD1* 1958G>A, *AMPD1* 34C>T, *ATIC* 347C>G

Abbreviation(s): AUC= area under the curve; *MTHFR*= methylenetetrahydrofolate reductase, AMPD1=adenosine monophosphate deaminase, ATIC= aminoimidazole, carboxamide ribonucleotide transformylase, ITPA= inosine triphosphate pyrophosphatase; MTHFD1= methylenetetrahydrofolate dehydrogenase and SNP= single nucleotide polymorphism

Optimalization of the clinical pharmacogenetic model to predict methotrexate treatment response: the influence of the number of haplotypes of *MTHFR* 1298A-677C alleles on probability to respond.

In the group of patients with a predicted intermediate response to MTX (n=74), the number of haplotypes of the *MTHFR* 1298A>C and 677C>T SNPs was assessed. The genotypes of the two single SNPs were not significantly associated with good clinical improvement at 3 months (data not shown). Subsequently, diplotype analyses demonstrated that none of these diplotypes in this specific group of patients appeared able to significantly enhance prediction of achieving good clinical improvement at 3 months (data not shown). Additionally, trend analyses were performed to explore differences in carrying the number of alleles of the 1298A and 677C haplotype (0, 1 and 2). The data revealed no significant differences between the number of *MTHFR* haplotype and good clinical improvement at 3 months (table 3).

	Good Clinical Improvement at 3 months		
	ΔDAS>1.2 (N=40)	ΔDAS≤1.2 (N=34)	P-Value[a]
2 vs. 0 or 1	4 vs 36	5 vs 29	0.395
2 or 1 vs. 0	21 vs 19	22 vs 12	0.205

Table 3. Number analysis of the MTHFR 1298A and 677C haplotype between responders and nonresponders in patients predicted with an intermediate probability of response to MTX (N=74)[a]

a) Fisher's Exact Test (One-sided)

Discussion

In this study, it is concluded that the predictive performance of the pharmacogenetic model to predict the efficacy of MTX therapy in this group of early RA patients is not improved when the *MTHFR* haplotype is included in the model. Moreover, the discriminative effect for the prediction of MTX efficacy including 1 or 2 or (solely) 2 copies of the 1298A and 677C haplotype was significantly smaller compared with the four SNPs *AMPD1* 34C>T, *ITPA* 94C>A, *ATIC* 347C>G and *MTHFD1* 1958G>A.

Furthermore, it is observed that incorporation of the number of alleles of the favorable *MTHFR* 1298A en 677C haplotype (0, 1 or 2) into the predictive model does not lead to improvement of the number of classifiable patients in those with an intermediate probability of response to MTX. However, some remarks have to be made concerning the analysis of the *MTHFR* haplotype.

Notably, current analyses were performed in Caucasian patients. In this way, our results can not easily be compared with studies representing other ethnicities. This is demonstrated in the study of Hughes et al (14), which revealed significant differences in haplotype distribution between Caucasians and African-Americans. Namely, alternative distributions in haplotypes between ethnic groups are caused by differences in allele frequencies of the two SNPs in these groups resulting in different values for D' (D-prime), a value ascribing LD. Specifically, Hughes et al (14) reported that the D' value for the two SNPs was 0.955, indicating strong LD. However, in African-Americans the D' value is much lower (0.408), indicating less linkage disequilibrium (www.hapmap.org). Alternative values of linkage disequilibrium could therefore explain the different results seen in the reports of Urano et al (18) and Taniguchi et al (16), which studied the influence of the haplotype on response in patients with Asian backgrounds. Furthermore, the degree of LD could be biased by the number of patients under study, since small numbers of included patients may lead to differences in haplotype distribution.

Also, to elucidate an additive effect of the number of risk haplotypes on treatment outcome, a gene dose effect is informative. Hereby, a gene dose effect compromises a linear relationship between the number of haplotypes and clinical response. In a part of above described studies, this specific trend is not seen. For example in study of Kurzawski et al (8), patients carrying 1 haplotype *MTHFR* 1298A-677C showed increased response when compared with patients carrying 0 or 2 haplotypes meaning that a gene dose effect was lacking. In this way, a clear biological explanation for involvement in MTX efficacy remains difficult to acquire.

Finally, it has been demonstrated that the pathophysiological consequences of *MTHFR* genetic variants (especially the C677T polymorphism) are significantly affected by demographic and environmental factors such as nutritional (folate) status age, smoking and alcohol intake, parameters that may bias genetic associations with therapy outcome to MTX (24-26). Therefore, multivariate analysis including these confounding factors is inevitable. Regarding our analyses, no significant changes in results are expected.

In conclusion, incorporation of the number of alleles of the *MTHFR* 1298A and 677C haplotype (0, 1 or 2) into the pharmacogenetic model did not lead to improvement of the model, since no associations with achieving good clinical improvement at 3 months (ΔDAS>1.2) were seen in patients with an intermediate probability of response to MTX. Moreover, the results presented in this paper suggest that a (leading) role for the *MTHFR* 1298A and 677C haplotype with regard to predicting efficacy of MTX monotherapy in early RA patients seems unlikely. Future research is necessary to elucidate the exact pharmacogenetic and biological role of *MTHFR* 1298A>C and *MTHFR* 677C>T and their haplotypes in the efficacy of MTX in RA.

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Part 2:

Adalimumab

Chapter 8

Pharmacogenetics of TNF inhibitors in rheumatoid arthritis

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Abstract

Etanercept, infliximab and adalimumab have shown clinical benefit in immune-mediated inflammatory diseases; however, the outcome of treatment with these tumour-necrosis factor inhibitors remains insufficient in 40–60% of individuals with rheumatoid arthritis. Moreover, their use is accompanied by adverse events and unintentional immune suppression. Pharmacogenetics has the potential to increase efficacy and ameliorate adverse events and immune suppression, and its application might be of clinical benefit for patients with rheumatoid arthritis. Pharmacogenetic studies have shown associations between single nucleotide polymorphisms in genes encoding enzymes related to the pharmacodynamics and pharmacokinetics of these drugs and treatment outcome. As we discuss here, replication and prospective validation are warranted before pharmacogenetics can be used in clinical practice.

Introduction

Although the pathogenesis of many autoimmune diseases including rheumatoid arthritis remains unknown, studies have shown that tumour-necrosis factor-a (TNFα) has a key role in the inflammatory process of these immune-mediated disorders. TNFα is known to play a leading role in stimulating cytokine (including its own) and chemokine production, in enhancing neutrophil, chondrocyte and osteoclast activation, expression of adhesion molecules. Also, it facilitates also as a co-stimulator of T-cell activation and antibody production by B cells (1;2).
Consequently, TNFα has emerged as an important target in novel therapeutic strategies used to treat rheumatoid arthritis. The anti-TNF targeting drugs currently used in daily clinical practice are etanercept (Enbrel®), infliximab (Remicade®) and adalimumab (Humira®). Etanercept is a human, soluble, dimeric TNF type II receptor linked to an IgG1 Fc half that binds to and inactivates TNFα. The chimaeric IgG1 monoclonal antibody infliximab and the complete humanized IgG1 monoclonal antibody adalimumab bind to TNFα with high affinity and
thereby inactivate it. The therapeutic effect of these biological agents is achieved by blocking the potential interaction of TNFα with the accessory TNF cell-surface receptors (3;4). In vivo and (more in) in vitro studies have demonstrated that this effects in neutralization and blockage (5;6); interaction with Fc receptor (cross-linkage) (7); initiation of reverse signalling, leading to blockage, increased apoptosis or growth arrest (8;9); reduction of inflammatory cytokine production and angiogenic factor expression (6;10-12); Mediation of complement-dependent cytotoxicity and antibody-dependent cytotoxicity (5;9); down-regulation or discontinuation of bone and cartilage destruction (13;14). In this manner, an expanding array of drug therapy options for the treatment of rheumatoid arthritis in the clinic has been established over the past decade (15;16).
However, high costs, adverse drug events and unintentional concomitant immune suppression, leading to serious (opportunistic) infections, present limitations that might prevent the prescription of these biological drugs (17;18). For example, Bongartz et al. (19) have provided evidence for a higher risk of serious infections (odds ratio (OR) 2.0) and a dose-dependent, increased risk of malignancies (OR 3.3) in patients with rheumatoid arthritis who are treated with anti-TNF antibody therapy. Another limitation is that the treatment outcome of the TNF inhibitors remains insufficient in 40–60% of patients with rheumatoid arthritis (20-22).
Pharmacogenetics has the potential to increase drug efficacy and to ameliorate adverse events and immune suppression. Its application might be of great clinical benefit for individuals affected with rheumatoid arthritis. Studies have shown associations between single nucleotide polymorphisms in genes encoding enzymes related to the pharmacodynamics of the anti-TNF drugs used to treat this disease and treatment outcome. The ultimate aim of using pharmacogenetic markers is to predict the probability of a wanted or unwanted drug response
in individual patients (23). Replication and prospective validation are warranted before pharmacogenetics can be used in clinical practice (24).
Here, we review the potential of pharmacogenetics and its impact on anti-TNF therapy outcome in individuals with rheumatoid arthritis.

Diagnostics and therapeutics of rheumatoid arthritis

Treatment of rheumatoid arthritis usually follows a stepwise approach that is based on the evaluation of disease activity and radiological progression of joint damage (21). The most commonly used measure to evaluate disease activity is the ‘28-joint disease activity score’ (DAS28), which includes

an assessment of 28 joints for swelling and tenderness, the erythrocyte sedimentation rate and a general health assessment using a visual analogue scale (25). To assess disease activity in clinical trials, specific improvement and response criteria have been developed (26). These American College of Rheumatology (ACR) improvement criteria are based on a perceptual improvement (20, 50, 70 and 90%) in disease symptoms (termed ACR20, ACR50, ACR70 and ACR90, respectively). The ACR criteria measure only change (dichotomous outcome), whereas the DAS28 measures both change and the extent of rheumatoid arthritis (continuous outcome). The validity of both methods for describing the results of randomized clinical trials is comparable (25;27). In this way, data integration is being performed by presenting both criteria in clinical trials. Radiological damage is evaluated using the Sharp–van der Heijde score, which assesses erosions and joint space narrowing of joints of hands and feet in rheumatoid arthritis (28).

In daily clinical practice, the design of a therapy plan would ideally be based on monitoring disease activity and strict treatment scheduling to prevent functional disability (29). First, patients would be treated with disease-modifying antirheumatic drugs (DMARDs), of which methotrexate is the first drug of choice (30;31). Should there be an unfavourable response, side-effects and/or drug toxicity, therapy would then be escalated to include biological agents, such as TNF inhibitors, either alone or in combination with DMARDs, and modifications of the dosing regimen (20;22;32). After the onset of disease, tightly scheduled management of treatment is required to maintain efficacy (33). Because high and variable disease activity results in joint damage, effective intervention with TNF inhibitors, either as a monotherapy or in combination with DMARDs, can halt the progression of radiological damage, which consequently translates into a slowing or cessation of functional decline (34;35).

Pharmacogenetics of TNF inhibitors

Pharmacogenetics holds the promise not only to explain interindividual variability in drug response, but also to predict efficacy and adverse drug events in different patients (23). Importantly, several studies have revealed that failure to respond to TNF blocking drugs is not a class effect, but instead is related to the individual drug. For example, the response rates to adalimumab have been evaluated in patients who were unresponsive to etanercept or infliximab (36). Remarkably, the response rates measured were similar to those in patients not previously exposed to TNF blocking agents, which makes a class effect of these agents unlikely (20). The results of several studies in which anti-TNF treatment has been switched underline the pharmacogenomic independency of these drugs (37;38). Therefore, pharmacogenetic results are presented in this review with annotation of the specific TNF inhibitor.

Studies have gathered considerable information on drug interaction with, and mediation of, the cytokine TNFα (2). The fact that these drugs target TNFα has led to interest in TNFα itself as a candidate gene for pharmacogenetic association studies. In recent years, many polymorphisms in genes encoding proteins related to TNFα have been identified that might be associated with treatment outcome. Such candidate gene polymorphisms have been investigated for their ability to predict treatment outcome in patients with rheumatoid arthritis receiving anti-TNF drugs.

The polymorphism in the promoter region of *TNF* SNP −308A>G is one of the most studied variation in *TNF* gene (Table 1). However, results considering an association of infliximab, etanercept or adalimumab efficacy with this polymorphism are inconsistent. For etanercept, six studies reported an association with this SNP (39-44) with half of these studies finding an association with clinical response. Specifically, patients genotyped for *TNF* -308 GG were likely to obtain a better response compared to patients with an A-allele genotype (40;42;44). This was further elucidated by a meta-

analysis performed by Lee et al in order to compare the results concerning the *TNF* –308A>G. After inclusion and analysis of six studies, it was seen that patients carrying an A allele have a poorer response to anti-TNF therapy than those with the G allele (45). This *TNF* –308A>G polymorphism was also studied in relation with treatment outcome and infliximab. The majority of thee studies also found a positive association of the GG genotype with infliximab efficacy (42;44;46-49).
However, regarding treatment with adalimumab, an association was found with the G-allele, as part of a single *TNF* haplotype (238G/-308G/-857C) and inefficacy (50). Additionally for the SNP -857 C>T in the *TNF* gene it was reported that T-allele carriers were associated with a positive response to etanercept therapy (41).
Interestingly, it is hypothesized that altered binding capacities of the two TNFα receptors (TNFRSF1A and TNFRSF1B) for TNFα is due to genetic variation in these receptors (51). Therefore, SNPs in genes encoding these receptors are linked to treatment outcome in several reports. Initially in one study, three SNPs within the *TNFRSF1A* gene were explored (at positions -609, -580, -383), but no associations with response to etanercept was seen (39).
More data are available about the SNP +676 T>G in the *TNFRSF1B* gene. Specifically, three reports demonstrated a link with the G-allele and inefficacy to infliximab and etanercept, whereas other studies reported no effect of this SNP on treatment outcome to all three TNF inhibitors (39;52-55).
Besides anti-TNFα neutralizing properties, TNF inhibitors may involve in effects via their IgG1 Fc fragments, for example by complement activation and binding to cellular Fc-gamma receptors (Fc-R) (56). Hypothetically, ligation of the low-affinity FcR type IIIA can induce apoptosis in synovial macrophages. However, significant effects of *FcR IIIA* -158V>F on the efficacy of infliximab and etanercept were not found (39;54;56). In contrast, increased efficacy was found with the -158 FF genotype in two smaller cohorts of patients treated with either of the three TNF inhibitors (57;58). Regarding the SNP -131R>H within *FcGR IIA* gene, an association was found with a positive clinical response and patients with the homozygous wild-type genotype (58).However the whole genetic region of the Fc-gamma receptors is characterized by extensive gene duplication and the presence of insertions and deletion. This all lead to a highly polymorphic locus and no studies have been published that that the full variability of the locus into account.
It is thought that the pro-inflammatory cytokine IL-1 enhances the inflammatory response in RA (2). This is also seen in clinical trials, in which RA patients were successfully treated with the IL1-receptor antagonist anakinra (59). Therefore, genetic variation within genes coding for IL1 or its receptor could potentially influence treatment outcome to TNF inhibitors. In three studies, SNPs within the genes *IL-1B* and *ILRN* were assessed (43;60;61). Only one association was found in a small cohort of patients: carriage of the number of tandem repeats within the *IL1RN* gene was associated with obtaining a better clinical response to infliximab therapy (61).
Notably, anti-inflammatory cytokines, such as IL-10, could also influence TNF-inhibitor therapy outcome. Indeed, it is demonstrated that the SNP *IL-10* -1087G>A is associated with an increased IL10 production and hereby anti-inflammatory response (62). However, this result was not detected in an association study with a clinical endpoint to measure efficacy in etanercept treatment (43). One other study focused on two *IL-10* promoter microsatellite polymorphisms, *IL10.R* and *IL10.G*, which have been shown to be related with IL-10 secretion (63). A positive response was associated with carriage of the R3 allele or R3-G9 haplotype, whereas the allele G13 and the haplotype R2-G13 were present in patients with moderate or no response (64).
For many years it is known that *HLA-DRB1* shared epitope (SE) is associated with susceptibility to RA. More recently, other SNPs, for example in the *PTPN22* gene, have been associated with susceptibility to RA. Patients with mutations in these genes are likely to have a more severe disease activity state and at baseline compared with patients not having mutations in these disease-susceptibility genes. In contrast,

an association between *HLA-DRB1* (SE) and treatment outcome was only reported in one of seven studies (39;41;50;60;65-67). In this study, two *HLA-DRB1* alleles encoding the SE were related to a positive clinical response to etanercept treatment (39).
Only recently, a genome-wide association study using a 300K-SNP array was performed to analyze response in anti-TNF treatment (etanercept, infliximab and adalimumab). Four SNPs were significantly associated with treatment outcome in this genome-wide analysis in loci *MAFB*, *IFNk*, *PON1* and *IL10* genes. However, replications of these SNPs in independent and larger data sets are required due to the small sample size used in this study (68) (not displayed in table 1).
Beside its potential to reduce disease activity leading to the reduction of inflammation and joint damage, the use of biologic DMARDs in RA has raised concern about the risk of serious and opportunistic infections. Specifically, Bongartz *et al.* (19) have provided evidence for a higher risk of e.g. serious infections (odds ratio of 2.0) in patients with RA who are treated with anti-TNF antibody therapy. Yet, common infections, such as upper respiratory infections and urinary tract infections, have not been studied in large prospective clinical trials. Only, one group performed an association study concerning this last topic (69). In this study, the SNPs *TNF* -238G>A, *LTA* +365G>C and *FCGR3A* +176F/V were significantly associated with experiencing a urinary tract infection during MTX and etanercept treatment. Additionally, the number of risk alleles (*TNF* -238 A-allele, *LTA* +365 C-allele and *FCGR3A* +176 F-allele) was correlated with an increased risk to this type of infection, demonstrating an additive effect (69).

Gene	Function	Genetic polymorphism(s)	Clinical effect on: Toxicity	Clinical effect on: Efficacy
TNF	TNFα production and regulation	-308 G>A	A-allele associated with increased TNFα levels after INF (49); No association with toxicity ETN (69)	GG associated with efficacy ETN and INF (40,42,44,47,48); In hp G-allele effect on inefficacy ADA (50); A-allele associated with inefficacy (45); No association with efficacy ETN, INF and ADA (39,41-43,53,60)
		-857C>T	*	T-allele associated with efficacy ETA (41); In hp C-allele effect on inefficacy ADA (50)
		-238G>A	A-allele effect on toxicity ETA (69)	G-allele associated with inefficact INF (42); In hp G-allele effect on inefficacy ADA (50); No association with efficacy INF (60)
		-1031T>C, -863 C>A, +488, +2018	No effect on efficacy or toxicity ETA, INF and ADA (39,41,42,50,69)	
TNF microsatellites	Linked to TNFα -308 A>G polymorphism	a,b,c,d,e	*	TNF α11 and β4 haplotype associated with efficacy INF (67)
TNFRSF1 A	TNFα soluble receptor type 1	-609, -580, -383	*	No effect on efficacy ETA (39)
TNFRSF1 B	TNFα soluble receptor type 2	676T>G	*	No effect on efficacy ETA, INF and ADA (39,55); GG associated with inefficacy ETA and INF (73) G-allele associate with inefficacy INF (53,54)
Lymphotoxin α (LTA)	Mediation of inflammatory actions	+319C>A, +177A>G, +249, +720	No effect on efficacy or toxicity ETA (39,41,69)	
		+365	C-allele effect on toxicity ETA (69)	No effect on efficacy ETA (39)
HLA DRB1, DRQ1 alleles (SE)	Antigen presenting molecules	See references	*	No effect on efficacy ETA, INF ADA (41,50,60,65-67); HLA-DBRB1 associated with efficacy ETA (39);
PTPN22	Involved in T-cell receptor signaling pathway	1858 C>T	*	No effect on efficacy ETA, INF and ADA (65)
FCGR (I,II,III)	Influence cell activation, apoptosis. Indirect target anti-TNF	131H/R, NA1/NA2, 212V/F	No effect on efficacy or toxicity ETA and INF (39,54,69) 131 RR associated with efficacy INF (58)	
		-158V/F	*	No effect on efficacy ETA and INF (56); FF associated with efficacy ETN, INF, ADA (57,58)
		176F/V	F-allele effect on toxicity ETA (69)	No effect on efficacy ETA (39)
Interleukine-10 (IL-10)	Anti-inflammatory cytokine	-1087G>A	*	No effect on efficacy ETA (43)
		Several microsatellites, see reference	*	IL-10- R3 and haplotype IL-10 R3-R9 associated with efficacy ETA (64)
Interleukine-1 (IL-1)	Pro-inflammatory cytokine	IL-1β +3954C>T	*	No effect on efficacy INF (60,61)

IL-1 receptor antagonist	Inhibits action of interleukine 1	IL-1 RN +2018 T>C	*		C-allele associated with inefficacy INF (60)
		IL-1 RN VNTR intron 2	*		IL1RN*2 allele associated with efficacy INF (61)
MIF	Pro-inflammatory cytokine, Modulation of macrophage and T-cell function	-173 C>C, CATT (7) repeat	*		No effect on efficacy INF (74)

Table 1. Pharmacogenetic association studies of TNF inhibitors with treatment outcome in rheumatoid arthritis

* = No information on association(s) with specific efficacy or toxicity was present regarding this SNP under study. Abbreviations and accessory full names of formal genes can be relocated in the NCBI gene database

Conclusion

TNF inhibitors have been demonstrated to be effective in the treatment of rheumatoid arthritis. Nevertheless, several patients fail to achieve a good response, develop serious side-effects and/or experience drug toxicity, which precludes further treatment with the drug. Unfortunately, interindividual differences in drug response cannot be predicted in patients and (genetic) markers are warranted to individualize and optimize drug treatment. Here, we have discussed mainly reports of associations between genetic polymorphisms in candidate genes and drug efficacy of TNF inhibitor treatment in rheumatoid arthritis, because clear data on associations between toxicity and TNF-inhibiting therapy and associations between genetic characteristics and discontinuation of TNF-inhibiting treatment are limited.

Most pharmacogenetic studies performed so far have an insufficient sample size (power) to detect expected differences in genotype frequencies between responders and non-responders.

Replication and validation in larger comparable cohorts are required before definitive conclusions can be drawn (70). From the studies that have been published, no conclusions can be made on the potential utility of genotyping for *TNF* -308 A/G, the *HLA-DRB1* shared epitope or *TNF* microsatellite haplotypes to predict treatment outcome in rheumatoid arthritis patients who are treated with infliximab. Similarly, on the basis of the levels of significance, the clinical use of genotyping rheumatoid arthritis patients who are treated with etanercept cannot be implemented as yet.

Several difficulties exist in interpreting and comparing the results in pharmacogenetic studies. For example, difficulties arise when genetic variations are known to be disease related, such as the *HLA-DRB1* shared epitope gene in rheumatoid arthritis (71). Patients with mutations in these genes are likely to have a more severe disease and thus a higher state of disease activity at baseline, as compared with patients lacking such mutations. Owing to regression to the mean, patients with high disease activity, in contrast to those with low disease activity, might show a higher response. Predicting a positive or negative treatment outcome is thus hampered by higher disease activity at baseline, rather than referring to an effect of variance in genotype.

In addition, owing to their mechanism of action, the dose of anti-TNF drugs should be considered when interpreting and comparing treatment outcome in pharmacogenetic studies. In theory, the cellular amount of TNFα and, thus, the amount available for inhibition by anti-TNF drugs, might depend on the genotype. The clinical consequence of the genotype might thus be dependent on drug dose, because increasing or decreasing dosage could have a similar net effect. In pharmacogenetic studies, therefore, it is important that baseline characteristics (disease activity state) and drug dosages between cohorts are kept at similar level to estimate adequately associations between genetic polymorphisms and treatment outcome. To avoid genetic variation in a population itself as a predictor for clinical response, the prevalence of a candidate gene in responders and non-responders and in controls must be compared in pharmacogenetic studies. In this way, a genuine gene–dose effect becomes visible (24).

Furthermore, the problem of potential functionality of a candidate gene, tested in vitro, remains because any functionality determined can have no relevance to the in vivo mechanism of drug action. Such genes can be in linkage with other loci, which have a true influence on the pharmacology of the drug (72). Lastly, the location of SNPs on chromosomes and the frequency of SNPs vary to a great extent between different populations; in the interpretation of any associations presented, the genetic variation between racial and ethnic groups has to be considered.

We conclude that pharmacogenetics of anti-TNF drugs in the treatment of patients with rheumatoid arthritis has the potential to optimize therapy and clinical outcome. In general, however, the current studies are too small and subsequent findings in larger studies often fail to replicate the original

data. Continued large-scale studies are essential before a pharmacogenetic approach will be applicable in daily clinical practice.

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Chapter 9

Criteria for the selection of single nucleotide polymorphisms in pathway pharmacogenetics: TNF inhibitors as a case study

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Abstract

Pharmacogenetics aims to identify genetic variation to predict drug response or to establish an individual optimal dose. Classically, explorative pharmacogenetic studies are performed concerning a limited number of SNPs in genes encoding enzymes involved in the drug's metabolic route. Alternatively, potential markers across the genome are elucidated by the performance of the hypothesis-free genome-wide method. Besides their successful use, both methods provide substantial disadvantages. A solution toward these difficulties is the pathway pharmacogenetic approach, which considers variability in the entire pathway without restricting the analysis to only one gene. In this article, we present selection criteria for this approach to effectively explore potential associating SNPs. As an illustration, the method is applied to the biological adalimumab as a case study.

Introduction

The concept of pharmacogenetics is that germline genetic variability causes variable drug response among individual patients. Knowledge about related genetic variants, mostly single nucleotide polymorphisms (SNPs), may help to predict drug response or optimal dose in the individual patient (1). Classically, explorative pharmacogenetic association studies are aimed at finding potential predictive SNPs. These concern a limited number of SNPs in genes encoding enzymes or proteins representing the drug's major metabolic route or target. For example, to explain variable drug response of the anticoagulant warfarin, association studies showed that bleeding time (INR) was associated with cytochrome P450 2C9 (the major metabolic route of warfarin) genotype and VKORC1 genotype (the pharmacodynamic target of warfarin) (2,3). Obviously, the selection of SNPs within the candidate gene is essential, because only some of them may be related to drug response whereas others are not.
This approach has its limitations, however, because of an incomplete knowledge of the pharmacology of a substantial number of drugs and the wide variety of SNPs in the human genome. Thus it may not be surprising that the candidate gene approach has led to poor reproducibility with regard to potential predictors of drug response. Therefore, systematic selection remains a challenge to scientists in obtaining
a potentially successful set of SNPs for predicting drug response.
In this article, SNP selection for pharmacogenetic association studies is discussed. Additionally, a pharmacogenetic pathway approach is presented, together with proposed criteria for systematic selection of SNPs. We have applied this method for the selection of potential interesting SNPs within genes related to the mechanism of action of TNF inhibiting drug adalimumab. This drug has been effective in the treatment of progressive rheumatoid arthritis (RA) by reducing inflammation and joint destruction (4). Approximately 40–60% of individuals with RA, however, do not respond adequately to this drug (5,6). Moreover, the use of TNF inhibitors is accompanied by adverse events and unintentional immune suppression. Pharmacogenetics has the potential to increase efficacy and ameliorate adverse events and its application can translate into clinical benefit for patients with RA (7)

Candidate gene method

Selection of SNPs in hypothesis driven pharmacogenetic association studies is based on their functionality, in which the genetic variant leads (or is predicted to lead) to alteration in protein
function and hence differences in drug response. This approach has led to the discovery of a substantial number of relevant SNPs in pharmacogenetics [3,8,9]. This approach, however, also demonstrated associations that could not be replicated by other investigators [10,11] and thus could result in possibly false-positive findings. Moreover, in a substantial number of studies, SNP selection is not systematically performed but seems to be arbitrary or extensions of previous findings. Also, because complex traits are mostly considered not to be monogenetic, selecting SNPs according to this hypothetical approach will repeatedly lead to a limited explanation of variance in drug response.

Genome-wide method

A more comprehensive, and more expensive, approach is the genome-wide method using SNP arrays (WGA). A clear advantage of this method is that it is hypothesis-free and that this may reveal unexpected SNPs related to drug response. Hence this method does not rely on current knowledge with regard to the metabolism and mechanism of action of the drug. Indeed, in the past two years genome-wide association studies have presented novel associations of SNPs with drug response

[12–14]. Moreover, novel information about the pathogenesis and progression of complex diseases, like RA and Crohn's disease, could be revealed using the genome-wide SNP approach [15–17]. An advantage of this approach is that complex traits can be explored, accommodating polygenetic variation. Yet, various remarks can be placed regarding clinical overvaluation of the results from this approach because of the overall limited effect sizes found [18]. Additional problems arise regarding the discrepancy between type I errors (false-positive results) and subsequently adjusted type II errors (false negative results) in detecting an associated SNP [19,20]. Specifically, the appliance of rigorous criteria for significance (owing to multiple testing) to oppose type I errors can eventually lead to type II errors (missing a real effect).

Pathway gene method
A third method is the pathway gene approach that combines the advantages of the candidate gene approach and the genomewide approach. Moreover, with this method fewer disadvantages are experienced. Namely, by applying the pathway gene approach fewer false-positive results will be found than with the genome-wide method owing to the limitation of multiple testing. A characteristic of the pathway gene method is that a set of SNPs is selected based on a description of pathways regarding the mechanism of action and pharmacokinetics of the drug under study. In this systems pharmacology approach, one considers variability in the entire pathway without restricting the analysis to a single gene, of which the impact on the drug's mechanism of action is unknown. With the candidate gene method, SNPs that are responsible for the rate limiting or extending step in mechanism of action are easily missed. For example, if SNPs in the signal transduction routes of the beta-adrenergic receptor are explored, a complex quandary of proteins come across which are involved in the signal transduction route. Assumably, for most drugs pharmacogenetics has the greatest potential to be clinically useful if information on multiple genes is used. In this context, the pharmacogenetics of most drugs is likely to be comparable to the genetics of complex diseases. In both cases numerous proteins are involved, and genetic variability in each might contribute to the overall variability observed clinically [21].

Before SNP selection in pathway pharmacogenetics

Exploration of the pathway and gene selection
Before SNP selection, an extensive literature search regarding the hypothetical mechanism of action of TNF inhibitors was performed to select candidate genes coding for involved proteins. Pubmed/National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) was searched for original research concerning in vivo and in vitro studies, published in the past five years, regarding this subject. This search was performed using the Mesh terms RA, TNF-alpha, pharmacology, monoclonal antibodies, etanercept, adalimumab and infliximab.
The anti-TNF drug adalimumab is a complete humanized IgG1 monoclonal antibody which binds to and neutralizes both soluble and transmembrane forms of TNF-alpha. Generally, a summary of seven groups can be created (table 1): neutralization and blockage [22,23]; interaction with Fc receptor (crosslinkage) [24]; initiation of reverse signaling, leading to blockage, increased apoptosis or growth arrest [25,26]; reduction of inflammatory cytokine production and angiogenic factor expression [23,27–29]; restoration of immune regulation (Treg cell) [30]; mediation of complement-dependent cytotoxicity (CDC) and antibodydependent cytotoxicity (ADCC) [22,26]; downregulation or discontinuation of bone and cartilage destruction [31,32]. These mechanisms of action have mainly been demonstrated in vitro and, to a lesser extent, in vivo [33,34]. In the defined pathway 124 genes related to the mechanism of action of TNF inhibitors were explored.

SNP sources
After candidate genes had been selected, a SNP search within these genes was performed. SNPs were assessed using the database of the NCBI (http://www.ncbi.nlm.nih.gov/SNP). The NCBI has compiled a dataset of over 10 million SNPs throughout the entire human genome resulting from publicly and privately funded genome sequencing projects in the dbSNP [35,36]. Other databases of SNPper/CHIP-Bioinformatics (http://snpper.chip.org/bio) and Snap/SNP Annotation platform (http://snap.humgen.au.dk/views/index.cgi), mainly related to the NCBI, were consulted. A total of 51,793 SNPs in 124 genes were available for the SNP selection procedure.

Criteria for the selection of SNPs

Primary selection aims to obtain SNPs, with a high probability to detect, as a result of reported heterozygosity frequencies and the number of previous genotyping techniques applied. When these two primary criteria are applied, fewer SNPs are included for the secondary selection with more extensive parameters. Because of this time-effective aspect, namely not analyzing each SNP in each gene, this two-step design was chosen for the selection process.

Primary selection

The primary selection of SNPs is based on the criteria:

- Genetic region and heterozygosity: introns with a heterozygosity between 0.400 and 0.480 and all exons with a heterozygosity of more than 0.095.
- Validation status: only SNPs with a validation status of 2 or more measurements as reported in the NCBI.

When these criteria were applied, 2629 SNPs out of the total of 51,793 SNPs in 124 genes were selected (Fig. 1).

Mechanism of action	Selected genes	N of genes	References
Neutralization and blockage	TNF, LTA, TNFRSF1A, TNFRSF1B, ADAM17, IL1A IL1B, IL1R1, IL1R2, IL1RAP, IL1RN	11	(22,23)
Interaction with Fc receptor (cross-linkage)	FCGR2A, FCGR2B, FCGR3A, FCGR3B	4	(24)
Initiation of reverse signaling, leading to blockage or increased apoptosis or growth arrest	TRADD, FADD, RIPK1, TRAF2, TANK, TNFAIP3, MAP3K7IP1, MAP3K7IP2, MAP3K7, IKBKG, CHUK, IKBKB, NFKB1, NFKB2, NFKB3, MAPK8, TP53, BAX, BAK1, CASP3, CASP7, CASP8, MAPK14, BCL2L1, BIRC2, BIRC3, XIAP, CFLAR	28	(25,26)
Reduction of inflammatory cytokine production and angiogenic factor expression	IL6, IL6R, CSF2, CSF2RA, CSF2RB, CSF1, CSF1R, CSF3, CSF3R, LIF, LIFR, OSM, OSMR, IL2, IL2RA, IL3, IL3R, IL7, IL7R, IL8, IL8RA, IL8RB, IL9, IL9R, IL12A, IL12B, IL12RB1, IL12RB2, IL18, IL18R1, IFNA1, IFNB, IFNG, IFNGR1, IFNGR2, IL15, IL15RA, CD11, CD28, CD40, CD40L, CD69, APOA1, IL4, IL4R, IL10, IL10RA, IL10RB, IL11, IL11RA, IL13, IL13RA1, IL13RA2, TGFB1, VEGFA, VEGFB, VEGFC, FIGF, KDR, FLT1, FLT4, SELE, ICAM1, VCAM1, vWF, PECAM1	66	(23,27-29)
Restoration of immune regulation (Treg cell)	FOXP3	1	(30)
Mediation of complement-dependent cytotoxicity (CDC) and antibody-dependent cytotoxicity (ADCC)	C1QA, C1QB, C1QC, CR1, C2, C3, C4A, C4B, C5, C5AR1	10	(22,26)
Down-regulation or discontinuation of bone and cartilage destruction	TNFRSF11A, TNFSF11, TNFRSF11B, TRAF6	4	(31,32)
Total number of genes		**124**	

Table 1. Candidate gene selection

Abbreviations and accessory full names of formal genes can be relocated in the NCBI gene database *(http://www.ncbi.nlm.nih.gov)*

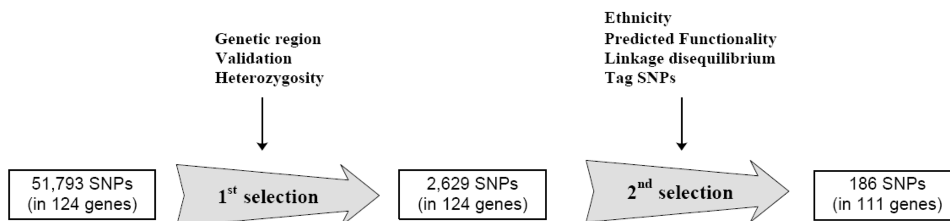

Figure 1. Design stepwise SNP selection
Abbreviation(s): SNP= single nucleotide polymorphism

Genetic region

In the NCBI SNP database, a subdivision is made between different regions of genes: 3' and 5' near a gene, introns and exons. Additionally, functional characteristics in these regions are: noncoding, nonsynonymous, frameshift, synonymous, promoter or untranslated. Important for SNP selection, on the basis of alteration of a gene product and in this way protein function, is the presence of SNPs in exons. Still, noncoding SNPs, like introns, which maybe, for example transcribed to noncoding RNA, could have functions in transcriptional interference and promoter inactivation, as well as indirect effects on transcription regulatory proteins and in genomic imprinting [37].

In the NCBI database, specific regions within each gene were examined. A subdivision was made into different regions: unknown, 3' and 5' near gene, introns and exons. Additionally, exons were subdivided into synonymous, nonsynonymous, 3'UTR and 5'UTR subgroups.

Heterozygosity

True associations in case–control studies depend on the precise definition of response criterion, power and sample size of the study. For the detection of small differences in allele frequencies, a study has to be sufficiently powered. Additionally, selecting SNPs with a low minor allele frequency (MAF) will require very large sample size cohorts to achieve an association which is statistically sufficiently powered [38]. Figure 2 presents examples of number of cases needed to detect significant differences in variable allele frequencies in a case–control (1:2) study design. Paired lines represent number of cases required to detect differences with significance level of 1.10-4 and 1.10-6 with 80% power depending on the MAF in controls and hypothetical odds ratios for obtaining good response in cases relative to controls. For example, to detect a significant difference with a MAF in controls of 0.3 with a hypothetical odds ratio of 2.0 for obtaining good response in cases relative to controls, at least 147 cases and 294 controls are needed.

A constructive tool in selection based on frequency is the usage of a SNP's heterozygosity, which is the frequency of the occurrence of heterozygous individuals for a particular SNP. To use a specific range of heterozygosity as a criterion, the heterozygosity can be calculated from a preferred MAF within a sample size regarding the power for an association study.

In this case study, SNPs were included on the basis of a total sample size of 400–500. In this way, for all SNPs, except exons, cutoff values regarding heterozygosity were chosen between 0.400 and 0.480. If heterozygosity was lower than 0.400 and higher than 0.480, SNPs were excluded, except for SNPs with a significant predicted functional change of protein (defined below). Because SNPs in exons are less abundant, cut-off values regarding heterozygosity were lowered. In this way, SNPs in exons with a heterozygosity of more than 0.095 were included.

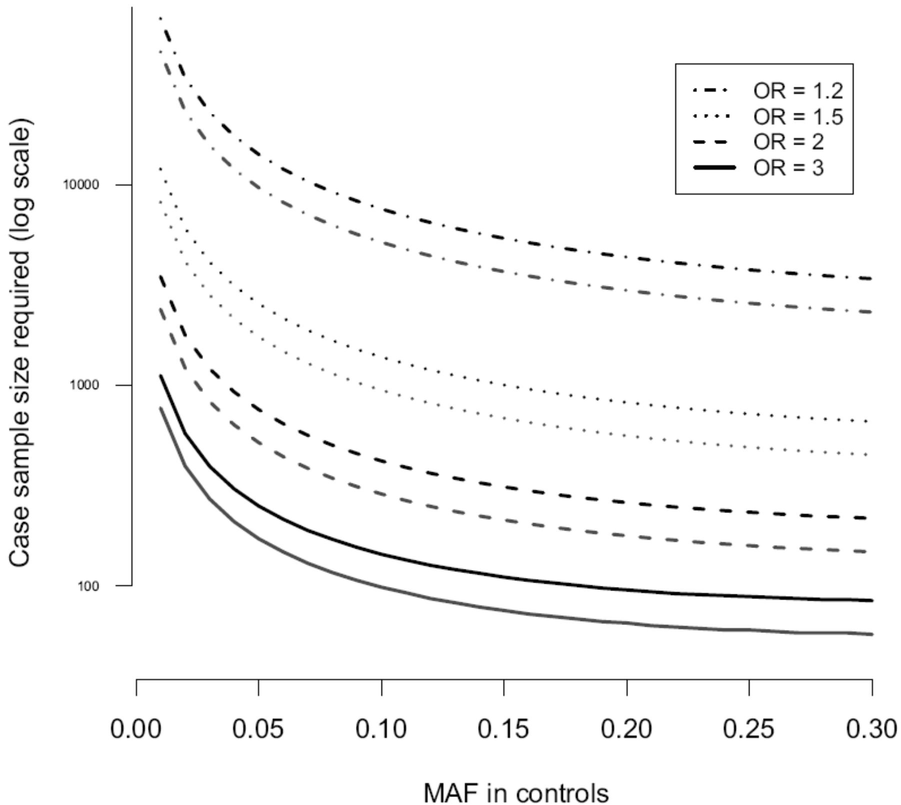

Figure 2. Schematic representation of the number of cases needed to detect significant differences in a case-control (1:2) study design[a,b]

a) Lines represent number of cases required to detect differences with significance level of 1.10^{-4} (lower light line of pair of hypothetical odds ratio- OR) and 1.10^{-6} (upper dark line of pair of hypothetical OR) with 80% power depending on the MAF in controls and hypothetical odds ratios for obtaining good response in cases relative to controls.
b) Abbreviation(s): MAF= minimal allele frequency, OR= odds ratio

Validation

The NCBI has created several descriptions of validation status for SNPs, which have been observed in individual experiments and accepted in this database without validation evidence. These descriptions are important in distinguishing high-quality validated data from unconfirmed data. Subsequently, this will lead to an increase in certainty of selecting a genuine polymorphic SNP. Validation status was assembled in six groups depending on the number of validation measurements:
- by multiple, independent submissions to the refSNP cluster,
- by frequency or genotype data: minor alleles observed in at least two chromosomes,
- by submitter confirmation regarding the SNP,
- all alleles have been observed in at least two chromosomes a piece,
- the SNP was genotyped by the HapMap project.

In this way, a validation score system (number of measurements) was created to distinguish high-quality validated data from unconfirmed data.

Secondary selection
The secondary selection of SNPs is based on three criteria:

- Predicted functionality
- Tag SNPs and linkage disequilibrium (LD)
- Ethnicity

When these criteria were applied, 223 SNPs out of 111 genes of the total remaining 2629 SNPs in 124 genes were selected.

Functionality
SNPs that affect gene expression occur in all regions of the genome. SNPs causing amino acid alterations (nonsynonymous SNPs) have been extensively studied. Less examined are variants located within the noncoding regions of the genome because mechanistic roles of noncoding genome sequences remain poorly defined. Moreover, the analysis of their functional consequences is complex [39]. While mostly regarded as nonfunctional, these variants can impact gene regulatory sequences, like promotors, to change gene expression and enzyme activity.
Another important feature is the exploration of possible functionally important regions in candidate genes within different species, which are identified within evolutionarily conserved sequences [40]. Several web software tools have been developed to assess these regions but this aspect is not further discussed in this article [41].
Functional change of a SNP was qualified and estimated using the Internet tools SNPs3D (http://www.snps3d.org/) [42] and/or PMut (http://mmb2.pcb.ub.es:8080/PMut) [43]. These resources provide a method of identifying those nonsynonymous SNPs that are likely to have a deleterious impact on molecular function in vivo.
For each SNP in the second step of the selection, predicted functionality according to the above resources was examined. If a predicted significant effect of a SNP was demonstrated, according to SNPs3D, this SNP was favorable to include in comparison with other SNPs with the same validation score, same heterozygosity for Caucasians and location within a gene region.

Tag SNPs and linkage disequilibrium
Tag SNPs usually occur in haploblocks or subregions. SNPs in different haploblocks or from different genes may, however, also be in LD. It is useful to search for both in association studies [44]. The degree of LD between alleles at two loci can be described with the correlation coefficient (r^2). This coefficient is informative in association analyses because it is inversely proportional to the sample size that is required for detecting a pharmacogenetic association given a fixed genetic risk [45]. An r^2 of 1 indicates full linkage, which means that there is no loss of power when using a marker Tag SNP instead of directly genotyping the disease causal variant. LD blocks (including tagged SNPs) can be relocated using the metric D0, which is closely related to r^2, and provides information about the recombination breakpoints of chromosomes. These parameters are required for the search of Tag SNPs in the HapMap database (http://www.hapmap.org). To limit the effort and costs of association studies, taking account of Tag SNPs is important [45].
Tag SNPs were explored in the database provided by the International HapMap Project [46]. Additional criteria were ethnicity (Caucasian, discussed below), $r^2 > 0.8$, MAF > 0.20 and maximum segment size of 250 basepairs.
Additionally, available software for the exploration of LD, are the HapMap database and WGAviewer (http://www.genome.duke.edu/centers/pg2/downloads/wgaviewer.php) [47]. This last tool provides an interface to automatically annotate, visualize and interpret the set of P-values emerging

from a whole genome association study [17]. HapMap data are used to identify nongenotyped polymorphisms that associate with the phenotype of interest through LD with genotyped variants. Regarding LD, SNPs with $r^2 > 0.7$ and $D0 = 1$ were regarded as SNPs in LD. Within a demonstrated LD, the most favorable SNP based on validation status and heterozygosity was selected. This was also the case for Tag SNPs: regarding SNPs in LD only the most favorable SNPs, based on validation status and heterozygosity, were included in the final selection.

Ethnicity

During a first exploration of frequency in SNPs the mean heterozygosity was assessed. Yet, it is also important to be aware of the differences in frequency mutation among ethnic populations [48,49]. In the NCBI, the MAF for each ethnic group is presented. Hereby, the consistency of the patient population under study should be examined, before accomplishing a SNP selection. For our case study we used the heterozygosity of each SNP for Caucasian population.

Characteristics of the selected SNPs

After applying these criteria, 186 SNPs were finally selected to analyze in RA patients treated with the TNF inhibitor adalimumab. Percentages of SNP selection according to mechanism of action are displayed in figure 3. The largest group of genes, 58% of all SNPs (N = 107), are located in genes involved in the reduction of inflammatory cytokine production and angiogenic factor expression. None of the SNPs within the gene coding for proteins related to immune regulation (Table 1) was finally selected.

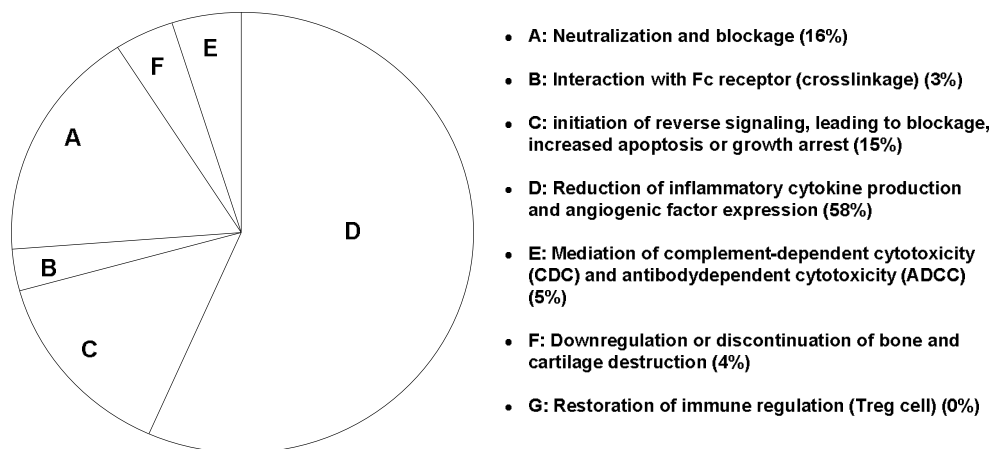

Figure 3. Percentages of selected SNPs according to mechanism of action.

In table 2, characteristics of the finally selected SNPs are presented. The majority of the selected SNPs are located in an intron or exon region (N = 170; 91.4%). In the exon region, 32 SNPs are thought to influence amino acid replacement, while 20 SNPs are substitutions that are synonymous.

More than half of the SNPs have a heterozygosity between 0.400 and 0.480. Of all available criteria scores, derived from the NCBI validation criteria, SNPs with several four criteria were abundant. Less effective criterion was the functionality of the finally selected SNPs using SNP3D. In 11 SNPs, a subdivision could be made based on deleterious (N = 3) and nondeleterious (N = 8). Additionally, 93 Tag SNPs were included for SNPs in a region of the genome with high LD, which facilitate a reduction of genotyping 467 SNPs, earlier selected in our primary selection. Finally, the number of SNPs selected with the capacity of representing a LD block was 20. Within our selection, these SNPs represent a total of 55 SNPs. During this selection, a large percentage within the criterion functionality remains unknown (94%).

Criteria	N of SNPs selected (%)
Gene region:	
- Unknown/N.A.	2 (2.2)
- 3' near gene	2 (2.2)
- 5' near gene	12 (6.5)
- Intron	84 (45.2)
- Exon:	86 (45.7)
o Synonymous	20 (10.8)
o Nonsynonymous	32 (17.2)
o 5'UTR	7 (3.8)
o 3'UTR	27 (14.5)
Heterozygosity:	
- Unknown/N.A.	1 (0.5)
- <0.400	37 (19.9)
- ≥0.400 or ≤0.480	118 (63.4)
- >0.480	30 (16.1)
Number of NCBI validation criteria:	
- 2	17 (9.1)
- 3	57 (30.6)
- 4	86 (46.3)
- 5	26 (14.0)
Functionality:	
- Unknown/N.A.	175 (94.1)
- Deleterious	3 (1.6)
- Non-deleterious	8 (4.3)
Tag SNPs	93 (50.0)
SNPs representative for LD	20 (10.8)

Table 2. Characteristics of finally selected SNPs according to defined criteria

Abbreviation(s): LD= linkage disequilibrium, N.A. = not available, UTR= untranslated region, SNP= single nucleotide polymorphism

Discussion

We present a rational approach for the selection of SNPs for pathway pharmacogenetic association studies. This method was applied in the presented case study by describing the pathways regarding the mechanism of action and pharmacokinetics of the TNF inhibitor adalimumab. This approach has several advantages over either the candidate gene approach or the genome-wide SNP analysis. First, because the rate-limiting step in the described pathway is unknown, this systems pharmacology approach provides a solution: variability in the entire pathway is explored. In fact, the relative contribution of the different SNPs in the pathway to the explanation of variability to drug response can be assessed. Second, this approach has an important statistical advantage: the chance of false-positive results is lower compared to the genomewide method, because of decreased multiple testing.

The next step would be to bring this pathway pharmacogenetic approach into practice. Namely, a pharmacogenetic study may be considered to validate the functionality of the selected SNPs in the pathway with respect to the therapeutic outcome to TNF inhibitors. Interestingly, an association study is projected by our group in the near future. Hereby, the efficacy of treatment with adalimumab in RA patients is linked with genetic variants systematically selected by this approach. Gene ontology analysis software may be useful in identifying novel pathways associated with mechanism of action of TNF inhibitors. One free-available software program is the Gene Ontology project, which is a large bioinformatics initiative to unify genomic databases and to increase convenient usage for biological scientists [50]. This software tool, however, was not used in our exploration of genes involved in the mechanism of action of adalimumab.

With the application of proposed criteria, objective selection of SNPs can be achieved. Defined steps were made to include 186 SNPs in 111 genes out of 51,793 SNPs in 124 genes in our case study. However, several crucial remarks can be placed.

Because the SNP selection is performed based on in vivo and in vitro studies concerning assumed pathways and targets in the mechanism of action of TNF inhibitors, there could be issues owing to limited understanding or changing opinion about the mechanism of action of the drug. For example, scientists thought that the drug imatinib was an inhibitor of several tyrosine kinases (TKs), like the BCR-Abl and platelet-derived growth factor (PDGF) receptor. Reports of inhibition of the c-kit signal transduction pathway by imatinib mesylate gave new insights into the mechanism of action of this drug [51,52]. Irrespective of whether or not the mechanism of action of the group of TNF inhibitors, such as infliximab, etanercept and adalimumab, is similar, clinical trials have demonstrated that the patient response differs within and between RA patients, as seen in results of several studies in which anti-TNF treatment has been switched [53,54]. Hypothetically, the variation in clinical results can be explained by differences in the mechanism of action. This makes a class-effect and a complete similar mechanical pathway less probable.

Although many SNPs have been reported in the past decade, only a very small minority of the genetic variants published have proven functional consequences. Generally, functionality remains an important SNP selection criterion if compared with other used criteria in our presented method. During our stepwise selection, however, a predicted functionality could be assessed in only 6% of the SNPs. Future research has to be performed to explore the functional ability of a SNP. Subsequently, more predictive tools for functionality may be available for scientists to use.

A third crucial remark is related to prognostic versus predictive nature of the biomarker. A substantial number of published SNPs have been described to potentially associate with drug therapy outcome and with disease susceptibility under study [55,56]. If a high qualitative association is demonstrated between a SNP and the susceptibility to RA, as is seen in genome-wide studies [16,57], these results may be of interest for pharmacogenetic studies. Moreover, next to a significant association of

a SNP with susceptibility to RA, a more than random chance of this SNP being related to treatment outcome could be intelligible. Likewise, a pharmacogenetic condition can have implications for understanding susceptibility of disease [58,59]. Still, despite the necessity of prospective validation of our approach compared with the other methods, so-called 'literature-SNPs' were not taken into account during our selection. Namely, significant results based on literature may influence the objectivity aiming at a systematical pathway gene method to obtain optimal, original and detectable SNPs. Interestingly, this is the case for the SNP TNFa -308A > G, which is extensively studied in association studies with responsiveness to TNF-alpha-blockers in RA. Because the heterozygosity of this SNP is 0.163 (according to NCBI) and its position is not within an exon region, this SNP would not be selected according to our objective criteria.
Finally, costs are an important limiting factor in the SNP selection process. Costs of assays are indirectly correlated with the number of SNPs that could be examined and leads to an unwanted constraint to objectively select SNPs [60].
In this paper we have presented a feasible pathway gene approach with defined selection criteria to effectively explore potential SNPs with adalimumab as a case study. The comparison of this approach with the candidate gene- and whole genome methods requires further investigation.

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Chapter 10:

Pharmacogenetic pathway approach detects associations with adalimumab efficacy in rheumatoid arthritis

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Abstract

Objective

Adalimumab efficacy in patients with RA is about 60-70% and predictors for response are yet unknown. This study aims at exploring predictors for adalimumab efficacy in RA patients by associating response with single nucleotide polymorphisms (SNPs) in: 1) the pharmacological pathway of adalimumab, and 2) disease susceptibility genes.

Methods

223 SNPs in 111 genes were analyzed in 325 genotyped rheumatoid arthritis (RA) patients treated with adalimumab. Treatment outcome was evaluated with the use of the 28-joint disease activity score criteria (DAS28) by the primary endpoints EULAR good response and remission and by the secondary endpoint relative change in DAS28. Initially SNPs were explored for associations under allelic and genotypic model using chi-square tests. Hereafter, SNPs were investigated in the most appropriate model with Cochrane-Armitage test for trend analysis and regression analyses under additive, recessive and dominant genetic model with the covariates age, gender, concomitant MTX therapy and DAS28 at baseline.

Results

19 SNPs, 11 SNPs and 8 SNPs were significantly associated with EULAR good response, EULAR remission and relative change in DAS28, respectively ($p<0.05$). Four SNPs, rs1126535 in *CD40LG*, rs682847 in *KDR*, rs1267067 in *TANK* and rs25648 in *VEGFA*, were significantly associated with adalimumab treatment outcome according to all three endpoints ($p<0.05$).

Conclusion

The SNPs, rs1126535 in *CD40LG*, rs6828477 in *KDR*, rs1267067 in *TANK* and rs25648 in *VEGFA* were found potential predictors for adalimumab efficacy. The results from this explorative study provide new insights regarding the potential of pharmacogenetics related to adalimumab efficacy in RA.

Introduction

TNF inhibitors have proven to be effective in a majority of rheumatoid arthritis (RA) patients, which had not experienced a positive response to traditional disease-modifying antirheumatic drugs (DMARDs) (1-3). Consequently, these biologicals have become important agents in therapy of severe RA. Still, a considerable proportion of 30 to 40% of these patients do not experience benefit from this treatment. If patients do not profit from TNF inhibitor therapy, disease progression and joint erosion proceeds while patients potentially experience serious drug related side effects. The idea of a priori prediction of drug response in RA patients is a highly relevant topic, as it will enable rheumatologists to readily identify those patients sensitive to certain drug regimens and thereby minimizing irreversible joint dysfunction existing in severe untreated RA (4-6).
Variation in treatment response for patients treated with TNF inhibitors could be expected, regarding interdifferential cytokine and receptor expression in patients leading to heterogeneity of cytokine presence and regulation in joints. Although this has not been studied intensively, indeed several polymorphisms have been studied over the years, which may associate with treatment outcome of TNF inhibitors in general (7-9).
It has been suggested that adalimumab and other TNF inhibitors target analogous functional pathways (10;11). Despite the awareness that the mechanism of action of TNF inhibitors may involve several pathways and is, therefore not monogenetic, so far, the majority of association studies have analyzed only genetic variants in single genes in relation with treatment outcome.
Alternatively, since a large group of enzymes and cytokines are likely involved in the pharmacodynamics of TNF inhibitors and inflammatory cascade of RA, an approach concerning reciprocal comparison of multiple genes encoding these proteins would be more logical. Characteristically, with this systems pharmacology approach one considers the variability in the entire pathway without restricting the analysis to only one candidate gene (12). Moreover, with the single candidate gene method, SNPs that are responsible for the rate limiting or extending step in mechanism of action are easily missed.
Therefore, the objective of this study is to explore whether the efficacy of treatment with adalimumab is associated with single nucleotide polymorphisms (SNPs) in genes related to the mechanism of action of TNF inhibitors and/or inflammatory process of RA by using a systems pharmacology approach. Similarly, SNPs, which were previously associated with genetic susceptibility to RA and/or treatment outcome to TNF inhibitors, were examined for association with treatment outcome.

Methods

RA patients

Clinical data of 325 patients enrolled in this study originated from a database of ApotheekZorg, which facilitates the Dutch distribution of adalimumab. In the Netherlands, adalimumab is prescribed according to the following protocol and is restrictive for reimbursement: 1) patients have used 2 DMARDs including MTX and 2) patients have a Disease Activity Score based on a 28-joint count (DAS28) of at least 3.2. Other inclusion criteria enclosed a diagnosis of RA according to the revised American College of Rheumatology criteria (ACR) (13); 18 years of age or older; an erythrocyte sedimentation rate (ESR) of at least 28 mm/hour; patient's global assessment of their general well-being measured on a 100 mm horizontal visual analogue scale (VAS), the left end representing as good as can be and the right end representing as worse as possible, of at least 20 mm.

The local ethics committee at our hospital (Leiden University Medical Center, The Netherlands) approved the study protocol. Patients gave written informed consent prior to inclusion.

SNP selection

The SNPs for analysis in this study were selected primarily according to a pathway selection method, as previously described (12). Briefly, a systematic approach was used including rational criteria for the selection of potential interesting SNPs within genes related to the mechanism of action of adalimumab. The criteria were based on the following characteristics of SNPs: heterozygosity frequency, validation status, ethnicity frequency, functionality based on alteration in protein, the extent of linkage disequilibrium and TAG SNPs. Included genes for SNP selection were genes encoding proteins involved in the mechanism of action of TNF inhibitors: neutralization and blockage (14;15); interaction with Fc receptor (cross-linkage) (16); initiation of reverse signaling, leading to blockage, increased apoptosis or growth arrest (17;18); reduction of inflammatory cytokine production and angiogenic factor expression (15;19-21); restoration of immune regulation (22); mediation of complement-dependent cytotoxicity (CDC) and antibody-dependent cytotoxicity (ADCC) (14;18); down-regulation or discontinuation of bone and cartilage destruction (23;24). Ultimately, with this pathway approach 186 SNPs in 111 genes out of 51,793 SNPs in 124 genes were selected.
Additionally, a set of SNPs was selected (N=37), which included SNPs previously studied in relation with efficacy of TNF inhibitors and/or susceptibility to RA.

DNA collection and genotyping

After inclusion and with patients' written consent, DNA was obtained from patients by using DNA from single 2 ml salivary samples. Specifically, a sample of whole saliva was collected from each patient using a Oragene™ DNA self-collection kit (DNA Genotek Inc., Ottawa, Ontario, Canada). Hereafter, DNA samples were isolated according to a standard laboratory protocol for manual purification of DNA designed to optimize DNA yield (OrageneTM DNA Purification Protocol). DNA from 323 patients was suitable for genotyping. In 2 patients DNA was of low quality, which was defined as a DNA yield of lower than 10ug and/or nucleic acid purity (260nm/280nm ratio) lower than 1.6.
Genotyping was performed using a custom designed array with Veracode GoldenGate GT assays on the Illumina BeadXpress platform. This array interrogated 384 SNP loci within a single well of a standard microplate.
Before analysis for association, quality control procedures were performed: SNP genotyping plots for each assay were checked for degree of clustering (GenTrain score of <0.4) and samples with a call rate of less than 90% and assays with a call rate of less than 95% were removed. During this quality process, 22 SNPs and 8 patient samples were excluded for analysis.
Additionally, genotype frequencies of each assay were calculated and these distributions were tested for Hardy-Weinberg equilibrium (HWE) ($p<0.05$). If deviation from HWE was observed, genotype distribution in our population was compared with allele frequencies presented in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP). Accordingly, 5 SNPs were removed for analysis.

Clinical evaluation

For association analyses, primary and secondary endpoints were chosen based on two scales: change in 28 joint Disease Activity Score (DAS28) between baseline and at 14 weeks. As primary endpoints, achievement of good response and remission at 14 weeks according to EULAR criteria were chosen (25). EULAR good response was defined as a change of DAS28 ≥ 1.2 and DAS28 at 14 weeks ≤ 3.2. EULAR remission was defined as achieving DAS28 at 14 weeks ≤ 2.6. As secondary endpoint a relative change in DAS28 was selected ($(DAS28_{baseline}-DAS28_{14weeks})/DAS28_{baseline}$).

Statistical Analysis

Initially, for the analysis of primary endpoints SNPs were explored for associations under allelic and genotypic model using chi-square tests. If a p-value of <0.1 was observed with either of these models, SNPs were fitted to the most appropriate model with Cochrane-Armitage test for trend- and logistic regression analyses for additive, recessive and dominant genetic model, respectively.

Secondly, if SNPs were significant SNPs (p<0.05) in the logistic regression analyses of either primary endpoints, the SNPs were candidates for analysis of the secondary endpoint. For this endpoint linear regression analyses for associations with relative change in DAS28 were applied.

Notably, with the analysis of SNPs previous associated with genetic susceptibility to RA and/or treatment outcome to TNF inhibitors, the reported genetic model (additive, recessive or dominant) was chosen. SNPs, which were reported under genotypic or allelic genetic model, were analyzed additionally according to methods described above.

All regression analyses were adjusted for age, gender, concomitant MTX therapy and DAS28 at baseline.

Since this study is explorative, no corrections for multiple testing were made. Therefore, p-values were presented without adjustments (p <0.05 was considered significant).

Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and GPlink software (http://pngu.mgh.harvard.edu/purcell/plink/) (26).

Power calculation

With the appliance of a power calculation it was demonstrated that for our study population of 325 patients, minimal allele frequencies (MAF) ranging from 10% to 50%, an exposure (response of) of 45% and a chosen type 1 error probability α=0.05, a power of >80% could be achieved to detect an odds ratio ranging from 1.4 (MAF of 50%) tot 2.0 (MAF of 10%). Notably, in more than 80% of the selected SNPs the MAF was higher than 30%

Results

Patient population

In total, 223 SNPs were genotyped in 323 patient samples, of which 196 (88%) SNPs were successfully genotyped in 315 (98%) patient samples after quality procedures and testing for HWE. Baseline demographic, disease characteristics and clinical response of the 325 initially included patients are presented in table 1. The average age of the cohort at the start of adalimumab therapy was 56 years with a mean disease activity (DAS28) at baseline of 5.9. Other patients received concomitant MTX with an average dose of 24.1 mg a week. In this cohort, 57 patients (18%) used adalimumab as monotherapy during evaluation period.
After 3 months of treatment with adalimumab, 174 (54%) and 103 (32%) of the patients responded according to the EULAR good response and EULAR remission criteria, respectively (table 1). No statistical differences in response rate between overall included patients and genotyped patients were observed (data not shown).

Characteristics	Value
Number of RA patients	325
Age -years (mean, sd)	56 (12)
Gender -female (%)	228 (71)
Concurrent MTX (%)	266 (82%)
MTX dose/week in mg (mean, sd)	24.1 (3.8)
Previous biological agent (%)	18 (6%)
DAS28 at baseline (mean, sd)	5.8 (0.98)
DAS28 at 3 months (mean, sd)	3.1 (1.1)
ΔDAS (mean, sd)	2.7 (1.0)
Relative change in DAS28 in % (mean)	46.1
Good response according to EULAR criteria (%)	174 (54)
Remission according to EULAR criteria (%)	103 (32)

Table 1. Study population characteristics[a,b]

a) Presented are numbers of patients (%) unless otherwise indicated.
b) Abbreviation(s): DAS28= 28 joint disaese activity score, MTX=methotrexate, EULAR= European League Against Rheumatism, sd= standard deviation

Primary endpoints

For EULAR good response, initial association analyses of 196 SNPs in 315 patients under allelic and genotypic model revealed 33 SNPs ($p < 0.1$) as candidates for further examination according to the best-fit genetic model. Additionally, multivariate regression analysis including covariates age, gender, concomitant MTX therapy and DAS28 at baseline, assessed for 19 SNPs significant associations with good response ($p < 0.05$) (table 2). In this group, 2 polymorphisms in the *TANK* and *TNFAIP* genes were most significantly associated (for both SNPs $p = 0.008$, table 2). When a multivariate analysis was performed with all 19 SNPs and covariates included, the SNPs *CD40LG* (rs1126535), *CSF1R* (rs10079250), *ICAM* (rs5498), *PECAM1* (rs4968622), *TANK* (rs1267067) and *TNFAIP3* (rs2230926) remained significant ($p < 0.05$, table 2)

Gene	SNP (rs number)	MAF	Best-fit genetic model	P-value[b]	OR (95% C.I.)[b]
ADAM 17	rs1048610 (C>T)	0.47	Additive: CC > CT > TT	0,035	1,4 (1,0-2,0)
C3	rs344543 (G>C)	0.41	Additive: CC > CG > GG	0,044	1,4 (1,0-2,1)
CD40LG[c]	rs1126535 (T>C)	0.17	Recessive: T-allele vs. CC	0,013	5,1 (1,4-18)
CSF1R[c]	rs10079250 (A>G)	0.07	Dominant: AA vs. G-allele	0.037	2.1 (1.0-4.2)
FCRL3	rs7528684 (A>G)	0.42	Dominant: AA vs. G-allele	0,029	1,7 (1,1-3,0)
ICAM1[c]	rs5498 (A>G)	0.42	Dominant: AA vs. G-allele	0,048	1,6 (1,0-2,7)
IL1A	rs1304037 (A>G)	0.33	Recessive: A-allele vs. GG	0,012	3,2 (1,3-7,9)
IL1RAP	rs13321840 (T>G)	0.29	Additive: TT>GT>GG	0,011	1,6 (1,1-2,4)
IL4R	rs1049631 (A>G)	0.47	Recessive: GG vs. A-allele	0,022	2,0 (1,1-3,6)
KDR	rs6828477 (T>C)	0.41	Dominant: TT vs. C-allele	0,041	1,4 (1,0-2,1)
PADI4	rs2240340 (G>A)	0.42	Dominant: GG vs. A-allele	0,013	1,9 (1,1-3,2)
PECAM1[c]	rs4968622 (G>A)	0.45	Dominant: A-allele vs. GG	0,020	1,9 (1,1-3,2)
TANK[c]	rs1267067 (T>C)	0.32	Additive: CC>CT>TT	0,008	1,7 (1,1-2,5)
TGFB1	rs1800469 (C>T)	0.28	Recessive: C-allele vs. TT	0,027	3,5 (1,1-10,5)
TGFB1	rs2241715 (G>T)	0.27	Recessive: G-allele vs. TT	0,028	3,4 (1,1-10)
TNFAIP3[c]	rs2230926 (T>G)	0.04	Additive: TT>GT>GG	0,008	4,0 (1,4-11)
TNFRSF11B	rs11573885 (G>A)	0.43	Additive: AA>AG>GG	0,036	1,5 (1,0-2,1)
TNFRSF11B	rs1485286 (T>C)	0.29	Additive: CC>CT>TT	0,036	1,5 (1,0-2,2)
VEGFA	rs25648 (C>T)	0.16	Additive: TT>CT>CC	0,021	1,8 (1,1-3,0)

Table 2. Association between SNPs and adalimumab efficacy (EULAR good response at 14 weeks) according to the best-fit genetic model (p<0.05)[a,b,c]

a) Abbreviation(s): EULAR= European League Against Rheumatism, MAF= minimal allele frequency, OR= odds ratio, 95% C.I.= 95 % confidence interval, SNP=single nucleotide polymorphism. Abbreviations and accessory full names of formal genes can be relocated in the NCBI gene database *(http://www.ncbi.nlm.nih.gov)*.
b) P-values and odds ratios with 95% confidence intervals are calculated by logistic regression analysis, adjusted for baseline DAS28, gender, age and concomitant MTX treatment according to best-fit genetic model.
c) When a multivariate analysis was performed with all 19 SNPs and covariates included, these SNPs remained significant (p<0.05)

Regarding the analysis of association with EULAR remission, 27 SNPs of the included 196 SNPs were further analysed (p<0.1) under additive, recessive and dominant genetic model. Within this set of potential SNPs, 11 SNPs were significantly associated with remission (p<0.05, table 3). Specifically, associations were observed with *CD69* (rs10844706, p= 0.035), *FLT1* (rs748253, p=0.031), *PTPN22* (rs2476601, p= 0.031), Region Chr1p34 (rs17534243, p= 0.010), *TANK* (rs1267067, p=0.006) and *VEGFA* (rs25648, p= 0.045) according to an additive genetic model; with *KDR* (rs6828477, p=0.016), *PECAM1* (rs12953, p=0.042), *RSBN1* (rs6679677, p=0.030) and *VWF* (rs1063856, p=0.022) according to a dominant genetic model and with *CD40LG* (rs1126535, p=0.048) according to a recessive genetic model (table 3). When a multivariate analysis was performed with all 11 SNPs and covariates included, the SNPs *KDR* (rs6828477), *PECAM1* (rs12953), Region Chr 1p34 (rs17534243), *TANK* (rs1267067) and *VWF* (rs1063856) remained significant (p<0.05, table 3).

Gene	SNP (rs number)	MAF	Best-fit genetic model	P-value[b]	OR (95% C.I.)[b]
CD69	rs10844706 (C>A)	0.36	Additive: AA>AC>CC	0.035	1.5 (1.0-2.3)
CD40LG	rs1126535 (T>C)	0.17	Recessive: T-allele vs. CC	0.048	1.9 (1.0-3.6)
FLT1	rs748253 (G>T)	0.40	Additive: TT>GT>GG	0.031	2.2 (1.1-4.4)
KDR[c]	rs6828477 (T>C)	0.41	Dominant: TT vs. C-allele	0.016	2.0 (1.1-3.5)
PECAM1[c]	rs12953 (A>G)	0.48	Dominant: AA vs. G-allele	0.042	1.8 (1.0-3.3)
RSBN1	rs6679677 (C>A)	0.17	Dominant: CC vs. A-allele	0.030	2.0 (1.1-3.7)
PTPN22	rs2476601 (G>A)	0.17	Additive: GG>AG>AA	0.038	1.8 (1.0-3.1)
Region Chr1p34[c]	rs17534243 (A>G)	0.23	Additive: GG>AG>AA	0.016	1.7 (1.1-2.7)
TANK[c]	rs1267067 (T>C)	0.32	Additive: CC>CT>TT	0.006	1.8 (1.2-2.7)
VEGFA	rs25648 (C>T)	0.16	Additive: TT>CT>CC	0.045	1.7 (1.0-3.0)
VWF[c]	rs1063856 (A>G)	0.34	Dominant: AA vs. G-allele	0.022	1.9 (1.1-3.3)

Table 3. Association between SNPs and adalimumab efficacy (EULAR remission at 14 weeks) according to the best-fit genetic model (p<0.05)[a,b,c]

a) Abbreviation(s): EULAR= European League Against Rheumatism, OR= odds ratio, 95% C.I.= 95 % confidence interval, SNP=single nucleotide polymorphism. Abbreviations and accessory full names of formal genes can be relocated in the NCBI gene database *(http://www.ncbi.nlm.nih.gov)*.
b) P-values and odds ratios with 95% confidence intervals are calculated by logistic regression analysis, adjusted for baseline DAS28, gender, age and concomitant MTX treatment according to best-fit genetic model.
c) When a multivariate analysis was performed with all 11 SNPs and covariates included, these SNPs remained significant (p<0.05)

Secondary endpoints

In total, 30 SNPs were examined for association with a relative change in DAS28 according to the SNPs' best-fit model. Linear regression analyses revealed 8 significant associations (p<0.05). Specifically, associations were demonstrated with *ADAM17* (rs1048610, p= 0.002), *TNFRSF11B* (rs1485286, p=0.019), *TANK* (rs1267067, p= 0.010) and *VEGFA* (rs25648, p= 0.025) according to an additive genetic model; with *FCRL3* (rs7528684, p= 0.004), *KDR* (rs6828477, p= 0.003), *PECAM* (rs12953, p= 0.026) according to a dominant genetic model and with *CD40LG* (rs1126535, p= 0.016) according to a recessive genetic model (data not displayed in table).
Notably, four SNPs were significantly associated with the analyses according to all three endpoints: *CD40LG* (rs1126535), *KDR* (rs6828477), *TANK* (rs1267067) and *VEGFA* (rs25648) (table 4).

	MAF	**EULAR Good response**		**EULAR Remission**		**Relative decrease of DAS28 in %**	
		P-value	*OR (95% C.I.)*	*P-value*	*OR (95% C.I.)*	*Response differences in %*	*P-value*
***CD40LG* (rs1126535)** Recessive genetic model: T-allele carriers vs. CC	0.17	0.013	5,1 (1,4-18)	0.048	1.9 (1.0-3.6)	46.8 vs. 36.9	0.016
***KDR* (rs6828477)** Dominant genetic model: TT vs. C-allele carriers	0.41	0,041	1,4 (1,0-2,1)	0.016	2.0 (1.1-3.5)	49.9 vs. 44.2	0.003
***TANK* (rs1267067)** Additive genetic model: CC>CT>TT	0.32	0,008	1,7 (1,1-2,5)	0.006	1.8 (1.2-2.7)	44.1>52.3>54.8	0.010
***VEGFA* (rs25648)** Additive genetic model: TT>CT>CC	0.34	0,021	1,8 (1,1-3,0)	0.045	1.7 (1.0-3.0)	44.9>54.0>57.9	0.025

Table 4. Association of SNPs in *CD40LG*, *TANK*, *VEGFA* and *KDR* according to EULAR criteria and relative change in DAS28[a,b]

a) Abbreviation(s): EULAR= European League Against Rheumatism, OR= odds ratio, 95% C.I.= 95 % confidence interval, SNP=single nucleotide polymorphism. B= regression coefficient, S.E.= standard error, KDR= kinase insert domain receptor, MAF= minimal allele frequency, TANK= TRAF family member-associated NFKB activator, VEGF= vascular endothelial growth factor, DAS28= 28-joint count disease activity score.
b) P-values, odds ratios with 95% confidence intervals and regression coefficients with standard errors are calculated by logistic regression analysis and linear regression analysis, respectively. In these analyses adjustments for baseline DAS28, gender, age and concomitant MTX treatment according to best-fit genetic model were made.

Discussion

In this explorative study, our aim was to determine whether SNPs in genes related to the mechanism of action of adalimumab were associated with efficacy of this biological DMARD. Furthermore, SNPs from previous research on pharmacogenetics of TNF inhibitors in RA and susceptibility to RA were included for analysis. It was demonstrated that 19 SNPs, 11 SNPs and 8 SNPs were significantly associated with EULAR good response, EULAR remission and relative change in DAS28, respectively. Four SNPs, rs1126535 in *CD40LG*, rs6828477 in *KDR*, rs1267067 in *TANK* and rs25648 in *VEGFA* showed consistent associations and, therefore, they appear to be the most predictive for adalimumab efficacy.

The pathway pharmacogenetic method used in this study has an advantage over the classical usage of the candidate gene approach and the whole genome approach. The main feature of this method is that a set of SNPs is selected based on a description of pathways regarding the mechanism of action of the drug under study (12). With the candidate gene method, SNPs that are responsible for the rate limiting or extending step in the mechanism of action of adalimumab are easily missed. Therefore, it seems likely that for most drugs and complex diseases, like RA, pharmacogenetics has more potential if information on multiple genes is used. Numerous proteins are involved in the mechanism of action and, therefore, genetic variability in each gene may contribute to the overall variability in drug response.

The four most potential SNPs, *CD40LG* (rs1126535), *KDR* (rs6828477), *TANK* (rs1267067) and *VEGFA* (rs25648) are rs numbers close to genes encoding proteins involved in the mechanism of action of adalimumab. 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 proinflammatory cytokines and chemokines after CD40-CD40LG ligation (27). CD40LG is an extracellular target for TNF inhibitors. This was also demonstrated in a study of Danese et al (28), which measured reduced levels of CD40LG with infliximab. 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 synonymous SNP rs1126535 in the gene encoding the CD40LG protein. Concerning our results, patients genotyped for CD40LG (rs1126535) CC may have less binding capacity to TNF inhibitors compared with patients genotyped carrying a *CD40LG* (rs1126535) T-allele (table 4).

Vascular endothelial growth factor (*VEGF*) is an important protein involved in angiogenesis. Since it is demonstrated that expression of *VEGF* is reduced in RA patients treated with TNF inhibitors, it is thought that these biologicals may interfere with the angiogenesis seen in the inflammatory process of RA (15). Regarding our selected SNP *VEGFA*-rs25648, patients genotyped for a homozygous mutant genotype (rs25648-TT) achieved a better response than patients genotyped for carrying the wildtype C-allele (rs25648-CT, rs25648-CC). Hypothetically, the mutant genotypes may assist TNF inhibitors in reduction of angionesis in RA by facilitating decreased expression of VEGFA enzyme.

Also, VEGF acts by binding with to tyrosine kinase receptors (VEGFR), of which KDR (kinase insert domain receptor) is the primary receptor for mediation of VEGF's action. In the study of Cañete et al it was observed that reduction of synovial angiogenesis after therapy with TNF inhibitor therapy in psoriatic arthritis patients could be related to modulation of molecular factors, like KDR, involved in this process (29). Hypothetically, regarding our results for rs6828477, indicate that carrying the C-allele may have a decreased modulation of KDR by adalimumab leading to a lower response compared with patients with a TT genotype.

In addition, TANK, a protein coding for TRAF family member-associated NFKB activator, is part of the TRAF family which mediates signals from cytokines signals through cell surface receptors and, hereby, activating downstream intracellular signaling cascades. TANK binds to TRAF1, TRAF2 and TRAF3. It is seen that the mechanism of action of TNF inhibitors includes the initiation of reverse intracellular signaling cascade by binding of antibodies to transmembrane TNF. This may lead to a decreased production of proinflammatory cytokines (like TNFα), increased production of anti-inflammatory cytokines (like IL10) and induction of apoptosis in cells (14;15;30). In our results, patients with a homozygous mutant genotype (rs1267067-CC) were more likely to achieve clinical response than patients carrying a wildtype allele (rs1267067-TT or rs1267067-CT). Hypothetically, this polymorphism may support the reverse signaling by less binding of TRAF1-TRAF3 to TANK. Generally, along with replication of these explored SNPs in other patient cohorts and determination whether these SNPs provide the strongest association compared with other adjacent variants in the gene, functional studies remain to be performed (31). In this way, beneficial- and/or risk genotypes involved in efficacy of TNF inhibitors could be biologically identified.

Concerning susceptibility to RA, GWA studies have provided new insights in the genetic risk profile of an individual RA patient. These genetic variants have strong evidence to be associated with RA and include SNPs in the *TRAF1-C5*, *OLIG3-AIP3* and *STAT4* gene regions. Consequently, genetic findings led to improved knowledge regarding the pathogenesis of this immune-mediated disease. Likewise, these findings could have implications on pharmacogenetics in RA. As is seen in other medical research areas, it seems plausible that a pharmacogenetic response in RA depends on the interaction of genes involved in anti-rheumatic drug metabolism and genes associated with RA's pathogenesis (32;33). However, several studies have been performed on the predictive value of these variants for response to therapy with TNF inhibitors, but with inconclusive results to answer the question whether in general genes contributing to disease susceptibility may play a role in determining response to treatment. In our analyses, associations were detected with *TNFAIP* (rs2230926), *PADI4* (rs2240340), *FCRL3* (rs7528684), *RSBN1* (rs6679677), Region Chr. 1p34 (rs17534243) (tables 2 and 3). Still, these results were not as consistent as the results for rs1126535 (*CD40LG*), rs6828477 (*KDR*), rs1267067 (*TANK*) and rs25648 (*VEGFA*) based on all three endpoints.

In our study, no adjustment for multiple testing was performed. Assessing a large number of SNPs for association creates potential false positive results and additionally the need for adjustment for multiple testing. Generally, the need for multiple testing arises from the assumption that the incidence of false positives is proportional to the number of test performed and level of significance. The most common method for dealing with multiple testing, the Bonferroni correction, involves adjusting the significance level of each test by the total number of performed tests (34). However this method has a conservative character, since interaction and cooperation between causative genes are not recognized (35;36). Consequently, studies may be willing to risk higher frequencies of false positives, instead of finding no associations at all due to false negative associations. Ideally, replication in a second comparable cohort of patients would be optimal, but not always feasible. Therefore, we have decided to present the p-values of this explorative study without adjustments to make the results accessible for clear interpretation.

Still, we would like to underline that we have performed multiple independent tests (e.g. variable endpoints) and that we recognize that the consideration of a cutoff value of $p=0.05$ for level of significance in this study is inaccurate. Therefore our results need to be marked as suggestive for association with adalimumab efficacy.

Generally, future research should be done to confirm our significant results. Particularly, replication of our results in patients treated with different TNF inhibitors remains a challenge. However, recently in a report from Bowes et al (7), stratified analysis for infliximab and etanercept was per-

formed. No significant differences were observed, suggesting that response was not subject to drug-specificity. Moreover, their results confirm the general applicability of our pathway pharmacogenetic approach, since this approach is based on the mechanism action of the class of TNF inhibitors.
In conclusion, primary analyses in this study have revealed new SNPs, rs1126535 in *CD40LG*, rs6828477 in *KDR*, rs1267067 in *TANK* and rs25648 in *VEGFA*, which may be involved in the efficacy of adalimumab. In this way, the results from this explorative study provide new insights regarding the potential of pharmacogenetics of adalimumab in RA.

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Chapter 11:

Summary

Rheumatoid arthritis (RA) is prevalent in approximately one percent of all types of populations. Characteristically, this immune-mediated disease is related with symmetrically inflammation, destruction of the joints leading to overall functional impairment and (serious) comorbidity.
Regardless of the increasing comprehension on the etiology and pathogenesis, a therapy resulting in remedy of the disease is not achieved to date. Consequently, in order to gain optimal benefit treatment is aimed at remission of disease by opposing the immune response with disease-modifying antirheumatic drugs (DMARDs). However, in practice suboptimal results are achieved with the use of DMARDs including methotrexate (MTX) and Tumor Necrosis Factor (TNF) inhibitors. Namely, highly differential response rates in overall clinical efficacy and/or toxicity have been observed in clinical trials with MTX and TNF inhibitors. Partly, pharmacogenetics is responsible for this variance in response. Therefore, the primary focus of this thesis is to assess the role of pharmacogenetics in the variation of treatment outcome in patients diagnosed with rheumatoid arthritis and treated with the DMARDS MTX and adalimumab.

Methotrexate

Initially, in the first part of this thesis an overview was presented of previously performed studies concerning genetic variability contributing to differences in response to MTX in RA treatment (**chapter 2**). Most pharmacogenetic studies have an insufficient sample size (power) to detect true associations with treatment response. In addition, other factors, like nongenetic factors, ethnicity and clear endpoints, influence treatment outcome. Therefore, definitive conclusions about the role of genetic prognostic factors in treatment outcome to MTX cannot be drawn from this literature study.
As it is generally accepted that MTX may act in RA through inhibition of folate pathway enzymes, other reports indicate that efficacy may also be related to the release of endogenous antiinflammatory adenosine. With this hypothesis, the relationship between SNPs in genes related to adenosine release and MTX treatment outcome in patients with recent-onset RA was explored in **chapter 3**. Results of this analysis did show an association between allelic variants in the adenosine monophosphate deaminase (*AMPD*), 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (*ATIC*), and inosine triphosphate pyrophosphatase (*ITPA*) genes and clinical response to MTX therapy in patients with recent-onset RA. Patients carrying the *AMPD1* T allele, the *ATIC* CC genotype, or the *ITPA* CC genotype were 2–3 times more likely to have a good clinical response, defined by a disease activity score (DAS) of ≤2.4, following 6 months of MTX therapy. Additionally, the rate of good clinical response increased substantially in patients carrying the 3 favorable genotypes. With regard to the occurrence of adverse drug events, the only association found was with the *ATIC* G allele. However, this association was not significant after adjustment for multiple testing. No associations between SNPs in the genes methionine synthase or methionine synthase reductase and MTX efficacy or toxicity were found.
So far, most genetic variants are selected for analysis based upon their hypothetical relation to the mechanism of MTX or inflammatory process in RA (**chapters 2 and 3**). Ideally, functional genetic variants are chosen because the alteration in protein function is thought to influence drug action and thus may explain interindividual differences in drug response. **Chapter 4** assessed the role of SNPs in genes with proven functional consequences on efficacy and toxicity of MTX in the BeSt cohort. It was observed that toxicity was potentially associated with *ABCB1* 3435C/T and *TLR4* +896A/G. However, none of these associations remained significant after (Bonferroni) correction for multiple testing. No significant associations of *DHFR* 829C/T, *ABCB1* 3435C/T, *ITPA IVS2* +21A/C, *HLA-G*

-/+14bp, *IMPDH2* +787C/T, *TGFB1* +869T/C and *TLR4* +896A/G with MTX efficacy were found. Additionally in this chapter, results from previous research according to reported endpoints were replicated in our cohort. Particularly, replication analyses are important, since pharmacogenetic studies have the potential to result in reporting false positive findings. However, no significant results were detected with these analyses in our cohort.

Previously, a clinical pharmacogenetic predictive model was developed for predicting the efficacy of MTX monotherapy in patients with recent-onset RA comprising the Dutch BeSt Cohort. The model consists of non-genetic factors sex, rheumatoid factor and smoking status, Disease Activity Score (DAS) before starting MTX and 4 genetic polymorphisms (*MTHFD1* 1958G>A, *AMPD1* 34C>T, *ITPA* 94A>C and *ATIC* 347C>G). With this model, a true positive predictive vale of 95%, true negative predictive value of 86% and categorization of 60% of the patients was achieved. In **chapters 5 and 6** the performance of the predicitve model was validated in a second Dutch cohort (chapter 5) and in a Swedish cohort (chapter 6).
In **chapter 5,** it was demonstrated that the clinical pharmacogenetic model to predict MTX monotherapy efficacy in patients with established RA does not perform as good as in DMARD naïve patients with recent-onset RA (BeSt cohort). Namely, it was found that the model had lower true positive and negative predictive values (47% and 81%, respectively) compared with the true positive and negative predictive values reported in the BeSt cohort (95% and 86%, respectively). Several explanations may be responsible for these findings: variation in RA disease duration and history of DMARD use; lack of (pharmacogenetic) association with components of the model in the second cohort; the presence of differences in tight control strategy of RA and, consequently, the variance in dosage between the two cohorts leading to different response rates.
In **chapter 6**, a model for predicting the efficacy of MTX monotherapy was validated in a cohort of DMARD naïve and early RA patients originated from the Swefot trial. Similarly as in **chapter 5**, both true predictive values observed in patients of the validation cohort were significantly lower compared to the values found in the original BeSt cohort (95% and 86%, respectively), since application of this pharmacogenetic model resulted in a true positive predictive value of 70% and a true negative predictive value of 68%. However, for the predictive values accuracy, number of patients classified and discriminative ability (AUC) the results were comparable between the Swefot cohort and BeSt cohort (for accuracy 48% vs. 53%: p=0.572; for number of patients classified 70% vs 60%: p=0.182 and for AUC 75% vs. 85%: p=0.111, respectively).
Overall, these validation data imply that efficacy of a substantial part of early RA patients treated with MTX could be predicted by this clinical pharmacogenetic model, but that this model may exclusively be applicable in DMARD naïve RA patients with short duration of disease. Additional replication and (ideally) performance of prospectively designed studies with this model in large cohorts is warranted to demonstrate the legitimate predictive value in rheumatology practice.

Chapter 7 evaluates the role of the haplotypes comprising the SNPs MTHFR 1298A>C and MTHFR 677C>T in treatment outcome to MTX in RA. Specifically, in this chapter optimalization of a previously designed pharmacogenetic model was aimed with addition of the number of haplotypes comprising MTHFR 1298A-677C alleles as additional criterion. Furthermore, the predictive value of the most predictive number haplotype is compared with the SNPs *AMPD1* 34C>T, *ITPA* 94C>A, *ATIC* 347C>G and *MTHFD1* 1958G>A involved in predicting MTX efficacy. It was observed that the predictive performance of the pharmacogenetic model to predict the efficacy of MTX therapy in this group of early RA patients was not improved when the *MTHFR* haplotype was included in the model. Moreover, the discriminative effect for the prediction of MTX efficacy including 1 or 2 or (solely) 2 copies of the 1298A and 677C haplotype was significantly smaller compared with the four SNPs

AMPD1 34C>T, *ITPA* 94C>A, *ATIC* 347C>G and *MTHFD1* 1958G>A. These results suggest that a (leading) role for the *MTHFR* 1298A and 677C haplotype with regard to predicting efficacy of MTX monotherapy in early RA patients seems unlikely. Future research is necessary to elucidate the exact pharmacogenetic and biological role of *MTHFR* 1298A>C and *MTHFR* 677C>T and their haplotypes in the efficacy of MTX in RA.

Adalimumab

At the start of this second part of the thesis an overview was presented of reports on associations between genetic variants and the drug efficacy of TNF inhibitors in RA (**chapter 8**). Similar to reports concerning pharmacogenetics of MTX in RA, the majority of pharmacogenetic studies were underpowered to detect accurate associations.
In **chapter 9**, SNP selection for pharmacogenetic association studies was discussed. Additionally, a pharmacogenetic pathway approach is presented together with proposed criteria for systematic selection of SNPs. These comprise the following genetic characteristics of SNPs: heterozygosity, validation, ethnicity, functionality, linkage disequilibrium and Tag SNPs. With the application of these criteria, an objective selection can be achieved: 186 SNPs in 111 genes out of 51,793 SNPs in 124 genes were included. Specifically, this method was applied for the selection of potential interesting SNPs within genes related involved in the mechanism of action of adalimumab and/or inflammatory process of RA. This approach has several advantages over either the candidate gene approach or the genome wide SNP analysis. First, because the rate limiting step in the described pathway is unknown, this systems pharmacology approach provides a solution: variability in the entire pathway is explored. In fact, the relative contribution of the different SNPs in the pathway to the explanation of variability to drug response can be assessed. Secondly, an important statistical advantage is present: the chance of false-positive results is lower compared to the genome-wide method, because of decreased multiple testing.

Chapter 10 put the presented systematically selection of SNPs in chapter 9 into practice: efficacy of treatment with adalimumab was associated with genetic variants selected by a pharmacogenetic pathway approach using a custom made anti-TNFα SNP array. Additionally, in this chapter SNPs from previous research on pharmacogenetics of TNF inhibitors in RA and susceptibility to RA were included for analysis. Results elucidated 19 SNPs, 11 SNPs and 8 SNPs, which were significantly associated with EULAR good response, EULAR remission and relative change in DAS28, respectively ($p<0.05$). Four SNPs, rs1126535 in *CD40LG*, rs6828477 in *KDR*, rs1267067 in *TANK* and rs25648 in *VEGFA* demonstrated the most evidence for potentiality in determining adalimumab therapy outcome, since these SNPs were significantly associated according to all three primary and secondary endpoints ($P<0.05$). Notably, p-values of this explorative study were presented without (Bonferroni) adjustments to make the results accessible for clear interpretation. Ideally, replication in a second comparable cohort of patients would be optimal, but not always feasible. Nevertheless, results from this explorative study provide new insights regarding the potential of pharmacogenetics of adalimumab in RA.

Chapter 12:

General discussion and future perspectives

Rheumatoid arthritis (RA) is a chronic autoimmune disease, which is characterized by inflammation leading to destruction and impairment of principally the joints. Treatment is aiming at limiting the progress of disease activity and involves the use of disease-modifying antirheumatic drugs (DMARDs) including methotrexate (MTX) and Tumor Necrosis Factor (TNF) inhibitors. Many clinical trials have demonstrated successful results with these classes of drugs underlining their suitability in the tight scheduled management of RA's disease progress. However, a considerable proportion of the patients do not experience a positive response. Especially, these patients are at risk of developing progressive and erosive RA. In the light of optimal management of individual patients, the idea of a priori prediction of drug response is considered an important achievement. It will enable physicians to readily select those patients sensitive to certain drug regimens and thereby minimizing irreversible joint dysfunction existing in severe untreated RA. Hereby, an important role is being played by pharmacogenetics as it is thought that drug response is, at least partly, a heritable trait.
Important progress has been made regarding pharmacogenetics in rheumatology in the last decade. Basically, explorative studies focusing on the pharmacokinetics and pharmacodynamics of antirheumatic agents have contributed to the introduction of new genetic markers. Still, consensus regarding their potential implication has not been reached. It is observed from previous research (**chapters 2 and 8**) and from the results presented in this thesis that definitive conclusions regarding the influence of genetics on treatment outcome to DMARDs can not be drawn. These differences in outcome are the result of a several factors, in which variance in study design plays an important role.
In this discussion a focus is placed on the interpretation and implication of potential pharmacogenetic findings. Hereby, answers are sought to the following questions: "What are the major points of concern in RA study design contributing to interpretation difficulties of pharmacogenetic results?" and "Which factors could be of influence in the implementation of proven genetic associations in rheumatology clinical practice?" Furthermore, an ideal study proposal for a prospective study concerning MTX and TNF inhibitors in RA is presented, in which strategy for therapy is guided by pharmacogenetics. In figure 1, a schematic overview of this chapter is provided.

Design and interpretation of pharmacogenetic studies

Power and sample size
In general, appropriate sample size is characterized by consideration of statistical power, which is the probability of correctly rejecting the null hypothesis in favor of the alternative hypothesis. Power analysis comprises selection of a sample size large enough to identify either a relation or effect size (1). Power calculation is optimally performed prior to analysis. In **chapter 10**, efficacy of treatment with adalimumab was associated with genetic variants related to the mechanism of action of TNF inhibitors and/or inflammatory process of RA. A power calculation analysis was performed to recognize the range of power in which the minimal allele frequencies (MAF) of the selected single nucleotide polymorphism (SNPs) would fall to find specific odds ratios (OR). In figure 2, this range is presented. With the appliance of a power calculation it was demonstrated that for a patient population of 325 patients, minimal allele frequencies (MAF) ranging from 10% to 50%, a response of 45% and a chosen type 1 error probability $\alpha=0.05$, a power of >80% could be achieved to detect an odds ratio ranging from 1.4 (MAF of 50%- line A) to 2.0 (MAF of 10%- line B) (figure 2). Line C represents a MAF of 5% indicating the lower chance of finding a reasonable odds ratio with the achievement of a power of >80%. Notably, in more than 80% of the selected SNPs the MAF was higher than 30%. Results elucidated 19 SNPs, 11 SNPs and 8 SNPs in the TNF pathway, which were

significantly associated for adalimumab with EULAR good response, EULAR remission and relative change in DAS28, respectively ($p<0.05$). In the majority of the associated SNPs odds ratios and corresponding MAF was observed to achieve a power of >80% (tables 1,2 and 3 of **chapter 10**).

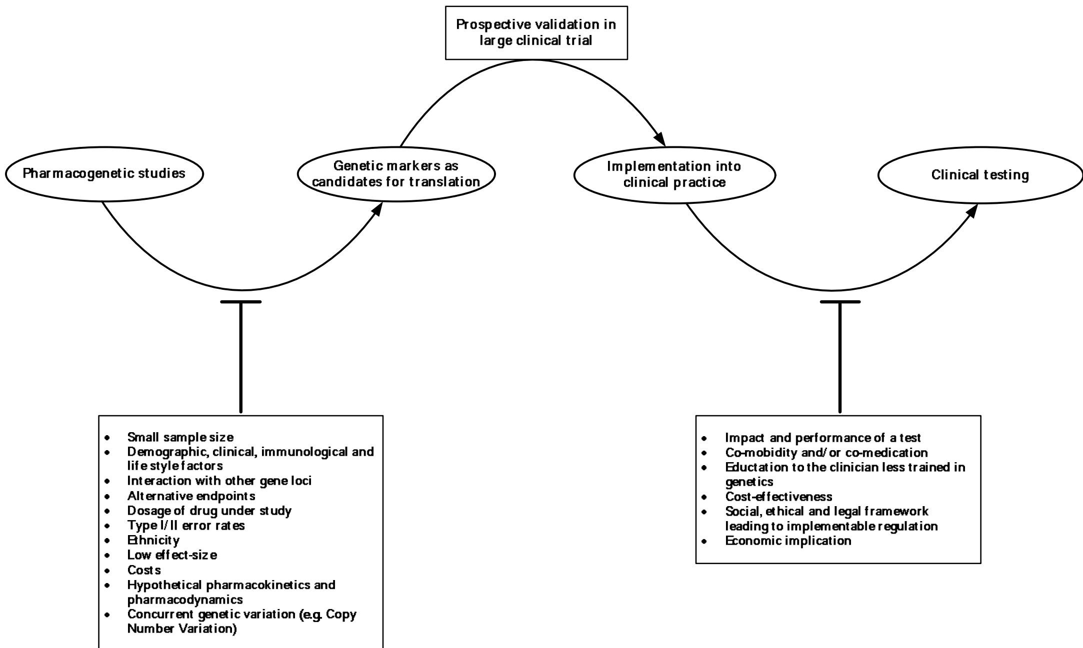

Figure 1. Stages towards implementation of pharmacogenetic markers into clinical practice

Stage 1: Before prospective validation in large studies of potential genetic polymorphisms can be performed, several aspects have to be noticed (see most left rectangle)
Stage 2: Next to general factors comprising cost-effective, regulatory and ethical aspects, several challenging factors appear before implementation of a pharmacogenetic marker as a tool in rheumatological clinical practice can take place (see most right rectangle)

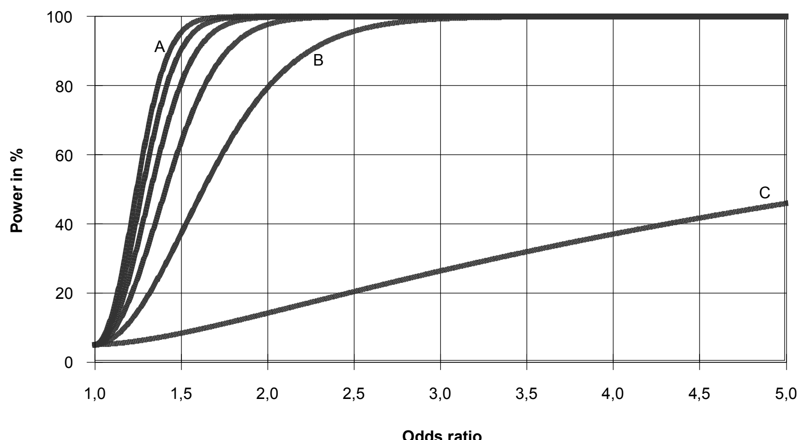

Figure 2. Power calculation analysis to recognize the range of power in which the minimal allele frequencies (MAF) differ to find specific odds ratios[a,b]

a) For a patient population of 325 patients, minimal allele frequencies (MAF) ranging from 5% to 50%, a response of 45% and a chosen type 1 error probability α=0.05, a power of >80% could be achieved to detect an odds ratio ranging from 1.4 (MAF of 50%- **A**) to 2.0 (MAF of 10%- **B**). Line **C** represents a MAF of 5% indicating the lower chance of finding a reasonable odds ratio with the achievement of a power of >80%. Curves between lines A and B represent MAFs of 40% (most left), 30% and 20% (most right).
b) Abbreviation(s): MAF= minimal allele frequency

In many pharmacogenetic studies concerning DMARDs low power is observed due to the use of small sample sizes. Consequently, reported p-values and effect sizes for efficacy and toxicity are difficult to interpret. Also, with the interpretation of underpowered studies a suboptimal reference is provided for future studies (2;3), since replication studies could expect finding smaller effect sizes than originally reported (4;5). For example, if a power calculation is performed to confirm an initial association with an odds ratio of 3.5, hypothetically, a smaller effect size of approximately 2.0 may be expected. Therefore, for the performance of replication studies a larger sample size may be considered to identify the smaller effect size (see also Figure 2 of **chapter 8** in this thesis).
If the calculated power turns out to be small, cooperation with other research groups is attractive. Yet, in practice cooperation is challenging because of regulatory and organizational problems to combine patient cohorts (6).

Ethnicity
Conflicting results may be explained by different frequencies of polymorphisms among ethnic populations, which makes association studies less likely to compare (34). For example, this is highlighted in a study in which an association of *MTHFR* 677C>T and MTX-related alopecia in only African Americans was demonstrated (35). Therefore, to compare results between studies, considering ethnicity of the patient population is appropriate. For *MTHFR* 677C>T the MAF of this SNP in the African American population is 0.098, compared with a MAF of 0.24 in the Caucasian population (NCBI database).
These differences in frequency has also consequences for haplotypes concerning these SNPs. Significant differences in haplotype distribution between Caucasians and African-Americans were observed in the study of Hughes et al (7). Hughes et al (7) reported that the D-prime (D') value, a value

ascribing linkage disequilibrium (LD), for the two SNPs was 0.955, indicating strong LD. However, in African-Americans the D' value is much lower (0.408), indicating less linkage disequilibrium (www.hapmap.org). **Chapter 7** evaluates the role of the number of haplotypes comprising the SNPs *MTHFR* 1298A>C and *MTHFR* 677C>T in treatment outcome to MTX in RA. Analyses were performed in mainly Caucasian patients, which were derived from the BeSt study. It was observed that the predictive performance of the pharmacogenetic model to predict the efficacy of MTX therapy in this group of early RA patients was not improved when the *MTHFR* haplotype was included in the model. No significant associations were seen when differences in number of haplotypes were considered. Alternative values of LD could explain the different results seen in the reports of Urano et al (8) and Taniguchi et al (9), which studied the influence of the haplotype on response in patients with Asian backgrounds. Therefore, if allele frequencies and corresponding haplotypes are substantially differently distributed between ethnic populations, a genuine pharmacogenetic effect is difficult to observe. More important is whether the cohort of patients under study is large enough to limit a random change in genetic variation and to limit a sampling effect.

Nongenetic factors

Besides genetics as factors for drug response, demographic and clinical characteristics of patients are important to include for analysis in pharmacogenetic association studies. For example, several studies linked nongenetic factors to therapy outcome in patients treated with TNF inhibitors. Likewise, concomitant MTX usage and low disability have been demonstrated to predict optimal response to TNF inhibitor therapy (10;11). Moreover, Disease Activity Score (DAS) at baseline determines to a large extend the response of RA patients treated with DMARD therapy as was demonstrated from previous studies (12;13) and **chapters 3-6.** Previously, reciprocal comparison in multivariate regression analyses of 17 polymorphisms and 24 nongenetic factors in the BeSt cohort DAS at baseline was observed as most predictive (13). Scores for prediction of response regarding DAS at baseline were approximately 3 times larger than the SNP *ATIC* 347 C>G (13). This is in correspondence with the results demonstrated in **chapter 5,** which compared the same factors in a different cohort. For DAS at baseline and *ATIC* 347 C>G beta regression coefficient were 0.77 and -0.23, respectively.

Moreover, this was not only the case for MTX therapy, since DAS at baseline was also an important covariate in the multivariate-analyses for response of adalimumab, as demonstrated in **chapter 10**. In general, the results of a large influence of a nongenetic factor on therapy outcome emphasise the necessity of multivariate-analysis in pharmacogenetic association studies. On order to analyze this predictive effect, studies require longitudinal clinical data regarding the effect of a DMARD on disease activity. Moreover, if a wider focus is applied, interactions between gene and nongenetic factors similar to the observed interactions of genetic studies concerning susceptibility to RA may be concerned (13).

Drug dosage

An important feature in any pharmacogenetic study is drug dosage. In **chapter 5,** it was demonstrated that with the clinical pharmacogenetic model to predict MTX mono-therapy efficacy in patients with established RA smaller predictive values were calculated, compared with the calculated values in the DMARD naïve patients (BeSt cohort). It was found that the model had lower true positive and negative response rates (47% and 81%, respectively) compared with the true positive and negative response rates reported in the BeSt cohort (95% and 86%, respectively). Partly, different control strategy of RA and, consequently, the variance in dosage between the two cohorts leading to different response rates may have been responsible for differences of the model's performance.
Furthermore, drug dosage is necessary for the interpretation and comparison of a functional pharmacogenetic effect on treatment outcome. For example, in theory the cellular amount of Tumor Necrosis Factor-alpha (TNFα) and, thus the amount available for inhibition by TNF inhibiting agents, might depend on the genotype of the *TNF* gene (14;15). However, a higher drug dosage may lead to inhibition of more TNFα and may, therefore, overshadow a genetic effect. In contrast, a genetic effect may be assumed, which may be due to a lower dosage of the TNF inhibitor under study. Future research need to be performed concerning levels of TNFα and TNF inhibiting therapy.

Alternative use of response criteria

Various use of disease activity parameters and/or cutoff levels for the definition of response may contribute to different results observed in pharmacogenetic studies. In order to optimally compare studies or perform meta-analyses, criteria regarding efficacy and toxicity are standardized. Examples are response criteria according to the American College of Rheumatology (ACR) improvement criteria, which are based on a perceptual improvement (20, 50, 70 and 90%) in disease symptoms (termed ACR20, ACR50, ACR70 and ACR90, respectively) and EULAR criteria (defined in **chapter 10**).
Regarding pharmacogenetics and treatment outcome measurements in RA, studying defined groups of patients is challenging. In clinical trials frequencies of response according to disease activity scores after drug therapy are measured. Based on a selected cut off value, several types of frequency distribution curves can be drawn to divide response into two groups. Rarely, the distribution of drug responses is ideal bimodal (16;17). Instead, the frequency distribution curve for measures of response is mostly unimodal distributed (16). A unimodal distribution is consistent with a multifactor configuration caused by effects of many genetic and environmental factors, in which no single factor has a clearly large effect on response. In this way, it is difficult to study a subset of responders and nonresponders by the effects of a single genetic locus (18;19).

Selection of genetic variants for association analysis

Methods of selection

A clear design in selection of genetic variants is relevant for the interpretation of results of pharmacogenetic association studies in RA. Predominantly, the presented candidate genetic factors in these studies are selected based on current knowledge of mechanism of action of the drug (20). With this approach functional genetic variants are chosen because the alteration in protein function is thought to influence drug action and, thus, may explain interindividual differences in drug response. This is demonstrated in **chapter 4**, in which 7 SNPs in genes with proven functional consequences were related to efficacy and toxicity of MTX in the BeSt cohort. Due to the fact that an exact mechanism of action of DMARDs is uncertain, a clear pathway for selection of genetic variation coding for enzymes influencing these agents is challenging. The results from **chapter 3** may indicate that MTX therapy

works via the adenosine pathway, since *AMPD1* 34T allele, *ATIC* 347CC, or *ITPA* 94CC were associated with clinical response, as defined by a DAS of <2,4 (OR [95% confidence interval] 2,1 [1,0–4,5], 2,5 [1,3–4,7], and 2,7 [1,1–8.1], respectively). However, **in chapter 6**, no significant associations of these three SNPs with efficacy were found with a Swedish validation cohort under study (p>0.05). Therefore, it remains unclear, 1] whether the three variants are true markers for MTX response, 2] whether other variants in the three genes are responsible for the effect on treatment outcome, 3] whether other genes are involved. Future research on the mechanism of action of MTX is therefore required.
Since the mechanism of action of DMARDs is considered being polygenetic, selecting SNPs in a single gene will by definition only lead to a limited extent of explained variance of drug response. A solution toward these difficulties is the pathway pharmacogenetic approach, which considers variability in the entire pathway without restricting the analysis to only one gene. This method has advantages over either the candidate gene approach and the genome wide SNP analysis, as highlighted in **chapter 9**. In the same chapter, selection criteria for this approach to effectively explore potential associating SNPs with adalimumab are presented. With the application of these criteria, an objective selection can be achieved: 186 SNPs in 111 genes out of 51,793 SNPs in 124 genes were included for analysis in **chapter 10**.
Genome-wide association (GWA) studies may be a promising method for pharmacogenetic studies. GWA concerns a broad approach which rapidly assesses markers across the genome to elucidate genetic variation in patients compared to controls or responders compared to non-responders. As a result, in the last two years GWA studies have presented novel associations with susceptibility to RA taking care of its polygenetic variation (21;22). For example, SNPs within the *TRAF1-C5* gene region have been demonstrated to influence the susceptibility for RA (22). These novel genes form a new source of genetic variation potentially explaining variability in disease activity and treatment response in RA patients (23;24). Ultimately, next to the direct detection of new markers for treatment response, a pharmacogenetic GWA study could give new insights into the mechanism of action of antirheumatic agents.
On the other hand, various remarks could be placed regarding the results from GWA studies. This is due to the overall found small effect sizes (25) and difficult balance between type I errors and type II errors in presenting new associations (26;27).

Concurrent functional SNPs
Genetic variation in metabolic processes may be a confounder for interpretation of pharmacogenetic results. For example, in patients and healthy volunteers with genetic polymorphisms in the cytochrome P450 drug metabolizing enzymes CYP2D6 and CYP2C9 variation in pharmacokinetics of drug therapy have been demonstrated (38-40). CYP2D6 and CYP2C9 are involved in the pharmacokinetics of therapeutics, like anticoagulants, nonsteroidal anti-inflammatory drugs and hypoglycaemic drugs (40). Also, cytochrome P450 enzymes play also a role in metabolism of physiological substrates (41;42). Although the DMARDs in this thesis are not substrates for CYP2D6 and CYP2C9, genetic variation within these enzymes could be relevant for the drug response as outcome. For example, hepatotoxicity could not only be caused by genetic variants encoding enzymes involved in the metabolism of MTX, but also could be enhanced by SNPs encoding the cytochrome P450 enzymes involved in the physiological and pathological processes of the liver.

Confounding genetic variation
Besides SNPs, other types of genetic variation exist which could have an effect on treatment outcome. For example, a factor which can be of influence is copy number variation (CNV). CNV is defined as DNA segments, which are 1 kb or larger and present at variable copy number in comparison

with a reference genome. These segments are collectively termed copy number variants (28). Even though these variants are far less abundant in the genome, CNV account for more nucleotide variation on average than SNPs (29). Subsequently, a SNP effect on treatment outcome could be misinterpreted due to a CNV effect in the same gene region (30). One of the selected SNPs on the custom made array presented in **chapter 10** was the functional SNP *FCGR3A* -158T>G (rs396991). In previous studies, this SNP was associated with treatment outcome to TNF inhibitors (31-33). However, with our analyses presented in **chapter 10** an association with efficacy was absent. Hypothetically, CNV may cause a different interpretation of genotypes resulting in altered findings. From genome wide studies it has been observed that in the FCGR3A gene region CNVs are present (34). In this way, alternative genotyping results of *FCGR3A* -158T>G may be due to CNV: high copy number (more than 2 alleles) may lead to the detection of an inaccurate number of heterozygous genotypes and low copy number (one allele) may lead to more homozygous genotypes (35).
In addition, epigenetics could also be a reasonable confounder in finding (or not finding) genetic associations with treatment outcome. The term epigenetics covers phenotypic changes which are not covered by mutations in DNA sequence. It comprises grossly three different areas in which alteration could lead to changes in gene expression and enzyme activity: methylation of DNA, modification of histones in chromatin and RNA mediated regulation of gene-expression (36).
In summary, it is difficult to assign differences in treatment outcome solely to SNPs. Future research have to be performed to exactly study the weight of SNPs in differences in efficacy and/or toxicity.

Adjustment for multiple testing
Along with the discovery of novel causative loci for treatment response with GWA studies, testing a large number of loci for association creates potential false-positive results and, therefore, the need for adjustment for multiple testing (37). Similarly, but to a lesser extent, adjustment is necessary in studies applying a candidate- or pathway gene approach. The need for multiple testing arises from the assumption that the incidence of false positives is proportional to the number of tests performed and level of significance. For example, If 10,000 genes are tested, 5% or 500 genes might be found significant by chance alone. For this reason correction is important: it adjusts the individual p-value for each gene in order to keep the false positive- rate to less than or equal to the p-value cutoff. **Chapter 10** presented associations between efficacy of adalimumab therapy and SNPs selected by the pharmacogenetic pathway approach (**chapter 9**). It was demonstrated that 19 SNPs, 11 SNPs and 8 SNPs were significantly associated EULAR good response, EULAR remission and relative change in DAS28, respectively ($p<0.05$) Moreover, 4 SNPs, rs1126535 in *CD40LG*, rs6828477 in *KDR*, rs1267067 in *TANK* and rs25648 in *VEGFA* showed consistent associations and, therefore, they appear to be the most predictive for adalimumab efficacy. In this chapter, no adjustment for multiple testing was performed. The most common method for correcting for multiple testing, the Bonferroni correction, involves adjusting the significance level of each test by the total number of performed tests (38). However, this method has a conservative character, since interaction and cooperation between causative genes are circumvented (37;39). Also, other adjustments could be applied, like permutation testing or false discovery rate, but also these methods have specific difficulties (not discussed) (37). Replication of genetic associations in a second comparable cohort of patients is essential, but not always feasible. Therefore, we have decided to present the p-values of this explorative study without adjustments to make the results accessible for clear interpretation. Still, we would underline that multiple independent tests were applied and these results imply suggestive associations with adalimumab efficacy.
All statistical adjustments are focused on a certain level of significance. However, more weight should be on reporting effect size and confidence interval instead of p-values. For example, a mean

decrease in DAS for a specific genetic variant of 1.2 with a 95% confidence interval of 0.8 to 1.6 illustrates a range of values for what the mean decrease might be if the entire population is studied. This range of values highlights the importance of clinical values instead of statistical outcomes. Consequently, effect size and confidence intervals encourage meaningful qualitative decisions about quantitative data. In other words, a rheumatologist becomes more involved in the data and may evaluate its own clinical decision making in e.g. the additive value of genotyping patients in practice.

Future perspectives

Prospective study design for validation of pharmacogenetics in RA

In recent years, pharmacogenetic studies have revealed numerous SNPs that associated with drug response but only a few of these have been introduced as candidates for clinical implementation. Especially, prospective pharmacogenetic studies are scarce. One such prospective study concerns adverse drug reactions to abacavir in HIV-treatment (40). In this large study, the HLA-B*5701 polymorphism was highly linked to hypersensitivity reactions in a cohort of Caucasians and successfully replicated in other but similar cohorts. It was calculated that 14 patients would have to be screened, to prevent one hypersensitivity reaction on abacavir therapy (40). Currently, this polymorphism is increasingly being used as a genetic biomarker in routine clinical practice. Similar studies are needed to demonstrate the value of prospective genotyping for antirheumatic therapy in clinical practice. In figure 3 an ideal study proposal for a prospective study concerning MTX efficacy in RA is presented, in which strategy for therapy is guided by pharmacogenetics.
For this hypothetical study proposal, adult patients with early RA and active disease are enrolled. First a randomization (figure 3) is performed to assign patients to undergo prospective pharmacogenetic screening or to undergo a standard-of-care DMARD treatment without pharmacogenetic screening. Patients assigned to prospective pharmacogenetic testing are divided in predicted responders and predicted nonresponders based on the pharmacogenetic test determining MTX monotherapy efficacy. Predicted responders are allocated to treatment with MTX monotherapy. Predicted nonresponders to MTX are given the alternative traditional DMARD sulphasalazine. Patients assigned to the control group (without pharmacogenetic screening) are given MTX monotherapy as standard-of-care therapy. Therapy is evaluated and adjusted after 6 months. Hereafter, patients with standard-of-care DMARD treatment are screened for the pharmacogenetic test.
Primary analyses are focussed on statistical differences in response percentages between patient groups allocated to pharmacogenetic screening and the patient group allocated to standard-of-care treatment. As secondary analyses, the performance of the pharmacogenetic tests is calculated in the control group with standard-of-care treatment.
Power calculation reveals that enrolment of 300 patients for evaluation of at least 100 patients per group is needed for this prognostic study to have a statistical power of 90% (with a chosen type 1 error probability α=0.05) to detect an improvement in response of 50% in the groups with pharmacogenetic screening compared to the control group without pharmacogenetic screening.
Likewise, a more complex study design could also be applied for a prospective study including the pharmacogenetics of TNF inhibitors.
In conclusion, results of above described study could demonstrate the beneficial value of prospective pharmacogenetic screening compared to current standard therapy. Application of pharmacogenetic tests could reduce inefficacious and unnecessary drug exposure and thus treatment delay and toxicity in clinical practice. However, even with promising results from prospective studies, several challenges appear before a genetic marker can be implemented as a clinical tool (figure 1).

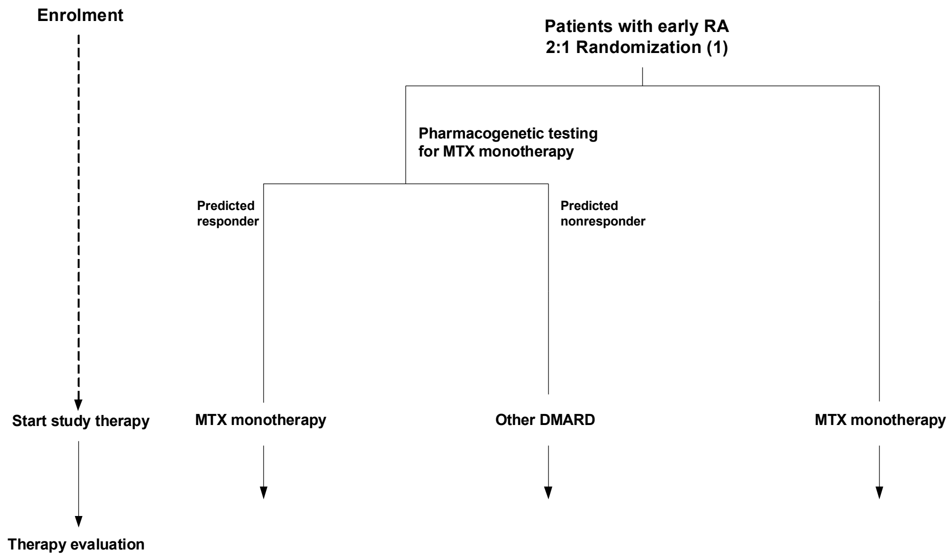

Figure 3. study proposal for a prospective study concerning MTX in RA, in which strategy for therapy is guided by pharmacogenetics[a]

a) Abbreviation(s): MTX= methotrexaat, RA= rheumatoid arthritis, TNF= tumor necrosis factor

Challenging steps towards clinical implication of pharmacogenetics

Social, ethical and legal implications of pharmacogenetics

It is demonstrated from the literature that pharmacogenetics holds the potential to improve therapeutic efficacy, to minimize adverse drug events, to enhance safety and to reduce the overall cost of management of disease, but needs further development for clinical implementation in the near future. Still, this development is not solely a challenge for genetic researchers and clinicians, since several social, ethical and legal implications form large obstacles for authorities, health care organizations, regulatory organizations and individuals.

For (pharmaco)genetic testing, privacy and informed consent may be essential in clinical usage of the genetic information. Personal information could be used adversely to a patient's interests. However, overall the knowledge on genetics is limited in individual patients. As a consequence, the autonomy of patients is reduced and the risk of involuntarily and abusively application of genetic data would be increased (41;42).

Also, it may be considered unethical not to employ pharmacogenetic testing in patients in order to avoid the exposure to the inefficacy and harmful side effects of drugs (43). On the other hand, with the performance of genetic testing the problem of handling 'by-catch' arises. This by-catch is the result of creating a genetic profile by the performance of e.g. whole-genome testing in which not only

searched information is present, but also unsearched information. This was studied by Henrikson and colleagues (44), who demonstrated that 53% of potential pharmacogenetic variants were reported to have a significant association with disease susceptibility. Hereby, genetic profiling could reveal susceptibility to e.g. serious diseases. In the light of self determination, is it mandatory to notify the patient on this by-catch? The psychological impact of this knowledge and concomitant responsibility could be difficult for the patient to handle in the future. This could include a change in health behaviour, quality of life and social surrounding (42;45).
The question remains how the health care insurers would act based on the pharmacogenetic results of their clients. The focus of the health care system on clients is likely to be shifting from a general population view to a more personalized view (46). Likewise, this attitude of the health care system could advance the inclusion of pharmacogenetics in to clinical practice. This could also lead to unwanted situations (46;47). For example, patients that are predicted non responder to conventional medication would be unfavourable to insure, since these patients would require more expensive medication and/or their nonresponse would result in chronic disease.

Economic considerations towards pharmacogenetics
In the short term, implementation of pharmacogenetic testing could result in higher drug related health care costs. Partly, investment in the development and evaluation of pharmacogenetic tests may lead to higher expenses. Also, higher costs could be due to a higher number of individual prescriptions written by confident clinicians, since choice of therapy is scientifically more gratified. In contrast, in the long term, the overall health care costs could be reduced, since e.g. unnecessary and unsuccessful expensive drugs are avoided. Moreover, overall drug related morbidity and mortality could be decreased (47;48).
As in the health care system, a more individualized trend towards the patient may be expected in the pharmaceutical industry. Ideally, application of pharmacogenetic testing can eventually enhance drug discovery and development process leading to market segregation, which is increasing the number of new drugs available on the market for a group of patients. Furthermore, it could result in more effective usage of existing drugs and could assess the efficacy of previously eliminated drugs, which had failed in clinical trials due to e.g. toxicity reasons. However, in practice for a pharmaceutical company this market segregation is difficult to cope, which has invested a substantial time and a significant amount of money in developing a new therapeutic agent for a small part of patients (47;49;50).

Impact of genetics
The impact of the pharmacogenetic test has to be considered. Specifically, in the case of effectively predicting effective drug therapy to antirheumatic agents, the additional value to conventional treatment has to be proven. For example, consulting a rheumatologist in an early phase of RA's disease progress and, hereby, achieving an optimal result on treatment in order to reduce joint damage may overshadow the genetic effects and/or demonstrate over-valuation of a clinical pharmacogenetic test.

Comorbidity, co-medication and adherence to therapy
Individualizing disease and medication of patients remain a problem for clinical usage of pharmacogenetics. If a test is based on a population of patients with RA, mostly the general well being or disease status is assessed. However, regarding the genetic aetiology of RA, previous findings demonstrated that two types of RA, ACPA-positive and ACPA-negative RA, have different genetic origins (51). In this way, estimating a chance of drug response and/or toxicity with a pharmacogenetic test could be limited by individual type of disease(s).

The same limitation arises for a specific test based on one type of drug instead of considering the use of other medication besides antirheumatic agents in RA patients. Indeed, it is demonstrated that statins may have moderate disease modifying effects in RA and these drugs might prevent or slow the development of RA (52). In this way, a clear effect of DMARDs on therapy outcome is difficult to observe. In order to adapt pharmacogenetic tests based on assembled patients, who will have an identical risk of response based on same type of disease, concomitant medication and environmental factors, remain a significant challenge.
Finally, a patients' suboptimal adherence to proposed therapy guidelines could be causative for errors in evaluation of interindividual variability in treatment outcome and hereby interpretation of genotypic effects on treatment outcome. Drug adherence could increase if patients would know they could benefit from their personal pharmacogenetic profile to improve the response to drug therapy.

Education to the clinician less trained in genetics
Along with a growing pharmacogenetic knowledge in rheumatology arises an increasing difficulty to explain to clinicians the use, benefits and pitfalls of pharmacogenetics and how to interpret a pharmacogenetic test. Therefore, additional education for clinicians is required for a successful choice and/or adjustment of drug therapy and, moreover, for an optimal explanation towards the patient.

Conclusion

Results from this thesis have elucidated potential genetic markers, which were associated with treatment outcome to MTX and adalimumab. Furthermore, a model for predicting the efficacy of MTX in patients with RA was validated in two cohorts indicating that predicting efficacy by a pharmacogenetic model is feasible in RA patients treated with MTX. Importantly, definitive conclusions about the role of genetic predictive factors in treatment outcome to DMARDS could not be drawn, since these results have to be further validated and replicated in future pharmacogenetic studies. Large randomized prospective studies should be planned to demonstrate its legitimate predictive and cost-effective value before a genetically individualized approach is applicable in daily clinical practice.

The potential role of pharmacogenetics in the prediction of efficacy and adverse events in RA patients treated with DMARDs is presented in this thesis. Hereby, new knowledge is added to the relatively young research field of pharmacogenetics, which may hopefully lead to a better treatment strategy for RA patients.

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Nederlandse samenvatting

Reumatoïde artritis (RA) komt in ongeveer 1 procent van de algemene populatie voor ongeacht etnische achtergrond. Kenmerkend voor deze immunologische ziekte is de aanwezigheid van een symmetrische ontsteking en schade aan de gewrichten, die leidt tot algehele functiebeperking en uiteindelijk tot evt. invaliditeit. Daarnaast RA patiënten een verhoogd risico op andere (ernstige) aandoeningen, zoals hart-en vaatziekten.
Ongeacht het toenemende inzicht in het ontstaan, verloop en ontwikkeling van reumatoïde artritis, heeft men een genezende therapie nog niet gevonden. Daarom is de huidige behandeling met het beste resultaat gericht op het verminderen van de ziekteactiviteit. Deze reductie kan worden behaald met behulp van geneesmiddelen als 'disease modifying antirheumatic drugs' (afgekort DMARDs) met een werkingsmechanisme, dat de immuunreactie betrokken bij RA onderdrukt. Toch zijn in de praktijk de resultaten suboptimaal bij het gebruik van DMARDs, zoals methotrexaat (MTX) en anti-TNFalfa middelen. Er worden namelijk grote verschillen in responsepercentages waargenomen in de klinische praktijk evenals in klinische studies, die de effectiviteit van DMARDs hebben onderzocht.
Farmacogenetica beschrijft de invloed van genetische factoren (DNA) op behandelingsuitkomst van geneesmiddelen. DNA is de drager van erfelijke informatie en bevat basen (chemische bouwstenen van het DNA) die coderen voor de productie van verschillende eiwitten waaronder enzymen. Bij de verschillende processen in het lichaam, waaronder de omzetting van geneesmiddelen of ontstaan van een ziekte, spelen eiwitten een belangrijke rol. De complete DNA volgorde van de mens bestaat uit 3.1 miljoen basenparen. Bij niet verwante personen is ongeveer 99.9% van deze basenparen identiek. Slechts 0.1% van de basenparen verschilt van mens tot mens en dit kan leiden tot ziekte of verschillen in uitkomst na behandeling met geneesmiddelen. De meest voorkomende variatie in DNA (90%) zijn zgn. 'single nucleotide polymorphisms' (afgekort SNPs), waarbij 1 base is veranderd. SNPs kunnen leiden tot een wijziging in de DNA sequentie waardoor alternatieve enzymen kunnen worden gevormd. SNPs, die deze veranderingen in enzymfunctie of anderszins in eiwitten veroorzaken, worden functionele SNPs genoemd. Aangezien enzymen/eiwitten betrokken zijn bij omzettingen en werking van geneesmiddelen, kunnen SNPs leiden tot verschillen in behandelingsuitkomst.

Het doel van dit proefschrift is om de invloed van genetica (SNPs) op de behandelinguitkomst bij patiënten met RA die behandeld worden met MTX en/of het anti-TNFalfa middel adalimumab, te onderzoeken. Hierbij is dit proefschrift ingedeeld twee delen: de rol van farmacogenetica bij de behandeling met MTX (deel 1) en de rol van farmacogenetica bij de behandeling met adalimumab (deel 2).

Methotrexaat

In het eerste hoofdstuk van dit deel werd een literatuuroverzicht gepresenteerd van onderzoeken die genetische variatie in behandelingsuitkomst van MTX in RA hebben onderzocht. Hiervan hebben de meeste onderzoeken slechts kleine patiëntenpopulaties bekeken, waardoor het eigenlijk niet goed mogelijk is om werkelijke associaties met therapie-uitkomst aan te kunnen wijzen. Ook andere factoren, zoals niet-genetische factoren, etniciteit of onduidelijke eindpunten bemoeilijken dit. Daarom kunnen definitieve conclusies over de rol van genetische factoren bij de respons op MTX niet worden getrokken op basis van de huidige literatuur (hoofdstuk 1).

Enerzijds is het werkingsmechanisme van MTX mogelijk te verklaren doordat MTX enzymen uit de folaat cyclus remt, anderzijds is gebleken uit studies dat MTX ook zou kunnen werken via het vrijzetten van adenosine, een anti-ontstekingsstof. Met deze hypothese werd in hoofdstuk 3 de relatie tussen SNPs in genen (DNA) coderend voor eiwitten, die betrokken zijn bij het vrijzetten van adenosine, en behandelingsuitkomst met MTX onderzocht in 205 patiënten met vroege (d.w.z. korter dan 2 jaar durende symptomen van) RA. Deze patiënten waren afkomstig uit het 'BeSt cohort'. De resultaten lieten associaties zien tussen genetische variatie in de genen coderend voor adenosine monophosphate deaminase (*AMPD*), 5-aminoimidazole-4-carboxamide transformylase (*ATIC*) en inosine triphosphate pyrophosphatase (*ITPA*) en klinische effectiviteit bij behandeling met MTX. Patiënten die namelijk drager waren van het *AMPD1* T-allel, het *ATIC* CC genotype en het *ITPA* CC genotype hadden een 2-3 maal grotere kans op een gunstige klinische response (gedefinieerd als 'disease activity score'- DAS ≤2.4 punten) na 6 maanden MTX therapie. Daarnaast verbeterden de responspercentages aanzienlijk wanneer patiënten alle drie de genotypen hadden. Daarnaast werd gekeken naar associaties met bijwerkingen als gevolg van MTX therapie. Een relatie werd gevonden met het *ATIC* G-allel dragerschap. Er werden geen associaties gevonden tussen SNPs in genen coderend voor methionine synthase of methionine synthase reductase en effectiviteit of bijwerkingen.
Tot nu toe zijn de meeste genetische varianten voor farmacogenetische analyse met MTX geselecteerd op basis van een mogelijk werkingsmechanisme van MTX of het ontstekingsproces bij RA (hoofdstukken 2 en 3). Maar in het ideale geval worden functionele SNPs gekozen, die verandering in enzym- of eiwitfunctie veroorzaken en op deze wijze de werking van MTX beïnvloeden. In hoofdstuk 4 is de relatie tussen functionele SNPs en de effectiviteit en bijwerkingen van MTX therapie bestudeerd in patiënten met vroege RA afkomstig uit het BeSt cohort. Het optreden van bijwerkingen na MTX therapie bleek geassocieerd met 2 SNPs in de genen *ABCB1* en *TLR4*. Geen associatie werd gevonden tussen de effecten van MTX en functionele SNPs in de genen *DHFR*, *ABCB1*, *ITPA* IVS2, *HLA-G*, *IMPDH2*, *TGFB1* and *TLR4*. Daarnaast werden resultaten van eerder onderzoek gerepliceerd in ons RA patiënten cohort. Deze replicatie analyses zijn erg belangrijk omdat farmacogenetische onderzoeken een hoge kans hebben op het vinden van vals positieve bevindingen. Maar de associaties, die in eerdere studies gevonden werden, konden wij niet bevestigen in het BeSt cohort.
Kort geleden is door onderzoekers van onze groep een klinisch farmacogenetisch predictiemodel ontwikkeld om de effectiviteit van MTX in patiënten met vroege RA te kunnen voorspellen. Het model bestond uit verschillende niet-genetische en genetische factoren waarvan was aangetoond dat ze van invloed zijn op de effectiviteit van MTX: geslacht, reumafactor, rookstatus, DAS bij aanvang van behandeling en 4 SNPs in de genen *MTHFD1*, *AMPD1*, *ITPA* en *ATIC*. Met dit model werd oorspronkelijk een positief voorspellend waarde van 95% en een negatief voorspellende waarde van 86% gevonden. In totaal kon op deze manier 60% van de patiënten gegroepeerd en dus voorspeld worden. In hoofdstukken 5 en 6 werd het model gevalideerd door replicatie in respectievelijk een ander Nederlands en een Zweeds cohort RA patiënten.
In hoofdstuk 5 werd gezien dat het farmacogenetisch model minder goed presteerde in patiënten met die al langer RA hadden dan in patiënten met vroege RA. De positief voorspellende waarde en negatief voorspellend waarde waren respectievelijk 47% en 81%. Het verschil ten opzichte van het oorspronkelijke onderzoek kan mogelijk worden verklaard door langere RA ziekteduur, DMARD gebruik in het verleden, de afwezigheid van bewezen associatie met de 4 SNPs in the model; de afwezigheid van nauwgezette behandelingstrategieën met RA en door lagere doseringen MTX en lagere responspercentages.
In hoofdstuk 6 werd het model getest in een Zweeds cohort met RA patiënten, die nooit eerder een DMARD hadden gebruikt, vroege (korter dan 1 jaar durende symptomen van) RA hadden en die afkomstig waren uit de Swefot trial. Evenals in hoofdstuk 5, waren de voorspellende waarden lager

dan in het oorspronkelijke onderzoek. De positief voorspellende waarde en negatief voorspellende waarden in de Zweedse groep waren 70% en 68%. Echter, de resultaten voor de accuraatheid, het aantal patiënten gegroepeerd en onderscheidend vermogens (gedefinieerd als area under the curve-AUC) waren overeenkomstig.
Over het algemeen laten de resultaten van hoofdstuk 5 en 6 zien dat de effectiviteit in een groot deel van RA patiënten met MTX therapie voorspeld zou kunnen worden met het klinisch-farmacogenetisch model, maar dat het model het best toegepast zou kunnen worden in patiënten zonder eerder DMARD gebruik en met een korte ziekteduur. Replicatie van deze resultaten in een onderzoek met een prospectieve studieopzet is (ideaal gezien) nodig om de meest betrouwbare resultaten te kunnen verkrijgen.

In hoofdstuk 7 wordt een relatie van het haplotype, bestaande uit de SNPs *MTHFR* 1298 A>C en *MTHFR* 677 C>T, met behandelingsuitkomst van MTX in het BeSt cohort bestudeerd. Een haplotype is een combinatie van allelen, die vaker dan door toeval in combinatie voorkomen in de algemene populatie. Eerder bleek dat de SNPs *MTHFR* 1298 A>C en *MTHFR* 677 C>T en hun haplotype bestaande uit de allelen MTHFR1298A en MTHFR 677C geassocieerd te zijn met effectiviteit van MTX therapie. Hieruit bleek dat met het dragen van 0, 1 of 2 haplotypen *MTHFR1298A-677C* belangrijke verschillen in response percentages werden waargenomen. Met behulp van het toevoegen van het aantal haplotypen (0,1 of 2), die bestaan uit de allelen *MTHFR1298A* en *MTHFR 677C*, werd geprobeerd om het farmacogenetisch predictiemodel uit te breiden en zo te verbeteren. Verder wordt in dit hoofdstuk het voorspellend vermogen ('discriminative ability') van het aantal haplotypen met de combinatie *MTHFR1298A-677C* geanalyseerd en vergeleken met de 4 SNPs in de genen *MTHFD1*, *AMPD1*, *ITPA* en *ATIC* uit het model. Resultaten lieten zien dat met de toevoeging van het aantal haplotypen als extra factor het model niet werd verbeterd. Ook bleek dat het voorspellend vermogen van het aantal haplotypen (0,1 of 2) minder was dan het voorspellend vermogen van de 4 SNPs in de genen *MTHFD1*, *AMPD1*, *ITPA* of *ATIC*. Deze resultaten suggereren dat een voorspellende rol voor MTX therapie door middel van verschillen in dragerschap van dit haplotype afwezig is. Toekomstig onderzoek zal uitgevoerd moeten worden om de exacte farmacogenetische rol van de SNPs *MTHFR* 1298 A>C en *MTHFR* 677 C>T nader te kunnen bepalen.

Adalimumab

Aan het begin van het tweede deel van dit proefschrift (hoofdstuk 8) werd een overzicht gepresenteerd van onderzoeken die genetische variatie in behandelingsuitkomst van anti-TNFalfa middelen (etanercept, infliximab en adalimumab) in RA hebben onderzocht. In overeenstemming met de studies die de relatie van genetische variatie met MTX beschreven, zijn ook de farmacogenetische studies met anti-TNFalfa middelen meestal uitgevoerd met te kleine patiëntgroepen om definitieve conclusies te kunnen trekken.
In hoofdstuk 9 werd het selecteren van genetische varianten voor farmacogenetische onderzoekingen beschreven. Aansluitend werd een farmacogenetische aanpak gepresenteerd waarbij met behulp van criteria op systematische wijze interessante SNPs geselecteerd kunnen worden. Deze criteria zijn gebaseerd op bepaalde eigenschappen, zoals bewezen kwaliteit en frequentie van SNPs. Met het gebruik van deze systematische aanpak kan op verbeterde wijze een objectieve selectie van SNPs worden bereikt. Op deze manier werden 186 SNPs in 111 genen uit een aantal van 51.793 SNPs in 124 genen gekozen. De genen waaruit de selectie is gemaakt, coderen voor enzymen en eiwitten die gerelateerd zijn aan het werkingsmechanisme van anti-TNFalfa middelen of het ontstekingsproces dat een rol speelt bij RA.

Deze benadering heeft een aantal voordelen boven de gebruikelijke selectiemethoden. Voornamelijk als het werkingsmechanisme (en de betrokken enzymen en eiwitten) van het desbetreffende geneesmiddel nog onbekend is, is deze methode bruikbaar.
Deze selectiemethode werd in de praktijk gebracht in hoofdstuk 10: de effectiviteit van adalimumab therapie werd geassocieerd met SNPs die geselecteerd waren volgens de beschreven wijze. Naast deze SNPs werden in dit hoofdstuk ook genetische varianten onderzocht die in eerder onderzoek bleken geassocieerd te zijn met de effecten van anti-TNFalfa middelen (etanercept, infliximab en adalimumab) en/of genetische varianten die waren geassocieerd met het ontstaan van de ziekte RA. Effectiviteit was onderverdeeld in de eindpunten: EULAR goede respons, EULAR remissie en een procentuele daling in DAS. Resultaten lieten zien dat 19 SNPs, 11 SNPs en 8 SNPs associeerden met respectievelijk EULAR goede respons, EULAR remissie en een procentuele daling in DAS. Vier SNPs in genen coderend voor CD40LG, KDR, TANK en VEGFA waren het sterkst gerelateerd met de effectiviteit van adalimumab therapie, omdat deze 4 SNPs associeerden met alle drie de gekozen eindpunten. De resultaten beschreven in dit hoofdstuk moeten gerepliceerd worden in een tweede onafhankelijk cohort om de waarde ervan voor het voorspellen van de effectiviteit van adalimumab therapie bij RA te kunnen bepalen.

Conclusie

Het in dit proefschrift beschreven onderzoek heeft geleid tot de identificatie van een aantal genetische varianten die geassocieerd zijn met de behandelingsuitkomst bij therapie met MTX en adalimumab in RA patiënten. De bevindingen zullen gerepliceerd moeten worden alvorens de resultaten in de praktijk toepasbaar zijn. Het eerder door ons ontwikkelde farmacogenetisch predictiemodel voor MTX bleek bij replicatie wisselend succesvol. Nader onderzoek is nodig om het voorspellend vermogen en dus de waarde van het predictiemodel te kunnen bepalen.
Tenslotte blijkt uit dit onderzoek dat genetische verschillen een rol kunnen spelen ten aanzien van de behandelingsuitkomst voor RA patiënten die met MTX of adalimumab worden behandeld. Daarmee is nieuwe kennis toegevoegd aan het relatief jonge vakgebied van de farmacogenetica dat hopelijk in de toekomst leidt tot een betere behandeling van patiënten met RA.

List of publications

Wessels JA, Kooloos WM, De Jonge R, De Vries-Bouwstra JK, Allaart CF, Linssen A, Collee G, De Sonnaville P, Lindemans J, Huizinga TWJ, Guchelaar HJ.
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Pharmacogenomics, Submitted

Kooloos WM, Wessels JA, van der Straaten T, Smit JH, Tan HL, Huizinga TWJ, Guchelaar HJ.
Pharmacogenetic pathway approach detects associations with adalimumab efficacy in rheumatoid arthritis.
Manuscript in preparation

Kooloos WM, Huizinga TWJ, Guchelaar HJ, Klareskog L, Padyukov L, Wessels JA, van Vollenhoven RF.
External Validation of the Clinical Pharmacogenetic Model for predicting MTX monotherapy efficacy using a Swedish Cohort of patients with recent-onset rheumatoid arthritis.
Manuscript in preparation

Nawoord

De periode als vreemde eend in de bijt is voorbij: De werkzaamheden als arts-onderzoeker in een ziekenhuisapotheek hebben geleid tot het proefschrift, dat voor u ligt. Ik ben blij dat ik de mogelijkheid heb gekregen dit onderzoek te mogen doen. Maar dit resultaat zou niet mogelijk zijn zonder hulp van vele kanten.
Allereerst ben ik de participerende patiënten, artsen en medewerkers betrokken bij de BeSt-, Swefot- en Humira studies erg dankbaar. In het bijzonder wil ik de medewerkers en apothekers van ApotheekZorg bedanken voor hun ruimte, tijd en inzet om het Humira-project te laten realiseren.
Ook mijn collega's van de Klinische Farmacie & Toxicologie ben ik erkentelijk voor hun hulp én gezelligheid. Ik vind het een goede ervaring dat ik als arts een kijk achter de schermen van een ziekenhuisapotheek heb mogen nemen.
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Tenslotte een laatste woord aan Evelien: Na dit proefschrift zal het niet echt rustiger gaan worden, maar laten we in ieder geval samen genieten van wat de toekomst zal brengen

Curriculum Vitae

Wouter Michiel Kooloos werd op 14 december 1982 geboren in Leiderdorp. Na het gymnasium diploma op het Stedelijk Gymnasium in Leiden in 2001 behaald te hebben, werd in dat zelfde jaar begonnen met de studie geneeskunde. Vanaf augustus 2005 tot en met januari 2006 heeft hij zijn afstudeeronderzoek bij de afdeling Klinische Farmacie en Toxicologie van het LUMC verricht onder leiding van dr. J.A.M. Wessels en Prof. Dr. H.-J. Guchelaar. Hiermee werd in 2006 de doctoraalfase van geneeskunde afgerond. Het afstudeeronderzoek vormde de basis voor het begin van het promotietraject in januari 2006. Voor deze werkzaamheden (het afstudeeronderzoek en de opzet van een promotietraject in de vorm van het schrijven van een onderzoeksprotocol en review) ontving hij de S.E. de Jongh award in 2007. Tenslotte werd in 2008 het artsexamen behaald. Vanaf maart 2008 tot en met mei 2009 was hij als arts-onderzoeker verbonden aan de afdeling Klinische Farmacie en Toxicologie (LUMC). Onder leiding van dr. J.A.M. Wessels, Prof. dr. H.-J. Guchelaar (hoofd afdeling Klinische Farmacie en Toxicologie van het LUMC) en Prof. dr. T.W.J. Huizinga (hoofd afdeling Reumatologie van het LUMC) is hij werkzaam geweest aan het onderzoek beschreven in dit proefschrift. Vanaf begin 2010 zal hij werkzaam als AIOS Radiologie in het Albert Schweitzer Ziekenhuis te Dordrecht (opleider T.R. Hendriksz).