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> ANTI-TNF PHARMACOGENOMICS IN RHEUMATOID ARTHRITIS: TOWARDS PERSONALIZED THERAPEUTICS

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift ter verkrijging van de graad van doctor aan de Radboud Universiteit Nijmegen op gezag van rector magnificus prof. mr. S.C.J.J. Kortmann, volgens besluit van het college van decanen in het openbaar te verdedigen op woensdag 3 maart 2010 om 13.30 uur precies, door

Erik Josephus Maria Toonen
geboren op 10 mei 1977 te Mook en Middelaar

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> CHAPTER 1

GENERAL INTRODUCTION

> RHEUMATOID ARTHRITIS, ETIOLOGY AND CLINICAL ASPECTS

Rheumatoid arthritis (RA) is a common chronic inflammatory disease mainly affecting the synovial joints. Approximately 1% of the world population is affected with RA and the disease has a threefold higher prevalence in females than in males (1). RA is a multifactorial disorder in which both genetic and non-genetic factors are involved not only in disease susceptibility, but also in the chronicity, severity, and a patient's response to therapy (2;3). Heritability of RA has been extensively studied and has been estimated at 50-60% (4-6). RA can result in joint and cartilage destruction (Figure 1), and loss of functionality. Apart from the articular manifestations there are also multi-systemic ones and the disease is furthermore associated with increased comorbidity, including a higher risk of coronary artery disease, infection and lymphoma as well as reduced life expectancy (7).

Figure 1 / Schematic presentation of a healthy joint and the features that characterize an arthritic joint

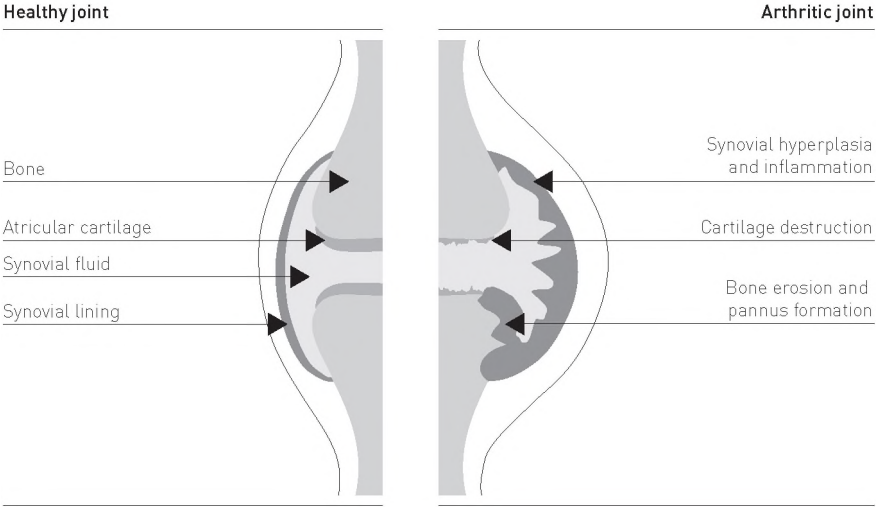


Figure was adapted, with permission, from the thesis of Marije Koenders; Interleukin 17 and its relation to IL-1 and TNF in experimental arthritis, 2007 (ISBN 978-90-9021074-2).

RA has a peak of onset in the fourth and fifth decades of life. The disease begins with fatigue, anorexia, generalized weakness and vague musculoskeletal symptoms in approximately 60% of the patients until synovitis becomes apparent. Joint involvement presents itself as joint inflammation which leads to pain, redness, tenderness, warmth and swelling. The disease course ranges from mild, self limiting arthritis to a progressive multisystem inflammation with profound morbidity and mortality. Most patients suffering from RA experience persistent but fluctuating disease activity. Although every joint in the body can be affected by the disease, joints of both the hands and feet are often involved and reflect the patient overall status (8). Pain in the affected joints, aggravated by movement, is the most common manifestation of RA. Inflammation is often accompanied by a generalized stiffness after a period of inactivity. A set of classification criteria was developed by the American College of Rheumatism (ACR) to diagnose RA reliably and distinguish it from disease with overlapping symptoms (9). These 1987 ACR criteria are nowadays most commonly used for the diagnosis of RA in the clinic, as well as for the definition of patients for research projects (Table 1).

Table 1 / 1987 American College of Rheumatism criteria for the classification of Rheumatoid Arthritis

Criterion	Short title	Definition
1	*Morning stiffness*	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2	*Arthritis of 3 or more joint areas*	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3	*Arthritis of hand joints*	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4	*Symmetric arthritis*	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5	*Rheumatoid nodules*	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6	*Serum rheumatoid factor*	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7	*Radiographic changes*	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

A patient is classified as having rheumatoid arthritis if he/she has satisfied at least 4 or these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded. PIP: proximal interphalangeal, MCP: metacarpophalangeal, MTP: metatarsophalangeal.

Currently, there is no cure for RA, treatment is directed at symptom reduction and minimization of disease activity and progression (10;11). Early therapeutic intervention is of upmost importance for minimizing joint damage and functional disability (12). Several studies have shown that a three months delay in the introduction of medication results in significantly more joint damage at five years (13;14). RA treatment is usually started with methotrexate (MTX) monotherapy administered weekly and follows a further stepwise approach. MTX is the most commonly used drug for RA treatment (12). When patients show insufficient response and/or adverse drug events, MTX can be replaced by another disease modifying anti-rheumatic drug (DMARD) or a second DMARD may be added to the MTX monotherapy. If a patient still does not respond to therapy it is also possible to switch to biologicals, a newer subgroup of DMARDs. These biologicals can be prescribed as monotherapy or in combination with another DMARD.

A number of standardized instruments have been developed to evaluate the disease activity and progression in a patient and judge his/her response to therapy. These instruments are used in the clinic as well as for the characterization of patients in research projects. A well standardized measure for assessing disease activity in RA is the Disease Activity Score 28 (DAS28). This score is calculated by a complex mathematical formula (Figure 2), which includes the number of tender and swollen joints (out of a total of 28), the erythrocyte sedimentation rate (ESR, a blood marker of inflammation), and the patient's assessment of global health (indicated by marking a 10 cm line between very good and very bad).

Figure 2 / DAS28 formula

$$DAS28 = 0.56 \bullet \sqrt{(t28)} + 0.28 \bullet \sqrt{(sw28)} + 0.70 \bullet Ln(ESR) + 0.014 \bullet GH$$

The following parameters are included in the calculation: tender joint counts (t28), swollen joint counts (sw28), erythrocyte sedimentation rate (ESR) and the patient's assessment of global health (GH).

A DAS28 score greater than 5.1 implies active disease, less than 3.2 well controlled disease, and less than 2.6 remission (15). To measure individual RA treatment response, the DAS based European League Against Rheumatism (EULAR) response criteria were developed. These response criteria use the individual change in DAS and the level of DAS reached through treatment to classify patients as good, moderate or non-responders (Table 2) (16).

Table 2 / EULAR response criteria

DAS28 at endpoint	Improvement in DAS28 from baseline		
	> 1.2	> 0.6 and ≤ 1.2	≤ 0.6
≤ 3.2	Good		
> 3.2 and ≤ 5.1		Moderate	
> 5.1			None

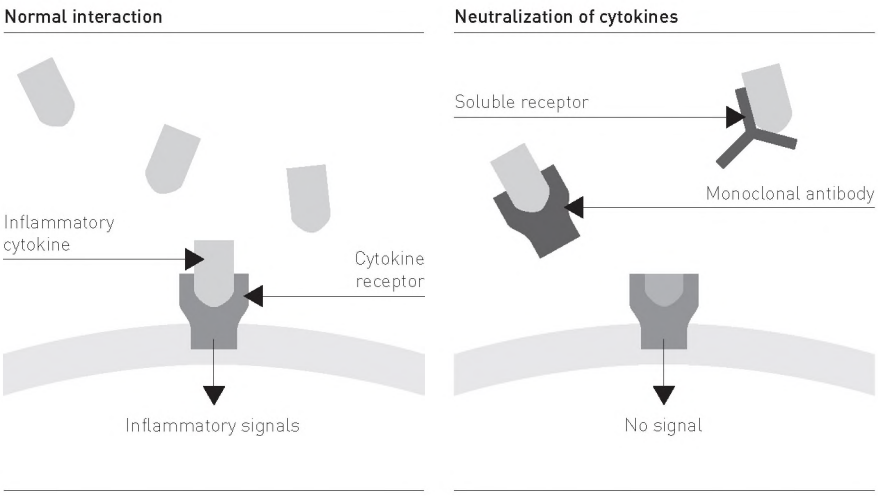
Damage in the joint due to destruction of cartilage and underlying bone is quantified to measure the severity and clinical course of RA. Joint (or radiological) damage is irreversible. There are a variety of methods for scoring radiological damage, the most frequently used instruments are the (modified version of the) Sharp's score (17) and Larsen's method (18). The modified Sharp's method uses a system in which each joint is scored both for space narrowing and for erosions. In Larsen's method the hands, wrist and feet are scored. It has a range from 0 to 200 and twenty joints in the hands and 10 in the feet are scored. The wrists are evaluated as a single joint. Scores ranges from 0 to 5 and the wrists are weighted by a factor of 5.

The next few paragraphs will first focus on the pro-inflammatory cytokine tumour necrosis factor (TNF) and its role in RA disease severity and as target for treatment (anti-TNF therapy). Next, the relationship between genetic variation and individual differences in RA severity and anti-TNF therapy response will be discussed, thereby introducing the terms 'pharmacogenetics' and 'pharmacogenomics' . In the last paragraph the outline of this thesis is discussed.

> TNF AND ITS ROLE IN THE TREATMENT OF RA

Even though the pathogenesis of RA is not fully understood, it is commonly accepted that the pro-inflammatory cytokine TNF has a key role in the inflammatory process (19). TNF neutralizing approaches have proven highly effective in the treatment of RA and other autoimmune diseases (20-23). Though in theory TNF neutralisation can be achieved at many levels, all TNF blocking agents available nowadays prevent cell signalling by preventing the interaction of TNF with its cell-surface receptors and/or by blocking membrane-bound TNF (Figure 3).

Figure 3 / Cytokine-TNFα receptor interaction



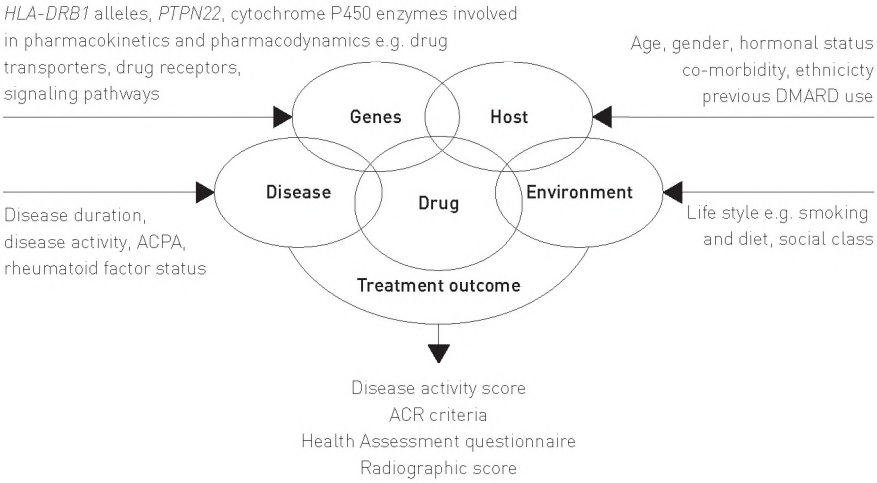
Binding of the cytokine TNFα to its cell-surface receptor leads to the production of inflammatory effector molecules. During anti-TNF therapy, TNFα is prevented from binding to the receptor by a monoclonal antibody or a soluble receptor. Figure was adapted, with permission, from the paper of Choy and co-workers (20).

Three anti-TNF agents are currently available: etanercept (Enbrel®), infliximab (Remicade®) and adalimumab (Humira®). Two new anti-TNF agents, golimumab (CNTO 148) and certolizumab pegol (Cimzia®), have been submitted to the European Medicines Agency (EMEA) requesting approval and will most likely be approved in 2009. Etanercept is a human, soluble dimeric TNF type II receptor linked to the Fc part of an IgG1 molecule that binds to and inactivates TNFα. The mouse-human chimeric IgG1 monoclonal antibody infliximab and the completely humanized IgG1 monoclonal antibody adalimumab bind to TNFα with high affinity and thereby inactivate it (24;25). The new anti-TNF agents golimumab and certolizumab pegol are a fully human monoclonal anti-TNF antibody and a humanized monoclonal anti-TNF antibody, respectively (26). More precisely, certolizumab pegol is the first PEGylated, Fc-free anti-TNF (27). In The Netherlands, patients will start their first course of a TNF-blocking agent when they have a DAS28 > 3.2 at baseline and failure on at least two disease-modifying antirheumatic drugs (DMARDs), one of which has to be methotrexate (MTX) (28).

Though TNF neutralisation is very effective in RA, long-lasting response does only occur in approximately 70% of the patients (28). Non-response can be classified in a primary response, if there is lack of efficacy already within weeks from therapy initiation, and a secondary response, if after an initial benefit from the therapy, response is lost with time (29-31). The causes for non-response is unknown. One explanation for non-response to anti-TNF treatment is the formation of antibodies directed to anti-TNF (human anti-chimeric antibodies, HACA and/or human anti-human antibodies, HAHA). These antibodies potentially reduce the efficacy of treatment and could lead to therapy discontinuation (32). Besides a lack of response, also adverse events can occur during anti-TNF treatment and can be the reason for therapy discontinuation. Adverse events reported during anti-TNF therapy include an increased susceptibility to tuberculosis and other coexisting infections, lymphoma and neutropenia. Most often reported adverse events are injection and infusion reactions (33;34).

Interindividual variability in drug response and the occurrence of adverse events can be due to genetic and environmental factors influencing pharmacokinetics and -dynamics, or to interindividual differences in disease pathogenesis and etiology (Figure 4) (35). Differences in therapy response and toxicities between individuals have become the most powerful drive for current pharmacogenetic research, especially for drugs that are highly costly and those that have a narrow therapeutic window.

Figure 4 / Interindividual variability in therapy response



Several factors (genetic, environmental and developmental) influence the response of an individual to therapy, as measured by the different scores, criteria and questionnaires. ACPA = anticitrullinated protein antibodies; ACR = American College of Rheumatology; DMARD = disease-modifying antirheumatic drugs. Figure is adapted, with permission, from the figure published by Wesoly *et al.* (35).

> PHARMACOGENETICS AND PHARMACOGENOMICS

Generally, genetic factors are estimated to be responsible for 15-30% of the observed interindividual differences in drug metabolism and response. For certain classes of drugs, genetic variation can account for up to 95% of variability in drug effects observed between patients (36-38). Pharmacogenetic research focuses on identifying the DNA sequence variants that influence drug response and/or toxicities. In this way, researchers hope to develop tools to predict treatment response prior to treatment start and determine the optimal treatment regimen for an individual patient. Single nucleotide polymorphisms (SNPs) are the most common forms of genetic variation in humans and occur with a mean frequency of one every few hundred nucleotides (39). Most of these SNPs are thought to be harmless for the organism since they are located in intergenic or intronic regions. However, a small percentage of SNPs is located in promoter regions and coding sequences and can have functional consequences if they result in e.g. alterations of DNA conformation, promoter activity, or RNA and protein quantity or quality. Therefore, certain SNPs may also play a direct or indirect role in therapy response and toxicities (35). Several studies of complex diseases already confirmed the relevance of SNPs in drug metabolizing enzymes, transporters, receptors and signalling pathways in altering drug response (36;40;41). SNPs and other forms of DNA variation are also likely to be (partly) responsible for non-response and adverse events observed in anti-TNF treatment in patients with RA, e.g., genetic factors may determine primary non-response as well as why some patients develop HACA or HAHA.

Non-response to anti-TNF treatment and adverse events observed in RA patients and the high costs of this therapy have driven the search for (genetic) markers that are able to predict treatment response (42). For the most part, two approaches are used to identify genetic factors underlying mechanisms of therapy (non-)response. The first approach is a hypothesis driven approach in which DNA polymorphisms in candidate genes are investigated for their effect on treatment response. For investigating anti-TNF therapy response, the most obvious candidate genes are the genes encoding TNFÐ and the TNF-receptors, since they are directly involved in TNFÐ signalling. Therefore, these genes have been investigated repeatedly for (43-48;48-63). The candidate gene approach is, however, hampered by the limited knowledge we currently have about pathways determining the kinetics and dynamics of anti-TNF treatment. This disadvantage can be compensated by a second approach. This so-called non-hypothesis driven approach uses genome-wide array techniques to identify genetic markers for

anti-TNF treatment outcome. In a genome-wide association study (GWAS) it is possible to investigate hundreds of thousands SNPs across the entire genome in one single experiment (64;65). Only one such GWAS has been published for anti-TNF treatment in RA, so far, in a very limited sized (66). Another technique to search for genetic markers in a non-hypothesis driven manner is the use of whole genome expression arrays. Using these arrays, it is possible to monitor the expression levels of most of the annotated genes. Genes that are differentially expressed in two groups (e.g. anti-TNF responders and non-responders) can be identified and further investigated for their (possible) role in therapy response (67).

> THE ROLE OF GENETIC VARIANTS IN DISEASE SEVERITY

As mentioned in the first paragraph, RA disease severity is often measured by using the disease variables disease activity (measured by the DAS28) and joint damage (measured by scoring radiologic joint damage). Disease severity is partly influenced by genetic factors, with the human leukocyte antigen (HLA)-DRB1 alleles identified as one of these factors (68-70). A second, more modest, association with disease severity has been identified for the protein tyrosine phosphatase non-receptor 22 (PTPN22) gene (71;72).

Importantly for this thesis, evidence from several studies suggests that genes involved in RA disease severity are also good candidates for influencing treatment response (49;53;73). This is especially the case for TNF-blocking therapy since this therapy interferes with the pro-inflammatory TNF pathway.

The most thoroughly studied polymorphism in disease severity is the -308G>A (rs1800629) promoter polymorphism located in the *TNFA* gene encoding TNFα itself (74-85). This functional polymorphism is suggested to be responsible for altering the regulation of cytokine production and may thereby not only affect the natural course of the disease but also the response to TNF blockade (53) (86-88). Results of the several studies are inconsistent: two studies, performed in 34 and 130 RA patients, suggested that the -308G allele is associated with radiologic joint damage progression (82;83), whereas another study reported a worse radiologic joint damage in patients carrying the A allele in a cohort of 189 RA patients (77). Finally in a larger sample of 283 patients Brinkman *et al*. could not confirm any association between either the G or A allele and radiologic joint damage (75).

As has been shortly mentioned above and will be discussed in more detail in the next chapter, obviously the -308G>A (rs1800629) promoter polymorphism has also been the prime target of studies directed at identifying genetic factors for anti-TNF treatment response. A number of additional genetic variants located in (inflammatory) genes have been investigated for their relation to disease severity, with convincing evidence for *HLA-DRB1* and *PTPN22* to be indeed associated with disease severity (Table 3) (44;69;71;72;89-104).

Table 3 / Genes investigated for their (possible) association with RA disease severity

Gene symbol	Gene name	Reference
ADAMTS13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	(105)
ATIC	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase / IMP cyclohydrolase	(106)
CARD8	Caspase recruitment domain family, member 8	(107-109)
CAT	Catalase	(110)
CD4	CD4 molecule	(111)
FCG2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)	(112)
FCG2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)	(113)
FCG3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	(112;114)
FCG3B	Fc fragment of IgG, low affinity IIIb, receptor (CD16b)	(112)
GSTK1	Glutathione S-transferase kappa 1	(115-117)
HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	(44;69;89-100)
HMOX1	Heme oxygenase 1	(118;119)
IL1	Interleukin 1	(120-126)
IL1RN	Interleukin 1 receptor antagonist	(53;122-124;126;127)
IL4	Interleukin 4	(126)
IL10	Interleukin 10	(126;128)
IL16	Interleukin 16	(129;130)
IL17	Interleukin 17	(131)
IL18	Interleukin 18	(132)
ITPA	Inosine triphosphatase	(106)
LTA	Lymphotoxin alpha	(44)
Mal/TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein	(133)
MBL2	Mannose-binding lectin (protein C) 2, soluble	(134;135)
MIF	Macrophage migration inhibitory factor	(136-138)
MTHFR	5,10-methylenetetrahydrofolate reductase (NADPH)	(106)
NLRP3	NLR family, pyrin domain containing 3	(107)
NOS2A	Nitric oxide synthase 2	(110;139)
PADI4	Peptidyl arginine deiminase, type IV	(140-143)
PTPN22	Protein tyrosine phosphatase, non-receptor type 22	(71;72;101-104)
SOD2	Superoxide dismutase 2	(110)
SOD3	Superoxide dismutase 3	(110)
TAP1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	(144)
TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	(144)
TGFB1	Transforming growth factor, beta 1	(53;145)
TLR2	Toll-like receptor 2	(146)
TLR4	Toll-like receptor 4	(133;146;147)
TNFA	Tumor necrosis factor	(74-85;87;110;148)
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	(149)
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	(45;49;149-151)
TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11	(89)
TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b 11b	(89;152;153)

Given their role in inflammation, these genes are also obvious candidates for influencing treatment response to anti-TNF, and many of them have been investigated for this phenotype, too (44;49;52;53;83;114;143). Therefore, studies investigating the genetic background of RA severity can also be used as a tool to identify markers for inter-individual response to anti-TNF treatment, since often the same inflammatory pathways are involved.

> AIM AND OUTLINE OF THE THESIS

The project leading to this thesis aimed at identifying genetic biomarkers that predict response to anti-TNF treatment in patients with RA. In the context of personalized therapeutics, we hope that pharmacogenetic research can lead to more tailor-made initial treatment decisions in patients with RA.

The first part of the thesis, described in chapters 2 to 6, is focused on the analysis of several SNPs and one variable number of tandem repeats (VNTR) polymorphism located in candidate genes for influencing disease severity and anti-TNF (non-) response (hypothesis driven approach), the latter after having given an overview of existing studies in this field.

The second part (chapters 7 to 10) assesses whether gene expression profiling is a viable (non-hypothesis driven) approach to identify markers or profiles able to distinguish anti-TNF responders and non-responders, after having given an overview of the published gene expression studies focusing on RA.

Chapter 2 reviews current literature concerning DNA polymorphisms investigated in candidate genes for anti-TNF therapy response. Different technologies and strategies for pharmacogenetic research are also discussed.

In chapter 3, a (GT)*n*-repeat, located in the promoter region of the heme oxygenase-1 (*HMOX1*) gene, is investigated. This repeat determines the level of induction of the heme-degrading HMOX1 enzyme. The enzyme protects cells against inflammatory and oxidative stress (154) and was recently shown to protect against the onset of RA (119). We investigated whether the length of the (GT)n-repeat is associated with RA disease severity and radiologic joint damage.

Chapter 4 focuses on the 676T>G (rs1061622) polymorphism in the tumour necrosis factor receptor superfamiliy member 1b (*TNFRSF1B*) gene and its relation to RA disease severity and anti-TNF response. This gene encodes one of the two TNFÐ receptors and therefore is a likely candidate for these phenotypes.

Chapter 5 and 6 focus on the -308G>A (rs1800629) polymorphism located in the promoter region of the tumour necrosis factor A (*TNFA*) gene. It is investigated if the polymorphism is associated with *TNFA* expression, disease severity and/or anti-TNF response. Chapter 5 describes the studies of the first two phenotypes. Chapter 6 is a meta-analysis of the involvement of the polymorphism in the response to anti-TNF treatment, including published and unpublished data from 14 international research groups.

In chapter 8 the published genome-wide expression profiling studies regarding RA susceptibility, severity and anti-TNF response are reviewed.

In chapter 9 we attempted to validate previously published expression signatures predicting anti-TNF response in an independent cohort. Secondly, a new expression profile able to distinguish anti-TNF responders and non-responders in RA is presented.

Chapter 10 discusses the signal regulatory protein B1 (*SIRPB1*) gene, which is identified as a gene showing differential expression in anti-TNF responders and non-responders by expression profiling. The expression differences turn out to be due to a frequent copy number variant (CNV) within the gene. This CNV is subsequently evaluated as a potential marker for anti-TNF response.

Finally, the findings described in this thesis are summarized and the potential for pharmacogenetics and pharmacogenomics in RA is discussed in chapter 11.

> CHAPTER 2

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PHARMACOGENETICS OF ANTI-TNF TREATMENT IN PATIENTS WITH RHEUMATOID ARTHRITIS

> ABSTRACT

Tumor necrosis factor alpha (TNFα) blocking strategies are widely used in the treatment of rheumatoid arthritis (RA). Three anti-TNF agents are registered for the use in RA; etanercept, infliximab and adalimumab. Although anti-TNF therapy is very effective in controlling disease activity and slowing down radiological damage, prolonged response is seen only in about 70% of the patients. The causes for non-response in the remaining patients have not been elucidated yet. Pharmacogenetic studies focusing on genes involved in RA etiology (and/or progression) and in the pharmacokinetics of TNFα blocking agents have identified markers associated with anti-TNFtreatment outcome. In the future, more exhaustive, less hypothesis-driven search strategies are expected to discover additional markers. Identification of these markers might be viewed as a the first step towards tailored TNFα blocking therapy for patients with RA. Nevertheless, replication and large prospective studies will be needed to demonstrate the validity of the identified genetic markers before implementation into daily clinical practice.

> INTRODUCTION

Tumor necrosis factor alpha (TNFα) is a key inflammatory mediator in rheumatoid arthritis (RA). Evidence for the central role of TNFα in the pathogenesis of RA has resulted in the development of therapeutic interventions reducing the excess of TNFα seen in these patients.

Three anti-TNF agents are nowadays commercially available. Etanercept (Enbrel®) is a fusion protein consisting of two p75 TNF receptors linked to the Fc part of human IgG1. This agent binds to TNFÐ and lymphotoxin alpha (LTA) thereby preventing binding of these molecules to their cell surface receptors and subsequent cell signaling. Besides etanercept, two monoclonal antibodies against TNFα are available; the chimeric antibody infliximab (Remicade®) and the fully humanized variant adalimumab (Humira®). These antibodies directly bind to TNFα leading to neutralization of the molecule.

Despite the clinical efficacy of all three anti-TNF strategies, 30% of the patients do not improve. Patients not responding to therapy can be divided in two groups, primary non-responders showing a lack of efficacy starting at the first administration of anti-TNF and secondary non-responders who show an initial benefit from the therapy which diminishes with time (29-31). In addition, some adverse events are observed in a small percentage of patients, including an increased susceptibility to tuberculosis and other coexisting infections, lymphoma and neutropenia. Injection and infusion reactions are the adverse events most often reported (155). Primary and secondary inefficacy of anti-TNF therapy in a considerable percentage of patients, adverse effects and the high costs of anti-TNF blocking agents have driven the search for markers that can predict treatment outcome. One area of such research involves genetics. Insight into the 'pharmacogenetics' of anti-TNF therapy will e.g. facilitate the choice for the most suitable anti-TNF agent for the individual patient and inform the decision on dosing strategies, resulting in an optimized treatment regimen in daily clinical practice.

In recent years progress has been made in the field of anti-TNF pharmacogenetics. The main focus has been on genetic variation in genes that can be directly linked to the mechanism of action of the biologicals and genes involved in RA in general. However these studies have not yet identified (sets of) genetic markers predicting outcome of anti-TNF therapy with high specificity and sensitivity.

This review will discuss the current literature concerning anti-TNF pharmacogenetics as well as technologies that can be used for the identification of additional genetic determinants of anti-TNF response.

Study cohorts

A systematic PUBMED search was performed using the following search terms 'etanercept', 'enbrel', 'infliximab', 'remicade', 'adalumimab', 'humira', 'polymorphism', 'association' and 'rheumatoid arthritis' alone or in combination. The studies had to match the following criteria, published after 2001, involving pharmacogenetics of anti-TNF therapy and including RA patients. In total 23 studies fulfilled these criteria; patient characteristics, the anti-TNF agent used and the investigated genes are summarized in Table 1.

The majority of the studies included patients meeting the American College of Rheumatology (ACR) criteria for RA (9). Two studies did not specify the criteria for the diagnosis of RA (43;156). The response criteria used in most of the studies were the two most widespread, validated (objective) methods to define response to therapy, those of the ACR and those of the European League Against Rheumatoid Arthritis (EULAR) (157;158). Both criteria require (a) three assessor-derived measures, tender joint count, swollen joint count and physician global assessment, (b) one laboratory test of either the erythrocyte sedimentation rate (ESR) or of C-reactive protein (CRP) levels and (c) three patient self-report measures of functional disability, pain and patient global assessment. However, a number of studies did not adhere to these methods (9;156) .

Table 1 / Study characteristics

Number of RA patients (% female)	Ethnic background	Anti-TNF agent: dose
n= 123 (81%)	NS	Etanercept: 2 x 25 mg/week
n=457 (72%)	NS	Etanercept: 2 x 25 or 10 mg/week
n=50 (76%)	German	Etanercept: 2 x 25 mg/week
n=70 (93%)	Korean	Etanercept: 2 x 25 mg/week
n=59 (78%)	NS	Infliximab: 3 mg/kg at t=0, 2, 6, 14, 22 weeks
n=78 (77%)	NS	Infliximab: 3 mg/kg at t=0, 2, 6, every 8 weeks thereafter
n=20 (95%).	Chilean	Infliximab: 3 mg/kg at t=0, 2, 6, 14 weeks
n=9 (NS)	NS	Infliximab: 3 mg/kg at t=0, 2, 6, every 8 weeks thereafter
n=22 (NS)	NS	Infliximab: 3 mg/kg at t=0, 2, 6, every 8 weeks thereafter
n=198 (75%)	French	Infliximab: 3 mg/kg at t=0, 2, 6, every 8 weeks thereafter
n=49 (95%)	Italian	Infliximab: dose not specified
n=58 (79%)	NS	Infliximab: dose not specified
n=20 (95%)	NS	Adalimumab: 40 mg every other week
n=30 (NS)	White, Hispanic, African-American, Asian	Etanercept, Infliximab, Adalimumab: dose not specified
n=54 (69%)	NS	Etanercept, Infliximab, Adalimumab: dose not specified
n=66 (94%)	Italian	Etanercept: 2 x 25 mg/week, Infliximab: 3 or 10 mg/kg every 4 or 8 weeks
n=282 (78%)	Swedish	Etanercept, Infliximab: dose not specified
n=1050 (77%)	UK	Etanercept, Infliximab, Adalimumab: dose not specified
n= 388 (78%)	French	Adalimumab: dose not specified
n=113 (78%)	Spanish	Infliximab: 3 mg/kg every 8 weeks
n=91 (89%)	Spanish	Infliximab: 3 mg/kg at week 0, 2 and 6
n=78 (68%)	French	Infliximab
n=105 (71%0	Italian	Etanercept: 2 x 25 mg/week, Infliximab: 3 mg/kg at week 0, 2 and 6 Adalimumab: 40 mg every other week

NS: not specified, ACR: American College of Rheumatology, EULAR: European League Against Rheumatism

Response criteria	Response (% responders)	genes investigated	Reference
ACR20, DAS28, EULAR	Based on ACR20 and DAS28: Good 76%, Non: 34%	*TNFA, IL10, TGFB1, IL1RN*	(53)
ACR20, ACR50, ACR70	ACR50: 84%	*HLA, TNFA, LTA, TNFRSF1A, TNFRSF1B, FCGR2A, FCGR3A, FCGR3B*	(44)
EULAR	Good: 50%, Mod.: 34%, Non: 16%	*IL10*	(159)
ACR20, ACR70	ACR20: 86%, ACR70: 20%	*TNFA, LTA*	(50)
EULAR	Resp.: 72%, Non: 28%	*TNFA*	(54)
EULAR	Resp.: 53%, Non: 47%	*HLA, MICA, TNF, BAT2*	(160)
ACR20, ACR50	ACR20: >85%, ACR50: >56%	*TNFA*	(55)
EULAR	Resp.: 48%, Non: 52%	*TNFA*	(56)
EULAR	NS	*TNFA*	(161)
ACR20, EULAR	ACR≥20: 66%	*HLA, IL1B, IL1RN, TNFA*	(58)
EULAR	Resp.: 29%, Non: 71%	*IL1B, IL1RN*	(162)
EULAR	Good: 47%, Mod: 31%, Non: 22%	*TNFR1, TNFR2, TNFA*	(43)
ACR20, EULAR	Resp.: 86%		(59)
Good Response: > 70% improvement in PhGA and the SJC and TJC and > 50% improvement in ESR or CRP levels and PGA or duration of morning stiffness.	Total group RA and psoriatic arthritis patients (n=35): Resp.: 66%, Non: 34%	*FCGR3A*	(156)
EULAR	NS	*TNFA*	(60)
EULAR	NS	*TNFR2*	(45)
ACR, EULAR	ACR20: 66%, ACR50: 35%, ACR70: 9%,	*FCGR3A*	(114)
DAS28, EULAR	EULAR: Good: 26%, Mod: 54%, Non: 20%, DAS28: NS	*TNFA, LTA, LST-1*	(52)
ACR50	Resp.: 40%	*TNFA, HLA*	(62)
EULAR	Resp.: 58.4%	*TNFA, HLA*	(63)
ACR20, ACR50, ACR70	ACR20: 45%, ACR50: 12%, ACR70: 3%	*FCGR2A, FCGR3A*	(163)
ACR20, ACR50, ACR70	ACR20: 56%, ACR50: 35%, ACR70: 22%	*FCGR3A, TNFRSF1B*	(47)
ACR20, ACR50, ACR70	ACR20: 32%, ACR50: 39%, ACR70: 28%	*TNFA, TNFRSF1B*	(48)

Pharmacogenetics of anti-TNF treatment

The mechanisms of action of anti-TNF agents have given direction to the pharmacogenetic studies performed so far (164;165). Although it has been demonstrated that patients not responding to a certain anti-TNF agent may benefit from treatment with a second TNF antagonist, which would suggest different mechanisms of action for the anti-TNF drugs and involvement of diverse genetic variants in clinical response (166;167), in a large study in 856 patients who switched to a second anti-TNF agent the reason for non-response to the first drug was related to the reason for stopping the

Table 2 / Genetic variants in genes studied for their association with anti-TNF response

Gene	Polymorphism
TNFA	-1031T>C (rs1799964), -863C>A (rs1800630)
	-857C>T (rs1799724)
	-308G>A (rs1800629)
	-238G>A (rs361525)
	488G>A (rs1800610)
	TNF haplotypes
IL1B	-511C>T (rs16944)
	3954C>T (rs1143634)
IL10	-1087G>A (rs1800896)
	Microsatellites IL10.R and IL10.G
TGFB1	915G>C (p.R25P, rs1800471)
IL1RN	VNTR in intron 2
	2018T>C (p.A39A, rs419598)
HLA region	Shared epitope
MICA	Microsatellite
D6S273	Microsatellite
BAT2	Microsatellite
D6S222	Microsatellite
LTA	249G>A (rs909253)
	365G>C (rs746868)
	177A>G (p.R13C, rs2229094)
	319(720)C>A (p.T60N, rs1041981)
	Ex-1A>T (rs2239704)
LST-1	Ex-5 (rs1052248), Int 2 (rs2256965)
TNFRSF1A	-609G>T (rs4149570), -580A>G, (rs4149621), -383A>C (rs2234649)
	36A>G (p.P12P, rs1139417)
TNFRSF1B	676T>G (p.M196R, rs1064622)
FCGR2A	550G>A (p.H166R, rs1801274)
FCGR3A	681G>T (p.V212F, rs396991)
FCGR3B	NA1/NA2

second drug (inefficacy or adverse events). This fact has lend validity to the investigation of the same genetic variants for all three anti-TNF agents (166).

The most obvious candidate gene, *TNFA*, coding for TNFα, the target of anti-TNF therapy, has been investigated in many studies in relation to all three anti-TNF agents (Table 1). Also the genes encoding TNF receptors, involved in the susceptibility to and severity of RA have been investigated for their association with anti-TNF therapy outcome (Table 1). The following paragraphs will provide an overview of the genetic variants studied in relation to the different anti-TNF biologicals. A summary of the investigated polymorphisms of each study and their possible function can be found in Table 2.

(possible) functional role of the genetic variant	References
Possible influence on TNFα production	(50-52)
Affects the transcription efficiency of TNFα	(43;50;168)
Influence on TNFα production	(43;44;48;50;52-63)
Possible influence on TNFα production	(43;44;50;52;58;59;62;163;169;170)
Unknown	(43;44;62)
Linked to TNFα -308G>A	(44;160)
Increased IL1B production	(162;171)
Possibly associated with IL1beta production	(58;162;172;173)
Associated with IL10 production	(53;174)
Associated with IL10 secretion	(159;175)
Influence on TGFß1 production	(53;176)
Associated with enhanced IL1 beta production	(53;162;173)
Unknown	(58)
Associated with RA susceptibility and severity and MTX response	(44;58;62;63;160)
Unknown	(160)
Unknown	(160)
Unknown	(160)
Unknown	(160)
LTA haplotype effect on in vitro TNFα production	(44;52;177;177)
LTA haplotype effect on in vitro TNFα production	(44;50;177)
LTA haplotype effect on in vitro TNFα production	(44;50;52)
LTA haplotype effect on in vitro TNFα production	(44;50;52)
LTA haplotype effect on in vitro TNFα production	(52)
Unknown	(52)
Unknown	(44)
Unknown	(43)
Effect on apoptosis	(43-48)
Effect on the affinity for the binding of human IgG	(44;163;178)
Effect on the affinity for the binding of human IgG	(44;47;114;156;163)
Effect on phagocytic capacity	(44;179)

Etanercept

A total of four studies has assessed the effect of genetic variation on response to etanercept (44;50;53;159). Three of these studies included single nucleotide polymorphisms (SNPs) in the promoter of the *TNFA* gene. Also polymorphisms in the genes encoding TNFα receptors and cytokines involved in RA as well as the HLA region and Fc receptors were investigated for their relation with etanercept response.

The first pharmacogenetic study related to etanercept treatment in RA patients focused on polymorphisms in genes encoding cytokines with an important role in the pathogenesis of RA (53). The hypothesis of the study was that patients displaying different cytokine levels in joints showed different responses to TNF blocking therapy. In each of the genes coding for the cytokines TNFα, interleukin 10 (IL10), transforming growth factor beta 1 (TGFß) one promoter SNP was analyzed for association with etanercept response, as was a variable number of tandem repeats (VNTR) in intron 2 of the IL1 receptor antagonist gene (*IL1RN*). All these polymorphisms were known to influence the expression of the respective gene. The authors also investigated the *HLA-DR* alleles, which are associated with RA course and the response to conventional disease-modifying anti-rheumatic drugs (DMARD) treatment. In a total of 123 patients no significant associations were found. Combined analysis of the SNPs in the *TNFA* and *IL10* genes, defining patients with a presumably lower propensity for an inflammatory response, showed that the -308 GG *TNFA* genotype together with the -1087 GG *IL10* genotype was more frequently encountered in responders than in non-responders after three months of therapy ($p<0.05$). Analysis of the combined genotypes for *IL1RN* and *TGFB1* (associated with strong inflammatory reactivity) showed that a combination of two-repeat alleles of the *IL1RN* VNTR with the +915 CG genotype of *TGFB* was significantly associated with poor response to etanercept ($p<0.05$).

The second study, including 50 patients, focused on two microsatellite polymorphisms in the *IL10* gene (*IL10.R* and *IL10.G*) (159). These polymorphisms had been related to IL10 production (175). The *IL10.R3* allele ($p=0.0113$) and the IL10.R3-G9 haplotype ($p=0.0212$) were associated with good response to etanercept therapy. The *IL10.G13* allele and the *IL10.R2-G13* haplotype were indicative of moderate or non-response.

Criswell and colleagues studied a group of 457 patients, 301 of these patients were treated with etanercept (44). The genes encoding TNFα, lymphotoxin alpha (*LTA*) which can both bind to etanercept and the genes coding for the two TNF receptors (*TNFRSF1A*; *TNFRI* and *TNFRSF1B*; *TNFRII*) were analyzed in this study. The authors also included *HLA-DRB1* alleles and the Fc receptor pathway (Fc gamma receptors 2A, 3A and 3B), involved in the degradation of etanercept-TNF complexes. In total 13 single nucleotide polymorphisms and 5 microsatellites were investigated. In single marker

analyses the only significant association was for the *HLA-DRB1* alleles and the ACR50 response (indicating 50% improvement in disease activity) at 12 months. Patients with one or two copies of the shared epitope were more likely to respond to etanercept therapy. Several studies have demonstrated that the association of HLA with RA susceptibility and outcome probably involves other loci closeby and is therefore difficult to analyze (180-182). To overcome this problem extended haplotypes were generated including *HLA-DRB1* and six polymorphisms in the *LTA* and *TNF* genes. Two of these haplotypes including *HLA-DRB1*0404*, the other *HLA-DRB1*0101*, both encoding the shared epitope, were associated with treatment response at 12 months. The association with etanercept response was also observed in a multivariate logistic regression focusing on the two associated haplotypes and including covariates (odds ratio (OR) 2.5 and 4.9 for the **0404* and **0101* containing haplotypes, respectively).

The involvement of polymorphisms in the *TNFA* and *LTA* genes in etanercept treatment outcome was thoroughly studied by Kang and co-workers (50). Five SNPs in the *TNFA* promoter region and 2 non-synonymous SNPs in *LTA* were tested for association with therapy response in 70 patients. Marginal association of the -857C>T SNP in the *TNFA* promoter with treatment response was found only when comparing ACR20 non-responders to ACR70 responders (p (corrected)=0.054). An association of the -857C>T polymorphism with the response to anti-TNF therapy may be explained by the fact that the OCT1 transcription factor can bind to the promoter of *TNFA* only when a C-allele is present, leading to higher TNFα production. In contrast to the study by Criswell no association with treatment response could be found for the shared epitope. This might be related to the ethnic background of the study populations, the alleles found by Criswell are rare in the Korean population investigated by Kang and colleagues (44), but might also be due to power issues

Infliximab

The effect of genetic variation has also been examined in relation to the response to infliximab in RA patients. Four studies focused on the -308G>A polymorphism in the *TNFA* promoter. The A-allele of this polymorphism is thought to be associated with increased TNFα production (54-57;161;183). Four other studies combined the analysis of this polymorphism with that of genetic variants in the HLA region and/or other candidate genes (43;58;63;160). Two other studies focused on polymorphisms located in other candidate genes for their possible influence on infliximab response: Canete *et al*. investigated polymorphisms in the Fc gamma receptor genes *FCGR2A* and *FCGR3A* (163) and Rooryck investigated polymorphisms in the genes *FCGR3A* and *TNFRSF1B* (47).

Mugnier and co-workers published the first study investigating the relation between the response to infliximab and the -308G>A SNP in the *TNFA* promoter in 59 RA patients (54). Patients with the GG genotype were twice as likely to respond to infliximab therapy as the group with the AG or AA genotype (p=0.0086).

Balog and colleagues performed a very small study analyzing the effect of the -308G>A polymorphism on response to infliximab (56). Due to the small patient number they did not perform a statistical analysis but could determine that the non-responders to therapy carried the A allele more often than the G allele.

Fonseca and co-workers demonstrated that patients with the GG genotype at the -308G>A polymorphism have a better response to infliximab than the patients with an AG genotype (57), thereby confirming the findings by Balog and Mugnier (54;56).

Cuchacovich and colleagues used a more hypothesis-driven approach (55). They first determined the genotype of the -308G>A SNP in 132 patients. Subsequently, they selected 10 patients with a GA and 10 patients with a GG genotype who were treated with 3mg/kg infliximab at initiation and at weeks 2, 6 and 14. No significant differences in the ACR20 and ACR50 improvement were found between genotypes at the diverse time points.

Pinto and coworkers investigated whether polymorphisms at position -238 and -308 at the promoter region of the *TNFA* gene, and the presence of the DR3 and shared epitope (SE) alleles, are able to predict response to longterm infliximab therapy (30 monthts) in a RA cohort of 113 patients. Their data did not support the concept that a certain combination of allelic forms was associated with response to infliximab (63).

Also Marotte *et al*. included the -308G>A promoter polymorphism of the *TNFA* gene in their study (58). In addition they investigated the –238G>A promoter polymorphism of this gene, the shared epitope and polymorphisms in the *IL1B* and *IL1RN* genes. All polymorphisms chosen for this study had previously been associated with RA severity. A total of 198 patients receiving infliximab therapy were included in the study. The authors could not detect an association of the response to infliximab with the investigated polymorphisms. However they demonstrated an association between the selection for treatment with infliximab and the shared epitope, in that patients with the shared epitope were more likely to be selected for infliximab treatment.

Martinez and co-workers investigated the response to infliximab treatment in relation to HLA and microsatellites in the nearby tumor necrosis factor genes (*TNFA*, *TNFB*, *TNFC*, *TNFD*, *TNFE*), MHC class I chain-related gene A (MICA) and HLA-B-associated transcript 2 (BAT2), as well as two microsatellites in the same region (D6S273 and D6D2223) in a sample of 78 RA patients (160). An increased frequency of the TNFa11;b4 haplotype was detected in the responder group (p=0.01) whereas the frequency of the D6S273_3 allele was decreased in this group (p=0.04). After Bonferroni correction for multiple testing the detected associations were no longer significant.

Tolusso and colleagues studied the *IL1* gene complex (*IL1A*, *IL1B* and the *IL1RN*) in a group of 49 patients treated with infliximab (162). They demonstrated that non-responders to infliximab therapy had a higher *IL1RN**3 allele frequency. The genotype combination *IL1RN**long/long, *IL1B* -511CC and *IL1B* +3953CC was exclusively found in patients not responding to infliximab therapy (p=0.05). The association with the *IL1RN* VNTR is likely due to a type I error, however, as it was not found by Marotte and colleagues using a much larger patient population (n=198) (58).

A recent study investigating SNPs in the genes encoding TNFα and TNF receptor (TNFR) I and II in 58 patients demonstrated that only a combination of SNPs was associated with treatment response (43). Good responders carried the *TNFR2* +676T allele and the *TNFA* -857C and +489G alleles more frequently compared with the poor responders (p=0.0008).

Canete and co-workers assessed the relationship between the polymorphisms 550G>A and 681G>T in respectively the Fc gamma receptor genes *FCGR2A* and *FCGR3A* and the response to infliximab in 91 RA patients. At week 6 of follow-up, the proportion of patients achieving ACR50 and EULAR good responses were significantly higher among homozygotes of the *FCGR3A* GG genotype when compared to the GT-TT genotype (ACR50: p=0.003; EULAR: p=0.04). At week 30, homozygotes of the *FCGR2A* GG allele had a better ACR20 response when compared to the AA-GA genotype group (p=0.035) (163).

In the study of Rooryck and colleagues is was determined whether two functional polymorphisms, 681G>T in the *FCGR3A*, and 676T>G in the *TNFRSF1B* genes correlate with rheumatoid arthritis susceptibility and response to anti-TNF-alpha therapy. The reported a significant correlation between 676G allele carriers and low response to infliximab therapy (47).

Adalimumab and studies focusing on more than one anti-TNF agent

Two study has been described thus far investigating the effect of genetic polymorphisms on the response to adalimumab (59;62). Six other studies focused on more than one anti-TNF agent, four of them including adalimumab (45;48;52;60;114;156).

Cuchacovich et al. investigated the –308G>A SNP in the *TNFA* gene in a group of 70 patients treated with adalimumab (59). They found a higher percentage of responders according to the DAS28 (disease activity score based on 28 items) in the GG genotype group at week 24 of treatment (p=0.05). Patients with the GG genotype also showed a significantly higher mean DAS28 improvement ($p<0.05$). No significant association was observed when using the ACR response criteria.

Miceli-Richard and coworkers investigated three polymorphisms in the *TNFA* locus (*TNFA* -238G>A, -308G>A and -857C>T) for their capability to predict therapy outcome in a cohort of 388 RA patients treated with adalimumab. No association was detected between the three polymorphism and the response to adalimumab when the polymorphisms were tested separately. However, haplotype construction of the *TNFA* locus revealed that the GGC haplotype (-238G/-308G/-857C) in a homozygous form was associated with response to adalimumab after 12 weeks of treatment (p=0.015) (62).

Seitz and colleagues also studied the –308G>A *TNFA* promoter polymorphism in 54 patients with RA. They combined all three anti-TNF agents in their analysis (60). The authors demonstrated an association between a positive anti-TNF treatment outcome and the -308 GG genotype ($p<0.001$) as well as the absence of the A-allele at position -308 ($p<0.001$).

Maxwell and co-workers investigated 7 known polymorphisms surrounding the TNF locus, including the genes coding for lymphotoxin alpha (*LTA*) and leucocyte specific transcript 1 (*LST-1*) for their association with response to all three anti-TNF agents in a cohort of 1050 patients. The following polymorphisms were included: *TNFA* -238G>A, -308G>A, -1031C>T, *LTA* exon-1, *LTA* intron-1, *LST-1* exon-5 and *LST-1* intron-2. They reported an association of the *TNFA* -308G>A polymorphism with anti-TNF response over the entire cohort (p=0.001). After stratification by anti-TNF agent, the *TNFA* -308AA genotype was associated with a poorer response to etanercept when compared to the *TNFA* -308GG genotype (p=0.001). No association was detected between the *TNFA* -308G>A polymorphism and infliximab therapy (p=0.8). Conversely, the *TNFA* -238GA genotype was associated with a poorer response to infliximab (p=0.028) but not to etanercept (p=0.6). The number of patients taking adalimumab was too small for meaningful analysis as a separate group (52)

Fabris and co-workers studied the *TNFR2* gene in 66 patients treated with etanercept or infliximab. They concluded that the presence of one G-allele at position +676 in *TNFR2* predisposes to non-response during therapy (45).

Tutunca and colleagues focused their study on the gene encoding Fc gamma receptor IIIA. The investigated polymorphism (valine 158 to phenylalanine (V158F)) has an effect on the affinity of the receptor for binding of human IgG (156). Thirty patients treated with one of the three anti-TNF agents were studied. The authors detected an association of the low affinity F/F genotype with therapy response compared to the combination of V/V and V/F genotypes ($p<0.01$).

Kastbom and co-workers invalidated the above association using a large patient sample (n=282) treated with either infliximab or etanercept (114). No significant association between response to treatment and *FCR3A* genotype could be found either using the ACR or the EULAR response criteria.

In the study of Ongaro and colleagues it was analyzed whether the polymorphisms 676T>G in the tumor necrosis factor receptor super family 1B (*TNFRSF1B*; *TNF* receptor II *TNFR-II*) gene and -308G>A in the TNFA promoter gene may influence the response grading to infliximab and adalimumab treatment in RA patients. They enrolled and genotyped 105 RA patients treated with etanercept (*n* = 55), infliximab (*n* = 40) and adalimumab (*n* = 10). Patients harboring the *TNFSF1B* 676TG genotype showed a significant lower ACR response, compared with the *TNFSF1B* 676TT, at three and twelve months. No association was observed between the *TNFA* -308G>T polymorphism and anti-TNF response (48).

Replication and meta-analysis

The pharmacogenetic studies performed so far mainly investigated relatively small patient groups. This resulted in a low power to detect differences between responders and non-responders to anti-TNF therapy (184;185). On the other hand, small sample sizes may also lead to overestimation of the strength of a certain association finding by for instance chance or sampling biases. Replication of association findings by different researchers and across diverse samples is essential to draw definitive conclusions about whether an association really exists. The *HLA-DRB1* alleles have been investigated in three studies. One of them encompassed 457 individuals suggesting that there is indeed an association between the HLA region and anti-TNF response (44;58;160). For the *FCGRIIIA* polymorphism the results are more convincing. Two large studies (including 301 and 282 patients, respectively) indicate a lack of association between this gene and anti-TNF response (44;114), whereas the study that did detect association between the *FCGRIIIA* gene and treatment response had a very small number of patients (30 patients) (156). Conflicting results have also been reported for the genes coding for the TNF receptor 2 as well as *IL1RN* (43-45;58;162). Comparison of studies focusing on the same gene is often complicated by the fact that different genetic variants in the genes are investigated; these can have different effects on treatment response (53;159). Furthermore, so far most genes have not thoroughly investigated. This might be the reason why an existing association between a genes and anti-TNF response is not detected, as the studies might not have focused on the right variant. Only the HLA region and the adjacent *TNFA* and *LTA* genes have been investigated more completely by creating haplotypes of diverse markers in the genes. Covering the genes with a substantial number of SNPs or other genetic markers can enhance the detection of associations with a phenotype that are either due to direct causal effects of the polymorphisms or due to the fact that they serve as proxies for unknown causal variants (linkage disequilibrium).

For the polymorphism that has been investigated most often, the –308G>A *TNFA* promoter polymorphism, a meta-analysis has been performed to overcome the problem of small studies (61). Meta-analysis is a statistical procedure integrating results from several studies to produce a single estimate with enhanced precision (186). Thus far one meta-analysis has been published combining data of the studies addressing. In total, data of 311 patients were included in the meta-analysis (50;53-57). Patients were treated with either etanercept or infliximab and standard disease activity scores (ACR or DAS28 scores) were used to assess the response to the anti-TNF therapy. The authors combined the rare AA genotype with the AG genotype. Analysis pointed out that the A-allele carriers were significantly less likely to belong to the responder group than patients with the GG genotype (OR=0.33. p=0.0008). This meta-analysis thus suggests that there is indeed an effect of the –308G>A SNP on the response to TNF therapy. However, a new (not yet published) meta-analysis regarding the –308G>A *TNFA* promoter polymorphism, which included over 2800 RA patients from 13 studies, stated that the polumorphism is not associated with response to anti-TNF therapy (chapter 6).

Genome-wide association studies

The focus of anti-TNF pharmacogenetic studies so far has been exclusively on polymorphisms in the most obvious candidate genes, which are those known to be involved in RA, implicated in TNF mechanisms of action or involved in the degradation of the anti-TNFagents. A less hypothesis-driven (pharmacogenomics) approach to the search for genes involved in anti-TNFresponse is the use of genome-wide screening of polymorphism for their association with anti-TNF respone.

Recently, the study of complex genetic diseases has undergone a dramatic revolution due to the development of genome-wide association studies (GWAS). Advances in technology have enabled hundreds of thousands of SNPs to be genotyped in thousands of samples in a single experiment, allowing studies to achieve good coverage of common variation in the human genome. An impressive GWAS was undertaken by the Wellcome Trust Case Control Consortium (WTCCC). This consortium, which is a collaboration of over 50 research groups within the UK, investigated 2000 case samples and 3000 controls for seven complex diseases, including RA. Two well-documented RA susceptibility genes (*HLA-DRB1* and *PTPN22*) and nine new variants were found to be associated with RA susceptibility in this study (187). This unbiased genome-wide approach is also been used to identify common genetic variation that could be responsible for differences in anti-TNF response. Liu and co-workers conducted a GWAS using the Illumina HapMap300 SNP array to investigate 89 RA patients for their response to anti-TNF treatment. Several SNPs

showed significant associations with loci including the *MAFB* (v-maf musculoaponeurotic fibrosarcoma oncogene homolog B) gene on chromosome 20. Also significant associations were reported for SNPs located within or nearby the genes *IFNk* (type I interferon) on chromosome 9, *PON1* (paraoxonase I) on chromosome 7 and *IL10* (interleukin 10) on chromosome 1 (66). Both *IL10* and *IFNk* are involved in immunity and reduced expression of *PON1* is associated with Crohn's disease (188), indicating that these are interesting candidate genes for further study on anti-TNF response and RA in general.

Other approaches to identify markers for anti-TNF response

Another less hypothesis-driven approach to identify genetic markers for anti-TNF response is the use of gene expression profiling strategies. Several studies analyzed differentially expressed genes using microarrays in responders and non-responders to anti-TNF therapy (73;189-192). These studies are described in more detail in chapter 8, since this chapter focuses on expression profiling in RA patients treated with anti-TNF.

Also at the level of the proteome possible markers for anti-TNF treatment outcome have already been investigated, though all studies have been limited to (single) proteins known to be involved in RA (193-196). Nowadays hypothesis free approaches to proteome analysis are also available, like two-dimensional gel electrophoresis or surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF) analysis. Using two-dimensional gel electrophoresis it has demonstrated that the MRP8/MRP14 heteroduplex might be a marker for anti-TNF therapy (197). SELDI-TOF analysis has already proven to be useful to identify proteins related to treatment outcome for other biologicals (198).

Polymorphisms in genes identified using these approaches might therefore be good candidates for future pharmacogenetic studies in relation to anti-TNF response.

Expert commentary

Although progress has been made in the search for genetic polymorphisms related to anti-TNF response (Table 1 and 2) so far only the –308G>A SNP in the *TNFA* promoter has been identified as a (weak) marker predicting treatment outcome. This may be attributed to the low power of most pharmacogenetic studies, the use of different ethnic groups and different outcome definitions and/or the limited number of genes studied so far. It would surely be worthwhile to search for genes involved in treatment response in a non hypothesis-driven manner, comparable to the gene expression profiling and proteomics studies.

Another such hypothesis-free method that has not yet been used in the search for predictors of anti-TNF response is the whole genome association approach (64). The availability of relatively inexpensive arrays allowing the analysis of hundreds of thousands of SNPs in one single experiment has recently made this approach feasible (64;65). Association is detected via a surrogate marker (SNP) in linkage disequilibrium with the true causal variant. Large, well-characterized patient samples will be needed for such an approach. Whole genome association studies represent a first step of analysis and need to be followed up by more focused studies searching for the causal variants in the regions of interest identified.

Of course, replication of the findings in additional patient samples is of utmost importance for all approaches that can be used for the identification of markers predicting anti-TNF response, as findings in one sample will not guarantee their validity and generalizability.

Combination of the above described techniques is probably the most powerful way to identify genes involved in the response to anti-TNF therapy. The integration of data will also provide us with a wealth of additional information about the way in which genetic factors exert their effects on treatment (e.g. via alterations in RNA expression and translation efficiency, protein folding or via secondary effects on other genes). It is highly likely that we will not be able to identify a single genetic variant that will predict treatment outcome in anti-TNF therapy with sufficient sensitivity and specificity. Rather it is to be expected that a set of genes and genetic variants in those will together form a marker for treatment response. In this respect it is important to take additive effects as well as interactions between genetic polymorphisms into consideration in pharmacogenetic studies. Furthermore, though SNPs are the most frequent polymorphisms in the human genome and are most amenable to high throughput genetic analysis, all types of polymorphisms (including repeat polymorphisms, insertion/deletions and large scale copy number variants) should be considered in the pharmacogenetic studies.

An additional factor needed to be taken into account in these studies is the presence of non-genetic factors influencing the response to anti-TNF treatment. For instance smoking is associated with non-response in patients with Crohn's disease and RA treated with infliximab (199;200).

Formation of antibodies directed to anti-TNF agents might also be a reason why patients do not respond to anti-TNF agents. An early study demonstrated that at least 8% of the patients treated with infliximab produced antibodies to this anti-TNF agent (30). This study suggested that there were no differences between the ACR20 response of patients with and without anti-infliximab antibody formation. However, other studies since have demonstrated that nearly half of the patients formed anti-infliximab antibodies upon treatment and that the formation of these anti-infliximab

antibodies can be correlated with the response to infliximab in the patients (201). In a pharmacogenetical sense researchers have already started to address this issue by investigating genes known to be involved in antigen presentation and degradation of antibodies. This investigation should be intensified as it might identify patients prone to the formation of antibodies directed against the anti-TNF agents. Which percentage of the non-response to the different anti-TNF agents will be explained by the formation of the anti-anti-TNF antibodies is not clear yet, but it seems safe to assume that additional mechanisms are involved.

In conclusion, a very detailed characterization of (large, homogeneous) patient samples will be necessary to arrive at an accurate prediction of the response to anti-TNF therapy.

Future perspective

Pharmacogenetic studies described so far constitute a first, cautious step towards the elucidation of the genetic variants involved in the modulation of anti-TNF response. The findings up to now already suggest that treatment response to anti-TNF is a multifactorial event, influenced by several (genetic and non-genetic) factors, with probably small effects of individual factors. As more and more patients are treated with anti-TNF agents the need for markers predicting treatment outcome with high sensitivity and specificity is strongly increasing to ensure effective treatment of individual patients, prevent unnecessary disease progression and keep treatment costs at a minimum. In the future the full spectrum of possibilities in genetic research as well as related disciplines need to be employed to identify markers for treatment outcome. With several clinical trials already registered at sites like www.clinicaltrials.gov it seems that indeed many efforts are currently ongoing to determine the factors influencing the response to anti-TNF therapy.

Large, very well-characterized patient samples will make it possible to use whole genome association approaches as well as transcriptomics and proteomics for the identification of markers that can predict treatment outcome in a substantial part of the patients. What is needed now more than ever is a good collaboration between different research groups and disciplines, to ensure fast discovery as well as efficient testing of validity and generalization of findings and serve the interest of the patient treated with anti-TNF best.

> CHAPTER 3

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HEME OXYGENASE-1 PROMOTER POLYMORPHISM MODULATES THE RELATIONSHIP BETWEEN DISEASE ACTIVITY AND JOINT DAMAGE IN RHEUMATOID ARTHRITIS

> ABSTRACT

Objective: The $(GT)_n$ -repeat in the heme oxygenase-1 (HMOX1) promoter determines the level of induction of the heme-degrading HMOX1 enzyme that protects against inflammatory and oxidative stress. Individuals with short $(GT)_n$ -repeats (SS; $n<25$) induce more rapidly higher levels of HMOX-activity than those with long repeats (LL; $n\geq 25$). Recently, it was demonstrated that HMOX-activity protects against the onset of rheumatoid arthritis (RA). The aim of this study was to determine whether the $(GT)_n$ -repeat length within the HMOX1 promoter region is associated with RA disease severity and radiologic joint damage.

Methods: A cohort of 325 well-characterized RA patients and 273 controls was investigated by DNA-fragment-length analysis for the association of $(GT)_n$ -repeats in the HMOX1 promoter region with RA disease susceptibility and severity.

Results: Although no significant differences in genotype or allele frequency was found between controls and RA patients, the odds ratios corresponded well to the previously described cohort. Among patients, those with the SS-genotype had a more favorable radiologic outcome over nine years than those carrying the LL-genotype. This was unexpected since no difference in disease activity between the genotypes or alleles was found.

Conclusion: Patients with the SS-genotype have a better long-time radiologic outcome despite bad prognostic markers at baseline and similar disease activity at follow-up. This suggests that the HMOX-system is involved in the uncoupling of disease activity and joint damage and may provide a novel target for the treatment of RA.

> INTRODUCTION

Although the exact etiology of rheumatoid arthritis (RA) remains elusive it is thought that oxidative and inflammatory stress is involved in RA joint damage. It has recently been demonstrated that heme oxygenase-1 (HMOX1), the enzyme that degrades heme into biliverdin/bilirubin, carbon monoxide and iron, is strongly induced in inflamed synovial tissue afflicted with RA (202).

HMOX1 attenuates inflammation and oxidative stress in a wide range of conditions, whereas inhibition of HMOX-activity exacerbates inflammation (154). This is exemplified by the observation that overexpression of HMOX1 in synovial tissues attenuates the expression of inflammatory cytokines whereas inhibition of HMOX-activity leads to opposite effects (202).

The length of a guanine-thymidine $(GT)_n$ -repeat polymorphism in the promoter region of the HMOX1 gene has been demonstrated to determine the level of HMOX1 induction (203). When given the same stimulus, people homozygous for a $(GT)_n$ -repeat $n<25$ express significantly more HMOX1 than people homozygous for a $(GT)_n$ -repeat $n\geq25$ (203). Of importance is that the size of this (GT)n-repeat corresponds clinically with differential susceptibility to various conditions. For example individuals homozygous for the short repeats are protected against emphysema and coronary artery disease, but have a higher risk for developing a melanoma (204). Recently, it has been demonstrated that people carrying a long $(GT)_n$ -repeat, have an increased susceptibility for developing RA, compared to people carrying a short $(GT)_n$ -repeat, and, subsequently increased HMOX1 expression (119).

Since HMOX-activity protects against inflammatory and oxidative injury we postulated that among RA patients, the people carrying a HMOX1 promoter polymorphism leading to less HMOX1 induction, would have worsened outcome than those harbouring a polymorphism resulting in high HMOX-1 levels.

> PATIENTS AND METHODS

Patient cohort

Genotyping was performed in patients with RA participating in an early inception study that started in 1985 and is still running at the department of Rheumatology of the Radboud University Nijmegen Medical Centre (RUNMC) in the Netherlands. The study included only those patients who met the American College of Rheumatology criteria for RA, had a disease duration of <1 year, and had no prior use of disease-modifying anti-rheumatic drugs or biological agents before inclusion. All patients in the early inception cohort are well characterized with regard to demographics and are regularly monitored for disease activity and outcome. Controls were recruited from anonymous healthy blood donors of the catchment area of the RUNMC. The study was approved by the local ethics committee of the RUNMC.

Demographic data such as gender and age at disease onset, as well as the presence of rheumatoid factor (RF), C-reactive protein (CRP), and erythrocyte-sedimentation rate (ESR) were included in the analysis. Disease Activity Score 28 (DAS28) was measured every three months, after which the average at baseline and after 3, 6 and 9 years of follow-up was used for our study (15). Radiological damage was assessed at baseline and during 3, 6 and 9 years of disease follow-up. The radiographs were scored by 1 blinded observer in accordance with the Ratingen radiological damage scoring system (205).

Genotyping of the $(GT)_n$-repeat in the HMOX1 gene promoter

Genomic DNA was extracted from leukocytes in peripheral venous blood from 325 RA patients that have been followed in time and 273 controls as previously described (206). The HMOX1 gene 5'-flanking region was amplified by PCR using either a FAM, VIC, NED or PET fluorescently-labeled sense primer (5'-label-AGAGCCTGCAGCTTCTCAGA-3') and an unlabelled antisense primer (5'-ACAAAGTCTGGCCATAGGAC-3') flanking the $(GT)_n$-repeat (207). PCR products were generated in a final volume of 10 µl containing 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands), 60mM Tris-HCL (pH 8,5), 15 mM $(NH4)_2SO4$, 1,5 mM $MgCl_2$, 0,25 mM of each dNTP, 5 pmol of each, primer and 50 ng genomic DNA. Amplifications were performed in a PTC 200 PCR apparatus (MJ Research, via Biozyme Landgraaf,

the Netherlands) using a 10 min denaturation period at 95 °C followed by 32 cycles of 94 °C for 1 min, 62,4°C for 1 min and 72°C for 1 min. A final extension step of 10 min at 72°C completed the reaction. PCR products, containing four different fluorescent labels, were pooled 1:1 for further analysis. Genotyping was performed using 1,5 µl of the pooled PCR product together with 9,7 µl formamide and 0,3 µl GeneScan-500 LIZ SIZE StandardTM (Applied Biosystems) on an ABI3100 Genetic Analyzer according to the protocol of the manufacturer (Applied Biosystems). Fragment-length determination was done using GeneMapper software version 4.0 (Applied Biosystems). Allelic repeats were divided into two subclasses based on previous studies (4). The short (S)-allele was classified as n<25 (GT) and long (L)-alleles with n≥25 (GT). The determination of the genotypes and documentation of the results was done by two independent researchers blinded for the clinical variables. For both the RA and control cohort, 5% of the samples were genotyped independently twice as intern controls.

Statistical analysis

X-ray data for the first three years of follow-up were available for 225 (69%) of the 325 RA patients of the inception cohort. Frequency of HMOX1 genotypes (LL, LS, SS) was the same in complete and sub-samples. Missingness was unrelated to clinical variables, specifically age, gender, rheumatoid factor positivity and the DAS28 score. Differences in baseline variables between patients grouped according to genotype were performed using chi-square, one-way ANOVA or Kruskal-Wallis tests as appropriate. An association between genotype and medication is a priori unlikely. Gender, age at diagnosis, and medication use was not significantly different between the genotype groups (data not shown). Joint damage progression was calculated after 3, 6 and 9 years as the difference with baseline. Differences in joint damage progression between genotype groups were analyzed using linear regression with dummy variables for genotype groups. To improve model fit, the analysis was repeated using root-transformed progression scores and these p-values were interpreted for between-group differences. To study differences in the trend over time the analysis was additionally performed using longitudinal regression, thus correcting for repeated measures over time (mixed models).

> RESULTS

HMOX1 genotype and allele frequency in RA patients and controls

In order to verify that the number of $(GT)_n$-repeats in the HMOX1 promoter is associated with RA disease susceptibility, as demonstrated in the studies of Rueda *et al*. (119), the number of GT-repeats of 325 patients and 273 controls was determined (Table 1). Five percent of the samples in both cohorts were genotyped twice as internal controls. We observed no genotyping errors in both the RA and control cohort. Repeat length analysis showed that the number of (GT)-repeats ranged from 13 to 41, with 23 and 30 repeats as most common, both in controls and in RA patients (data not shown). In both the patient and control group, the homozygous L-genotype was most frequently present. The SS-genotype was rarest in both patients and controls (Table 1). Although comparison of the genotype and allele frequencies between patients and controls did not show statistically significant differences (Table 1), the Odds Ratios were similar to those in Rueda's larger study (119).

Table 1 / HO-1 $(GT)_n$ microsatellite distribution in RA patients and controls*

$(GT)_n$ microsatellite	RA patients n (%)	Controls n (%)	p-value	OR (95% CI) Present study	OR (95% CI) Rueda *et al* (119)
Genotype frequency					
SS	27 (8)	28 (10)	0.44	0.7 (0.4-1.3)	0.6 (0.4-0.9)
SL	119 (37)	107 (39)	0.30	1.2 (0.6-2.1)	1.1 (0.8-1.3)
LL	179 (55)	138 (51)	0.33	1.3 (0.7-2.4)	1.1 (0.9-1.3)
	Total 325	**Total 273**			
Allele frequency					
S	173 (27)	163 (30)	0.22	0.8 (0.6-1.1)	0.8 (0.7-0.9)
L	477 (73)	383 (70)	0.22	1.2 (0.9-1.5)	1.2 (1.0-1.4)

Association of HMOX1 promoter polymorphism with joint damage in RA patients, but not with disease activity

We investigated the HMOX1 $(GT)_n$ repeat in relation to RA disease activity and radiologic joint damage in a cohort of 225 patients (Figure 1; Table 2). No differences in medication use between the subjects carrying different HMOX-1 promoter polymorphisms were found. The median (p25-p75) number of disease-modifying anti-rheumatic drugs used was 3 (2-5) in all three groups. In particular, there were no significant differences in the percentage of patients between the three genotype groups (SS, SL or LL), who used methotrexate, sulfasalazine, oral prednisone, methylprednisolone, or anti-TNF. No significant differences existed between the genotypes or alleles with respect to disease activity, CRP, RF (Table 2), and ESR (data not shown).

Linear regression analyses (Table 2) showed that after 9 years of disease follow-up, there was significantly less damage in patients homozygous for the S-allele when compared to homozygous L-allele (P=0.047). The course over time of X-ray scores demonstrated that indeed the RA patients with the SS-genotype had significantly less severe joint damage over time when compared to the LL- or SL-genotype (Figure 1). The regression coefficients are interpreted as the differences of LL- and SL-genotypes with the SS-genotype (genotype analysis) or the difference between L- and S-alleles (allele analysis) in progression of joint damage. In a longitudinal regression analysis the progression of joint damage (Figure 1) was significantly less for SS- compared to LL-patients (p=0.022). Differences between LL and SL (p=0.066) and between SS and SL (p=0.26) failed to reach statistical significance. A box-plot analysis of the erosion scores at year 9 demonstrated a skewed but unimodal distribution of the SS-genotype (Figure 1B).

In addition to genotype distribution within the RA patient group, we also performed allele analysis (Table 2). After 9 years of disease duration, the radiologic joint damage was significantly lower in patients carrying an S-allele when compared to patients with an L-allele (Table 2; P=0.013). Also after 3 and 6 years the joint damage was less marked, although this failed to reach statistical significance.

Longitudinal regression demonstrated statistically significant differences in joint damage progression between S-allele and L-allele carriers (p=0.0096). All models were corrected for RF-positivity, age at diagnosis and mean DAS28.

Table 2 / Inflammatory parameters and joint damage progression in RA patients

RA patient Characteristics	Genotype			p-value
	LL	SL	SS	
N	122	81	22	
RF positivity	86 (70%)	63 (78%)	19 (86%)	0.21
DAS28 baseline	5.2 (1.6)	5.1 (1.1)	4.9 (1.3)	0.68
Average DAS28 year 0-3	3.9 (1.2)	3.9 (1.1)	4.1 (0.9)	0.60
Average DAS28 year 3-6	3.6 (1.3)	3.5 (1.1)	3.5 (0.5)	0.69
Average DAS28 year 6-9	3.6 (1.3)	3.6 (1.1)	3.5 (0.9)	0.84
CRP baseline (mg/L)	5 (0-22)	3 (0-21)	3 (0-20)	0.90
Average CRP year 0-3	5 (1-15)	5 (0-21)	6 (1-11)	0.93
Average CRP year 3-6	4 (0-12)	3 (0-13)	5 (1-12)	0.67
Average CRP year 6-9	3 (0-8)	2 (0-8)	5 (1-12)	0.49
X-ray score > 0 baseline	48 (40%)	33 (44%)	8 (40%)	0.84
X-ray score baseline	0 (0-2)	0 (0-2)	0 (0-1)	0.75

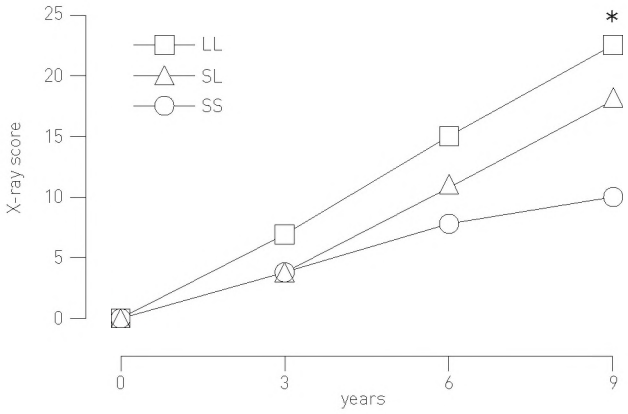
Differences in joint damage progression		Year 0-3 (n=225)			Year 0-6 (n=154)			Year 0-9 (n=117)		
		Estimate	SE	p-value	Estimate	SE	p-value	Estimate	SE	p-value
Genotype	LL	**4.4**	**2.4**	**0.26**	**6.5**	**4.2**	**0.36**	**12.1**	**5.9**	**0.047**
	SL	**2.9**	**2.4**	**0.88**	**3.3**	**4.3**	**0.86**	**8.2**	**6.1**	**0.39**
	SS*	0	--	--	0	--	--	0	--	--
Constant		0.1	2.6	0.065	0	4.6	0.066	-2.2	6.6	0.27
Allele	L	**2.2**	**1.1**	**0.078**	**3.5**	**1.8**	**0.16**	**5.9**	**2.7**	**0.013**
	S*	0	--	--	0	--	--	0	--	--
Constant		2.0	1.4	0.0001	2.2	2.4	0.0001	3.1	3.6	0.0023

Values are number (%), mean (SD) or median (P25-P75). P-values are from univariate parametric or non-parametric analyses, as appropriate.

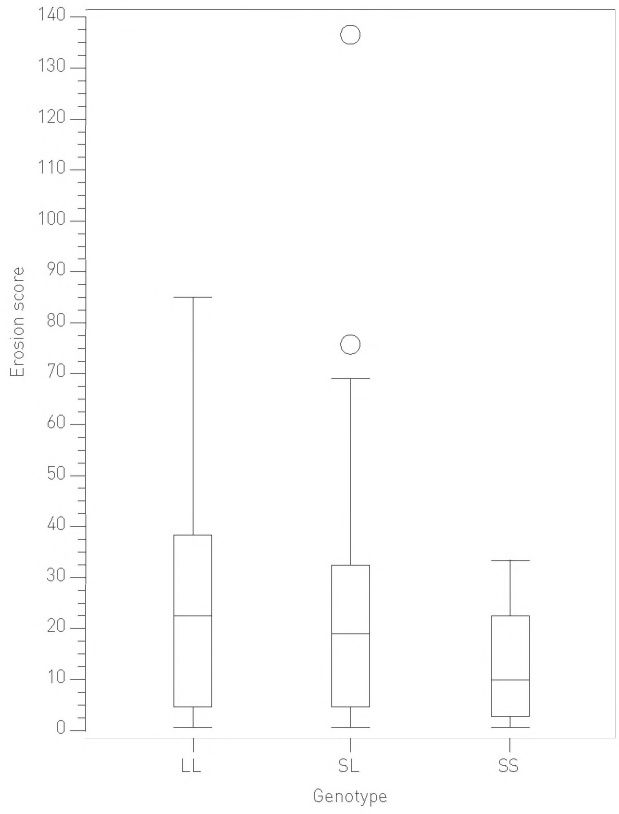
SS is the reference category for genotype analysis and S is the reference category for allele analysis. The differences between LL and SS, and SL and SS (genotype analysis) and between L and S (allele analysis) are printed bold and these are the differences to be interpreted. Both genotype and allele analyses are corrected for rheumatoid factor positivity, mean DAS28, baseline joint damage and age at baseline. P-values are for root-transformed progression scores.

DAS: disease activity score; RF: rheumatoid factor; CRP: C-reactive protein; SE: standard error.

Figure 1 / Effects of HMOX1 promoter genotype on joint damage in RA patients.



A > Severity of joint damage progression (X-ray score) in time within the RA patient cohort for the differential presence of HMOX1 genotypes.



B > A boxplot of the X-ray scores demonstrates in more detail the distribution of the different genotypes at year 9. The two open circles depict outliers and were only present within the SL-genotype. Note: Although cross-sectionally only statistically significant changes were found between SS-genotype and SL- or LL-genotype after nine years (P=0.047), patients with an SS-genotype had less joint damage during the disease in longitudinal analysis (P=0.022).

> DISCUSSION

In this study the $(GT)_n$-repeat length in the promoter region of the human HMOX1 gene was found to modulate RA disease susceptibility and joint damage, but not disease activity. The distribution of genotype and allele frequency and the odds ratios corresponded well with previously published observations in a Spanish cohort (119), strengthening the results.

Statistical analysis demonstrated that within the patient cohort, subjects with the SS-genotype had significantly less joint damage progression after nine years of follow-up as compared to patients with the LL-genotype. Since the mean disease activity over time was not different between the diverse genotypes, it appeared that patients homozygous for the S-allele tended to be better protected against joint damage than patients with an SL- or LL-genotype. These differences were significant according to the longitudinal regression on the complete monitored course of the disease up to 9 years (P=0.022). Also allele analysis showed significantly less joint damage progression after 3 and 9 years for the S-allele when compared to the L-allele, supporting our hypothesis that the SS-genotype is protective in patients with RA. Differences in treatment did not explain the results.

Our findings are in line with previous reports demonstrating that HMOX-activity protects against a wide range of injurious insults (154). Studies by our group have shown that HMOX-activity decreases inflammatory adhesion molecules and leukocyte infiltration (154). Moreover, Kobayashi *et al*. demonstrated that HMOX1 is expressed in RA synovial tissues and attenuates inflammation (202). In synovial cells it was found that HMOX1 induction inhibited the production of inflammatory cytokines, whereas inhibition of HMOX1 using RNAi enhanced this expression (202). Interestingly, RA patients to which high levels of the HMOX-effector molecule bilirubin was administered, had attenuation or complete resolution of joint pain and swelling, underscoring the importance of HMOX1 induction in protecting against the inflammatory, and oxidative injury. Other studies using animal models of arthritis demonstrated that induction of HMOX-activity by cobalt protoporphyrin (CoPP) attenuated inflammatory damage (208;209) Interestingly, also inhibition of HMOX-activity by the pharmacological inhibitor stannic protoporphyrin (SnPP) has been reported to mediate beneficial effects via decreased angiogenesis (208).

Interestingly, we found no differences between the genotype groups in terms of RF positivity, CRP and DAS28 at baseline, but SS-genotype patients had less joint damage (Figure 1; Table 2). Accumulating data in patients with RA support the proc-

ess of uncoupling between disease activity and radiologic joint damage (210;211). The unexpected lack of effect on inflammation by differences in HMOX1 genotype warrants further investigations. However, for the progression of erosive disease is synovial inflammation likely less important compared to osteoclastogenesis. In fact, Zwerina *et al*. demonstrated that HMOX1 suppresses osteoclastogenesis both in vitro and in vivo (212), supporting a role for HMOX1 in suppressing bone erosion in RA patients. It was also found that high concentrations of HMOX1 are present in synovial tissue of patients with RA, especially in the macrophages and fibroblasts (212). This could indicate that individuals with a high HMOX1 responsiveness better suppress bone resorption than individuals with lower enzyme activity.

Taken together, HMOX-1 induction offers not only protection against the onset of RA (119), but also protects against the progression of joint damage. We propose that since patients carrying the short GT-repeats exhibit less progression of joint damage, HMOX-1 induction may provide a novel therapeutic and preventive target in the treatment of RA.

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> CHAPTER 4

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THE TNF RECEPTOR SUPERFAMILY MEMBER 1B 676T>G POLYMORPHISM IN RELATION TO RESPONSE TO INFLIXIMAB AND ADALIMUMAB TREATMENT AND DISEASE SEVERITY IN RHEUMATOID ARTHRITIS

> ABSTRACT

Objective. The purpose of this study was to assess the effect of a functional polymorphism (676T>G, M196R) in the Tumor Necrosis Factor Receptor super family 1b (*TNFSF1b*) gene on disease activity, radiologic joint damage and response to infliximab and adalimumab therapy in patients with rheumatoid arthritis (RA).

Methods. Two RA patient cohorts were genotyped for the 676T>G polymorphism (rs1061622) in exon 6 of the *TNFSF1b* gene by restriction fragment length polymorphism analysis. One cohort (n=234) included patients from the Dutch Rheumatoid Arthritis Monitoring (DREAM) registry with detailed information on their response to anti-TNF therapy (infliximab and adalimumab), the other concerned patients from a long-term observational early inception cohort at our center (n=248).

Results. The 676T>G polymorphism was not associated with anti-TNF response after three or six months of therapy. Linear regression analysis showed no significant difference in the progression of radiologic joint damage during the first three and six years of disease between the three genotype groups (TT, TG and GG). No difference in mean disease activity between genotypes was observed after three and six years of disease, either.

Conclusion. Despite its demonstrated functionality, the 676T→G polymorphism in the *TNFSF1b* gene does not play a major role in either the response to anti-TNF therapy or in the disease severity or radiologic progression in RA.

> INTRODUCTION

Tumor necrosis factor alpha (TNFα) plays a central role in the pathogenesis of rheumatoid arthritis (RA). This is confirmed by the beneficial effects observed during treatment with TNF blocking agents though unfortunately approximately 30% of the patients do not respond to these agents (42). Pharmacogenetics has the potential of increasing drug efficiency by identifying genetic factors responsible for lack of treatment response or toxicities (213). In the case of TNF blocking agents, variation in genes involved in the mechanisms of action of these agents and the pathogenesis of RA have been investigated (42).

The most investigated polymorphism in relation to anti-TNF therapy is the –308G>A single nucleotide polymorphism (SNP) in the gene encoding TNFα., showing association with anti-TNF therapy response even after meta-analysis (61). However, it is not likely that the –308G>A SNP alone is responsible for anti-TNF response. Other interesting candidate genes include the TNF receptor superfamily, member 1a encoded by *TNFRSF1a* (*TNF-RI*, *p55*) and the TNF receptor superfamily, member 1b, the product of *TNFRSF1b* (*TNF-RII*, *p75*) (45;149-151;214;215). Both TNF receptors can exist as membrane bound or soluble proteins, comprising only the extracellular domains. These soluble proteins are derived from the membrane-bound forms by the proteolytic actions of a disintegrin metalloproteinase called TNFα-converting enzyme (TACE) (150;216). After cleavage, soluble TNF receptors (sTNFRs) retain their ligand-binding capacity and can act as natural inhibitors of TNFα. sTNFRs levels are elevated in the serum and synovial fluid of RA patients (217;218) but these levels seems to be insufficient to prevent chronic inflammation promoted by TNFα (218).

In the *TNFRSF1b* gene, most studies have analyzed a single nucleotide polymorphism (SNP) located in exon 6 (rs1061622, 676T>G). This polymorphism causes a non-conservative amino acid substitution (methionine (M) to arginine (R)) at codon 196 (M196R) within the fourth cysteine-rich domain of the extracellular domain and might affect the binding site for TACE (150). This suggests a disturbed processing of membrane-bound *TNF-RII* by this enzyme and might affect the levels and function of TNFR (150;151;215). sTNFR levels can be important in the response to anti-TNF treatment as the receptor acts as an endogenous TNF antagonist (45). Several studies have suggested that genes involved in response to anti-TNF may also be involved in RA severity (53;213). The 676T>G polymorphism has not been associated with radiological progression or disease activity in previous studies (149;215). The present study is aimed to test whether the *TNFRSF1b* 676T>G polymorphism is associated with anti-TNF response, disease activity and/or radiologic joint damage in a large sample of RA patients.

> MATERIAL AND METHODS

All patients in this study fulfilled the ACR criteria (9), attended the Department of Rheumatology of the Radboud University Nijmegen Medical Centre or the St. Maartens hospital in Nijmegen and had given informed consent.

For the analysis of the response to anti-TNF treatment, DNA of 234 patients treated with the monoclonal antiboedies infliximab or adalimumab was available from the Dutch Rheumatoid Arthritis Monitoring (DREAM) registry (www.dream-registry.nl) (219). This register encompass patients who start their first course of a TNF blocking agent according to the indication in the Netherlands (DAS28 > 3.2 and failure on at least two disease-modifying antirheumatic drugs (DMARDs), one of which has to be methotrexate (MTX)). The DAS28 scores at treatment start and after 3 and 6 months were used to calculate the clinical response according to the EULAR criteria (16).

The cohort used for the analysis of disease activity and radiologic joint damage included 248 RA patients from a long-term observational early inception cohort (220) from whom DNA was available. Patients in this cohort have a disease duration ← 1 year at enrollment and no prior use of DMARDs. Radiographs of hands and feet at baseline and after 3 and 6 years were scored using the Ratingen method. This is a modification of the Larsen score and evaluates 38 joints separately. The amount of surface destruction for each joint is graded from 0 to 5 (maximum score 190). Each grade represents 20% of joint surface destruction (205).

Peripheral blood samples (10 ml) collected in EDTA tubes were obtained from each patient and were stored at -80°C. Genomic DNA was extracted from leucocytes in peripheral venous blood according to standard protocols and restriction fragment length polymorphism (RFLP) analysis was used to distinguish between the 676T and 676G alleles as described previously (221).

Hardy-Weinberg equilibrium (HWE) was tested in both cohorts using the Chi-square test, no deviations from HWE were observed. To assess the effect in anti-TNF response, a two-tailed Fisher's exact test was used in comparison between the three genotype groups and the EULAR response criteria in a 3 x 3 table. For analysis at the level of alleles, the Fisher's exact test was used, using the two alleles and the EULAR response criteria in a 2 x 3 table. Linear regression modelling was used to test whether a polymorphism genotype or allelotype was associated with the course of joint damage. Bonferroni correction was used to correct for multiple testing.

> RESULTS

Clinical response to anti-TNF treatment

Table 1 shows patients' characteristics, anti-TNF therapy distribution, mean disease activity (DAS28) at baseline and DAS28 improvement after 3 (n=234) and 6 (n=207) months of treatment according to the three categories of the EULAR response criteria (good, moderate or no response). Table 2 shows the genotype and allelotype distribution for the three categories after 3 and 6 months of treatment. There was no association between genotypes and anti-TNF therapy response after 3 (p=0.22) and 6 (p=0.79) months in this sample. Although the TG group was slightly overrepresented in the moderate responder group compared to the homozygotes after 3 months of therapy, this did not reach statistical significance. Also in the analysis at the allele level no differences in anti-TNF response between the two alleles were observed after 3 (p=0.5) and 6 (p=0.9) months. Moreover, there was no association between the genotypes and the decrease in DAS28 after 3 (p=0.4) and 6 (p=0.1) months.

Table 1 / Baseline characteristics, disease activity and DAS28 improvement for the DREAM cohort according to the EULAR response criteria

	Good response	Moderate response	Non-response
N (baseline and 3 months follow-up)	54 (23%)	115 (49%)	65 (28%)
Female gender	37 (69%)	76 (66%)	45 (69%)
Age (mean ± SD)	54 (11.6)	56 (12.1)	59 (12.2)
RF positivity	43 (83%)	92 (81%)	60 (87%)
Infliximab	40 (74%)	73 (63%)	44 (68%)
Adalimumab	14 (26%)	42 (37%)	21 (32%)
DAS28 baseline (mean ± SD)	5.1 (1.0)	6.0 (1.1)	5.6 (1.3)
DAS28 decrease after 3 months of anti-TNF therapy (mean ± SD)	2.1 (0.9)	1.8 (0.8)	0.1 (0.8)
N (6 months follow-up)	51 (25%)	96 (46%)	60 (29%)
DAS28 decrease after 6 months of anti-TNF therapy (mean ± SD)	2.7 (1.0)	2.0 (1.0)	0.0 (0.8)

Baseline characteristics, mean DAS28 at baseline and DAS28 improvement after 3 and 6 months of anti-TNF therapy are indicated. Results are number (percentage) or mean (SD). Percentages are expressed in relation to the total number of patients for each response group except for the total number of patients.

Table 2 / 676T>G genotype and allele distribution according to the EULAR response criteria for the DREAM registry

	Good response	Moderate response	Non-response
Response after 3 months (n=234)			
Genotype			
TT	30 (56%)	54 (47%)	38 (58%)
TG	18 (33%)	55 (48%)	23 (36%)
GG	6 (11%)	6 (5%)	4 (6%)
Allele			
T	78 (72%)	163 (71%)	99 (76%)
G	30 (28%)	67 (29%)	31 (24%)
Response after 6 months (n=207)			
Genotype			
TT	27 (53%)	49 (51%)	32 (54%)
TG	19 (37%)	43 (45%)	23 (38%)
GG	5 (10%)	4 (4%)	5 (8%)
Allele			
T	73 (72%)	141 (73%)	87 (73%)
G	29 (28%)	51 (27%)	33 (27%)

Genotype and allele distribution after 3 and 6 months of anti-TNF therapy are indicated. Percentages are expressed in relation to the total number of patients for each response group.

Disease activity and radiologic joint damage

Table 3 shows the main characteristics of the patients from the early inception cohort according to genotype, as well as mean disease activity and joint damage at baseline, and three and six years later. There were no statistically significant differences between genotype groups with regard to baseline characteristics or disease activity at all three time points. Linear regression analysis shows a significant difference in progression of joint damage between the three genotype groups during the 3-years follow-up (p=0.02, Table 3) which lost significance after correction for multiple testing (corrected p value for 3 tests; p=0.06). Differences between the three groups were not significant after 6 years (p=0.29).

Table 3 / Baseline characteristics, disease activity and joint damage over time for the Early Inception Cohort

Characteristics	Genotype			P-value
	TT	TG	GG	
N (baseline and year 3 follow-up)	143 (58%)	87 (35%)	18 (7%)	-
Female gender	93 (65%)	56 (64%)	10 (55%)	NS
Age (mean ± SD)	55 (12)	52 (15)	48 (10)	NS
RF positivity at baseline	110 (77%)	63 (72%)	13 (72%)	NS
DAS28 baseline 0-3 (mean ± SD)	5.3 (1.3)	5.0 (1.5)	5.5 (1.7)	NS
DAS28 mean year 0-3 (mean ± SD)	4.0 (1.1)	3.9 (1.2)	4.1 (0.9)	NS
DAS28 mean year 0-6 (mean ± SD)	3.9 (1.1)	3.9 (1.1)	4.0 (1.0)	NS
Joint damage at baseline (mean + range)	0 (0-3)	1 (0-3)	4 (0-14)	NS
Joint damage progression after 3 years (mean + range)	4 (0-14)	7 (1-15)	13 (3-20)	0.02*
N (year 6 follow-up)	99 (58%)	57 (33%)	16 (9%)	-
Joint damage progression after 6 years (mean + range)	12 (1-25)	12 (5-22)	15 (2-27)	NS

NS: not significant. Results are number (percentage) or mean (SD). Percentages are expressed in relation to the total number of patients for each genotype group. Joint damage is according to the Ratingen score at baseline, year 3 and year 6 (mean and range). P-values of root-transformed scores at baseline and root-transformed progression rates between 0-3 and 0-6 years, corrected for baseline Ratingen score, age, gender, RF positivity and mean DAS28 are shown.

*Analysis at year 3 showed that the contrast between TT and GG was statistically significant (p=0.01), the TT and TG contrast (p=0.09) and the TG and GG contrast (p=0.12) were not. The p-values for the uncorrected models were slightly less significant, but did not lead to other conclusions.

> DISCUSSION

In the present study we set out to clarify the relation between the 676T>G (M196R) polymorphism in the *TNFRSF1b* gene and response to anti-TNF treatment, disease activity and progression in RA. We could not replicate the previously reported association between the *TNFRSF1b* 676T>G SNP and response to anti-TNF treatment (45). Furthermore, we were unable to detect an association with disease severity. We found nominal association between the polymorphism and radiologic joint damage progression within the first three years but not after 6 years of disease course. The association after the 3-years time point was lost upon correction for multiple testing. This lack of association is supported by other large studies with disease follow-up varying between 1 and 4 years (149;215). Constatin and co-workers reported association between the 676T>G polymorphism and functional but not structural severity in patients with early RA (215). Polymorphisms in TNFR genes are ideal candidate genes for disease severity in RA, as the receptors mediate the actions of TNFα. The 676T>G polymorphism has been pointed out as a marker for anti-TNF response in an earlier study (45). Fabris and co-workers reported a threefold higher chance of responding to anti-TNF therapy in the TT group with respect to the TG/GG group using a cohort of 66 patients receiving infliximab (n=47) or etanercept (n=19) (45). We could not confirm this finding in a large (n=234) and well characterized cohort of patients treated with monoclonal anti-TNF antibodies. This also might be due to ethnically differences between the two investigated cohorts. Although both cohorts were Caucasian, Fabris and co-workers investigated Italian Caucasians whereas our cohort consisted of Dutch Caucasians. Thereby, it is known that small sample sizes can lead to spurious findings (222). Another reason for not confirming the results of Fabris *et al.* might be the fact that we did not include patients receiving etanercept whereas Fabris and co-workers did. We focused on patients treated with anti-TNF monoclonal antibodies (infliximab and adalimumab), as these probably have similar working mechanisms (223). Differences in pharmacokinetics between monoclonal antibodies and the soluble receptor (etanercept) may exist. Therefore, it is possible that the 676T>G polymorphism is associated with response to etanercept but not with response to infliximab and adalimumab. It would be interesting to investigate this hypothesis in the near future. Thereby, it is unlikely that one independent polymorphism is responsible for anti-TNF response. Chatzikyriakidou and co-workers suggested that a combined study of polymorphisms 676T>G (*TNFRSF1b* and -857C>T (*TNFA*) could predict anti-TNF response in patients with RA (43). In conclusion, despite its potential functionality, the *TNFRSF1b* 676T>G polymorphism probably does not has a major effect on anti-TNF response and RA severity or radiologic progression.

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> STATEMENT

> CHAPTER 5

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TNFA -308G>A PROMOTER POLYMORPHISM INFLUENCES DISEASE SEVERITY IN PATIENTS WITH RHEUMATOID ARTHRITIS

> ABSTRACT

Aim of the study. To investigate whether the -308G→A promoter polymorphism in the tumor necrosis factor alpha (*TNFA*) gene is associated with disease severity in patients with rheumatoid arthritis (RA).

Methods. A long-term, observational, early RA inception cohort (n=208) with detailed information about disease severity and radiologic damage after 3, 6 and 9 years of disease was genotyped for the *TNFA* -308G>A promoter polymorphism (rs1800629) using a TaqMan SNP genotyping assay. To learn more about the mechanism behind the effect of the polymorphism, RNA isolated from peripheral blood of 66 RA patients was used for *TNFA* gene expression analysis by quantitative PCR.

Results. To our knowledge, this report describes the effects of the *TNFA* -308G>A promoter polymorphism in one of largest samples of patients with RA.

A longitudinal regression analysis showed a significant difference between GG and GA+AA groups in joint damage (p=0.002). No significant differences in *TNFA* gene expression were observed for the different genotypes.

Conclusion. Our data confirm that the *TNFA* -308G>A promoter polymorphism is associated with radiologic joint damage in patients with RA. This does not seem to be mediated by differences in TNF expression between genotypes.

> INTRODUCTION

Tumor-necrosis factor (TNF) plays a key role in the pathogenesis of rheumatoid arthritis (RA) and other auto-immune diseases (20). Several genetic variants in the *TNFA* gene, which codes for TNFα, have been investigated in relation to disease susceptibility and severity (74;75;77;80;83;84) but none of them appears to be highly specific and/or sensitive. The most thoroughly investigated *TNFA* polymorphism is the –308G>A (rs1800629) polymorphism located in the promoter of the gene (74-85) studies focusing on this polymorphisms have yielded conflicting results (74;75;77-80;82-85). Concerning the association of this polyporphism with radiologic joint damage two studies (82;83) performed in, 34 and 130 RA patients, respectively, showed an association of the G allele with a more pronounced radiologic joint damage, though they did not provide information concerning radiologic joint damage progression. Another study reported a worse radiologic joint damage in patients carrying the A allele in a cohort of 189 RA patients (77). Finally in a larger sample of 283 patients Brinkman *et al*. could not confirm any association between either the G or A allele and radiologic joint damage (75).

As the -308G>A variant is located within the *TNFA* promoter, several studies have also investigated the polymorphism in relation to TNFA transcription which could influence TNFα production and in turn disease activity and severity (86-88). Five studies, (224-228), suggested that the A allele is associated with an increased *TNFA* transcription whereas three other studies, (229-231), did not. Another important study by Knight *et al*., using the haploChIP method for high-throughput screening of common DNA polymorphisms that (might) effect gene regulation *in vivo*, also reported that the -308G>A polymorphism was not associated with *TNFA* expression in human B-cells (232). Similar conflicting results have been reported for the relation of the -308G>A polymorphism and *in vitro* TNFα protein production in cultured whole blood cells or peripheral blood mononuclear cells (PBMCs) (86;161;233-239).

In order to clarify these contradictory results, we investigated the possible associations of the –308G>A promoter polymorphism with *TNFA* expression and with disease activity, radiologic joint damage after 3, 6 and 9 years follow-up in a large (n=208), well characterized early RA patient cohort (240).

> MATERIAL AND METHODS

Patients

All patients in this study fulfilled the American College of Rheumatology (ACR) criteria for RA (9), had given informed consent and attended the Department of Rheumatology of the Radboud University Nijmegen Medical Centre or the St. Maartenshospital in Nijmegen, The Netherlands. The study was approved by the local ethics committee. The impact of the *TNFA* -308G>A polymorphisms on disease severity was analyzed using DNA isolated from blood of 208 patients from a long-term observational early RA inception cohort (220). Patients in this cohort have a disease duration <1 year at enrollment and no prior use of DMARDs. Disease severity was assessed using the disease activity score (DAS28) (15) at each patient visit. For this study we used the 3, 6 and 9 years averaged DAS. Radiologic joint damage at baseline and after 3, 6 and 9 years of disease duration was measured using radiographs of hands and feet, read in chronological order according to the Ratingen score. The latter is a validated modification of the Larsen score and evaluates joint surface destruction, graded from 0 to 5, in 38 hand and feet joints, separately (range 0 – 190) (205).

TNFA expression analysis was assessed in RNA from blood of a subset of 66 patients with active RA as defined by a DAS28 of >3.2. All patients visited the same hospital and blood for RNA isolation was always collected between 9 and 10 o'clock in the morning to avoid potential circadian fluctuations.

Molecular analysis

All DNA and RNA analyses were performed in a CCKL (Coördinatie Commissie ter bevordering van de Kwaliteitsbeheersing van het Laboratoriumonderzoek) -accredited laboratory at the department of Human Genetics at the Radboud University Nijmegen Medical Centre in Nijmegen, The Netherlands.

Genotyping of the -308G>A *TNFA* polymorphism

Genomic DNA was extracted from peripheral venous blood (10 ml/sample) according to standard protocols (206). Both patients cohorts, thus a total of 274 patients were genotyped for the -308G>A (rs1800629) promoter polymorphism using a TaqMan SNP genotyping assay (ID. C7514879_10) on the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Results were analyzed using the Allelic Discrimination software version 1.4 (Applied Biosystems).

RNA isolation, synthesis of cDNA and quantitative PCR

RNA was isolated from whole blood within 0.5 hours after collection, using the RNeasy midi kit (Qiagen Benelux B.V. Venlo, The Netherlands). The quality and quantity of the purified RNA was checked on a NanoDrop spectrophotometer (Nanodrop technologies, Montchanin, DE, USA). Degradation of RNA was controlled for by agarose gel electrophoresis. Per sample 1 µg of total RNA was reverse-transcribed using 200 ng random hexanucleotides (Invitrogen, Breda, The Netherlands) and 200 units of M-MLV Reverse Transcriptase (Invitrogen). For quantitative expression analysis a predesigned and validated gene-specific probe-based TaqMan gene expression assay (ID: Hs00174128_m1) was used according to the protocol of the manufacturer (Applied Biosystems). Samples were run on the 7500 Fast Real-Time PCR System (Applied Biosystems) using standard protocol. The general housekeeping gene *B2M* (Beta-2-microglobulin, TaqMan gene expression assay ID: Hs99999907_m1) was used as endogenous control. Threshold cycle numbers (referred to as Ct) were obtained using the 7500 System SDS software version 1.4 (Applied Biosystems). All samples were measured twice, duplicate samples with a standard deviation (SD) larger than 0.5 were excluded from the analysis. The relative quantity (RQ) of the gene-specific mRNA was calculated from the average value of the ΔCt ([*TNFA* Ct) – (endogenous control gene Ct)]). Differences in expression between two groups of samples were calculated by the $2^{\Delta\Delta Ct}$ method (241).

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was tested in both patient samples using the Chi-square test. Baseline differences between genotype groups were analyzed using a chi-square test, a Student T-test or a Wilcoxon test, as appropriate. Longitudinal analysis of radiologic joint damage was done using generalized linear regression models with random coefficients (mixed models). The joint damage score was the dependent variable, genotype the independent variable, under addition of time, time squared, and confounders. As the distribution of joint damage scores is skewed, the analysis was also performed using a square-root transformation of the joint damage scores. Regression assumptions were checked by evaluating the distribution of residuals and by a plot of observed and predicted joint damage scores. Mean differences in *TNFA* expression (ΔCt; (*TNFA* Ct) – (endogenous control gene Ct)) were analysed using the independent Student's T-test. A p-value of <0.05 was considered statistically significant in each situation. Analyses were performed using SPSS for Windows, version 14.0 (SPSS, Chicago, IL, USA) and SAS version 8.2 (SAS Institute Inc., Cary, NC, USA).

> RESULTS

Relation of *TNFA* -308G>A with disease activity and radiologic joint damage

The frequency of the A allele of the TNFA -308G>A polymorphism in the inception cohort participants was 18.3%, genotype frequencies were 66.3% for the GG group, 30.8% for the GA group and 2.9% for the AA group. Due to the small AA genotype group, the GA and AA genotype groups were merged and analyzed as one group. Demographic and disease characteristics were similar in the GG and GA+AA genotype groups at baseline (Table 1). No significant differences in disease activity were observed between genotype groups.

Table 1 / Demographics inception cohort (N=208).

	n	GG (n=138)	GA+AA (n=64+6)	P-value
Age		52 (13)	50 (13)	0.21
Female gender		89 (64%)	50 (71%)	0.32
RF positivity		105 (76%)	55 (79%)	0.69
DAS28, mean (SD)				
Baseline	208	5.2 (1.4)	5.3 (1.3)	0.38
3 year average	208	4.0 (1.1)	3.9 (1.0)	0.85
6 year average	159	4.0 (1.0)	3.7 (1.0)	0.10
9 year average	115	4.0 (1.0)	3.7 (1.0)	0.12
Patients with erosions at baseline, nr (%)	208	76 (55%)	44 (63%)	0.28
Ratingen score, median (range)*				
Baseline	208	0 (0-2)	0 (0-2)	0.17
At 3 years	208	7 (0-15)	4 (0-10)	0.08
At 6 years	160	12 (2-26)	10 (1-21)	0.18
At 9 years	116	22 (10-38)	14 (3-24)	0.07

Results are mean (SD), number (percentage), or median (range). Percentages are expressed in relation to the total number of patients for each genotype group. *Between-group differences in erosion score were tested using Wilcoxons' test.

In a longitudinal regression analysis (mixed model) with radiologic joint damage score as the dependent variable, corrected for gender and average DAS28, a significant difference between GG and GA+AA groups in progression of joint damage was observed (p=0.002), with more pronounced joint damage in patients with the GG genotype (Table 2 and Figure 1). However, the progression of joint damage *over* nine year follow-up was similar in both genotypes (p=0.61). After confounder correction for gender and DAS28 in the regression model, a correction for rheumatoid factor and age was unnecessary.

Table 2 / Longitudinal regression of joint damage and *TNFA* -308G>A genotype (N=208).

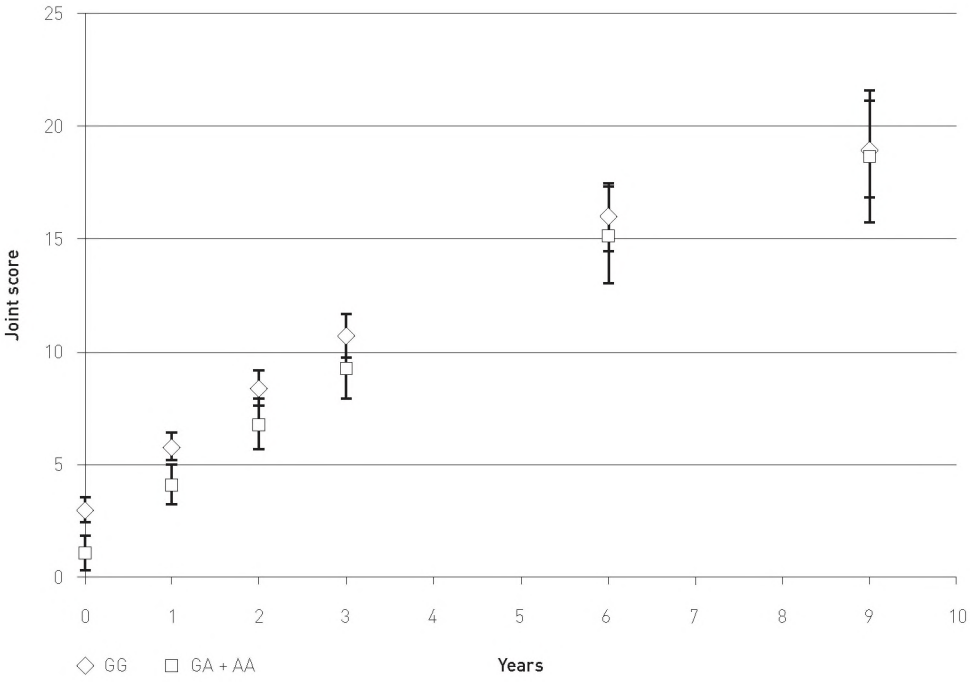
Effect	Estimate	SE (estimate)	P-value
Intercept	5.8	1.18	<0.0001
Genotype GA+AA	-5.0	1.62	**0.0020**
Year	1.6	0.38	<0.0001
Year^2	-0.13	0.020	<0.0001
Genotype GA+AA* Year	0.17	0.34	**0.61**
Male gender	-1.18	0.79	0.14
Averaged DAS28	-0.59	0.20	0.0039
Averaged DAS28 * year	0.34	0.063	<0.0001
Genotype GA+AA * Averaged DAS28	0.77	0.30	0.0094

Shown are the variables in the longitudinal regression analysis over 9 years, with joint erosion score as the dependent variable and TNFA -308G>A and TNFA -308G>A * Year as dependent variables of main interest. Gender and time-averaged DAS28 of the previous period between two radiographs were confounders, rheumatoid factor and age were not. Year^2 allows for a non-linear course of joint progression score over time.

Using a square-root transformation of the joint erosion score as the dependent variable to minimize potential effects of skewing showed similar results, with a significantly more joint damage in the GG group (p=0.0031), but no significant difference (p=0.34) in radiologic progression between the groups. Also the other models to study the influence of the skewed score distribution of the Ratingen scale confirmed the assumptions made for the regression model used: The residuals of the regression analysis with the untransformed skewed joint damage score showed a Gaussian distribution (not shown). In figure 2, the joint erosion scores predicted by the regression

model plotted against the actually observed joint erosion scores showed a high degree of linear correlation. Since the differences in radiologic damage could not be explained by differences in disease activity, we also assessed potential differences in the use of TNF and IL-1 blocking agents genotype groups. These agents can uncouple disease activity and joint damage (242). The number of patients with past or present treatment with TNF and/or IL-1 blocking agents was generally low and similar in both genotype groups, 14% and 17% in the GG and GA+AA groups, respectively.

Figure 1 / Development of corrected joint scores over time showing differences between the GG and GA+AA genotypes, separately

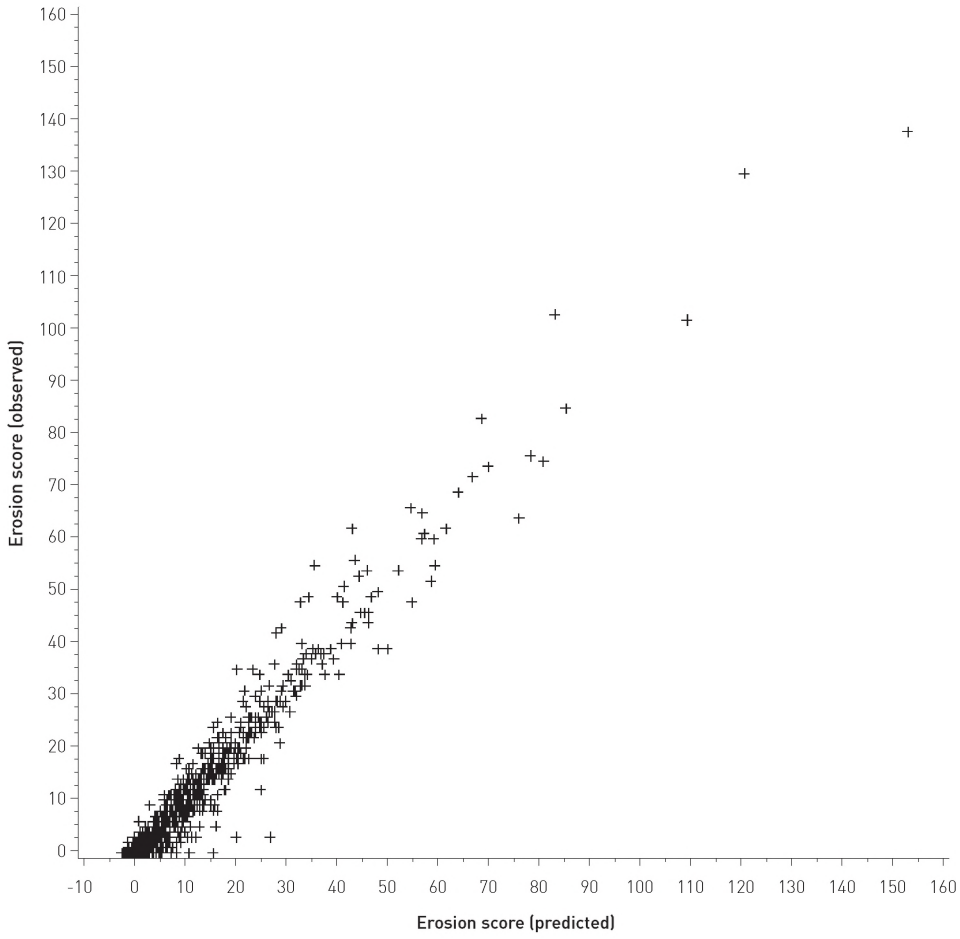


Data points are least-square means and standard errors derived from the longitudinal regression model.

TNFA expression, role of the -308G>A promoter polymorphism

To assess whether the difference in radiologic damage between G homozygotes and A allele carriers, could be explained at the level of expression of this pro-inflammatory cytokine, we measured the RNA expression levels of *TNFA* dependent on -308G>A genotype in a sample of 66 patients with active RA. Again, the GA and AA genotypes were merged and analyzed as one group. No differences in *TNFA* expression was observed between the GG group when compared to the GA+AA group (p=0.420).

Figure 2 / Predicted erosion scores versus observed erosion scores (N=280)



Erosion scores that are predicted by the model in Table 2 are plotted against the observed erosion scores in the patients of the cohort (N=280).

> DISCUSSION

Based on earlier literature, we tested the hypothesis that disease severity, defined by average DAS28 and radiologic joint damage, may be influenced by the *TNFA* -308G>A genotype. We were able to show that the GG genotype was associated with a worse radiologic outcome than the GA + AA group though the radiologic progression *over time* was not different between the two genotypes. This association between the GG genotype and worse radiologic joint damage could not be explained by differences in either disease activity, levels of TNF expression or biological therapies. Though many studies show that persistent disease activity in patients with RA will result in worse radiologic outcome, other data support an uncoupling between disease activity and radiologic joint damage (210;211;243). The course of the disease activity and radiologic progression observed during the long-term follow-up in our study reflects daily practice and is similar to other long-term observational studies in other cohorts (13;49;244). Nonetheless this is far from the present ideas of tight and adequate disease control (11). Our results cannot predict whether the latter, can overrule the impact of this and other genetic factors.

Several other studies investigated the –308G>A promoter polymorphism and its (possible) relationship with joint damage progression (74;75;77-80;82-85;226), but results are inconsistent. Reasons for the contradictory results could be differences in study design, mean duration of RA, sample size and ethnicity. With regard to sample size, given the generally small effect sizes observed in multifactorial disorders, this factor is expected to have a large impact on study results. A meta-analysis could help to clarify these contradictive results whenever similar disease activity scores and joint damage scoring methods would be used throughout the studies.

Our study shows that the effect of the -308G>A variant on radiologic joint damage is not explained by differences in allele-dependent expression. Many other studies have investigated allele specific expression of the *TNFA* gene and TNFα production but also here results are inconclusive (229-231;235-239). Again differences in sample size, power, study design and ethnicity might be of influence in these discrepancies. Linkage disequilibrium (LD) may be an overall factor which can (partly) explain differences in results, if we assume that the -308G>A variant is not functional, itself. In fact, LD is strong in this area of the genome and it is difficult to study the role of an SNP located there in isolation. The *TNFA* -308A allele is known to be in strong LD with the shared epitope (SE) (180). Unfortunately, the patients are not genotyped for the SE. Therefore we could not stratify the patients according to their SE genotype. However, the results

of the study of Khanna and co-workers indicate an effect of the -308G>A polymorphism on joint damage progression independent of the SE (77).

To conclude, we showed an association of the *TNFA* -308GG genotype with increased joint damage but no relation with radiologic progression or TNF expression. The effect of this polymorphismis probably modest and the suitability of the polymorphism as a marker for disease severity is questionable. We conclude that testing for this polymorphism alone is not cost effective.

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> CHAPTER 6

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TNF ALPHA -308 G>A POLYMORPHISM IS NOT ASSOCIATED WITH RESPONSE TO TNF-ALPHA-BLOCKERS IN PATIENTS WITH RHEUMATOID ARTHRITIS: SYSTEMATIC REVIEW AND META-ANALYSIS

> ABSTRACT

Objective. There is a need for biomarkers that are able to predict anti-tumour necrosis factor (anti-TNF) treatment outcome in patients with rheumatoid arthritis (RA). Several studies have suggested that the rare A allele of the tumour necrosis factor alpha (*TNFA*) -308G>A polymorphism could be associated with a poorer response to anti-TNF therapy. Nevertheless, these results remain controversial. We performed a meta-analysis to determine whether the *TNFA* -308G>A polymorphism is associated with response to anti-TNF treatment in patients with RA .

Methods. A bibliographic search identified studies in which the *TNFA* –308G>A gene polymorphism was investigated in RA patients treated with anti-TNF agents. Complementary data were requested when the DAS28 was not used as the primary outcome measure. Odds ratios (OR) for response based on DAS28 and standardized mean difference (SMD) for mean improvement of DAS28 were calculated to assess the potential association between *TNFA* -308 genotypes and response to anti-TNF agents.

Results. The bibliographic search yielded twelve studies that met the inclusion criteria. Those were supplemented with the data from a large Dutch cohort (n=426). The OR based on twelve studies including 1721 patients was 1.24 (95% CI 0.98-1.56) and the SMD based on eleven studies including 2576 patients was -0.15 (95% CI -0.38-+0.07). Sub-group analysis based on the two classes of anti-TNF agents did not demonstrate any association between TNFA -308 genotypes and anti-TNF treatment outcome

Conclusion. According to this meta-analysis, the *TNFA* -308 polymorphism is not a predictor of the clinical response to anti-TNF treatment in RA.

> INTRODUCTION

Although the pathogenesis of rheumatoid arthritis (RA) is not yet fully understood, it is clear that tumour necrosis factor alpha (TNFα) plays a key role in the inflammatory process of this common autoimmune-mediated disorder (20;21). In the last decades, TNF has emerged as one of the most important targets for therapeutic interventions in RA and other autoimmune diseases. Three anti-TNF agents are currently used in daily clinical practice, etanercept a human, soluble, fusion protein consisting of two p75 TNF receptors linked to an IgG1 Fc part and two monoclonal antibodies infliximab a chimaeric IgG1 antibody and the fully humanized IgG1 antibody adalimumab. All three anti-TNF drugs bind to TNF with high affinity leading to inactivation of TNF by preventing binding of TNF to its receptor (12;42). Though, TNF neutralization is highly effective and widely used in RA, there is a large heterogeneity in response and approximately 30% of patients treated with TNF-blocking agents fail to show clinical improvement (28). The latter, together with the potential toxicities and the high costs of TNF-blocking agents has driven the search for genetic markers to predict treatment outcome (61;66). To date, several variants in genes potentially involved in the action of anti-TNF therapy and/or in the pathogenesis of RA have been analysed (42) but no highly specific and/or sensitive genetic predictors of anti-TNF therapy outcome have been identified, yet.

The most thoroughly investigated polymorphism is the –308G>A (rs1800629) polymorphism in the promoter of the tumour necrosis factor alpha (*TNFA*) gene which codes for TNFα (53-55;58-61;245). The relation between the -308G>A promoter polymorphism and anti-TNF response has been investigated by several groups with inconsistent results (44;50;52-57;59-63;234;245-247). Two meta-analyses, performed by Lee *et al*. in 2006 (61) and O'Rielly *et al*. in 2009 (248), including data of 311 and 692 patients respectively, suggested that RA patients carrying the rare A allele had a poor response to anti-TNF therapy than those carrying the common G allele. However, these meta-analysis pooled data with varying response criteria and curiously, did not include studies that did not support a significant effect of this polymorphism on anti-TNF response (43;44). Furthermore, the meta-analysis from O'Reilly *et al*. did not include three recently published studies accounting for 1551 additional RA patients treated with anti-TNF agents (52;62;63). Therefore we considered that, a new meta-analysis of all published data could help to draw more definitive conclusions on the role of the -308 G>A *TNFA* polymorphism in predicting anti-TNF treatment outcome in patients with RA. The present meta-analysis included a total of 2576 patients, which is 4 times more than the previous meta-analysis published in 2009 (248).

> MATERIALS AND METHODS

The meta-analysis was performed according to the recommendations of the Cochrane Collaboration (249).

Search strategy

A bibliographic search was performed on the Medline (January 1966 to May 2009), EMBase and Cochrane database by two investigators (SP and ET) using the medical subject heading key words: polymorphism genetic OR single nucleotide polymorphism AND rheumatoid arthritis AND (TNFR-Fc fusion protein OR adalimumab OR infliximab). Bibliographic references contained in the articles and abstract publications from the American College of Rheumatology (ACR) Annual Scientific Meeting (2006–2008) and the European Leaque Against Rheumatism (EULAR) Annual European Congress of Rheumatology (2006-2008) were also included. Results from all searches were combined and duplicate references excluded. Inclusion and exclusion criteria were checked for every article independently by two reviewers (SP and ET). In case of disagreement, articles were re-examined and discussed until consensus was achieved.

Study selection

In order to be included in this meta-analysis, studies had to 1) be or consist of clinical trials using anti-TNF treatment for at least 3 months, 2) include RA patients according to the ACR criteria and 3) investigate the role of the *TNFA* -308G>A gene polymorphism. Studies on non-Caucasian patients were excluded from this analysis in order to evaluate a more genetically homogenous population.

Data collection

The primary outcome measure of the meta-analysis was the number of patients achieving clinical response to anti-TNF treatment defined as was the number of patients that achieved an improvement of the DAS28 (Disease Activity Score 28) (16) superior or equal to 1.2 after at least 12 weeks of treatment. This is a dichotomous variable i.e.

response/non-response. The secondary outcome measure was a continuous variable consisting of the mean improvement of the DAS28 in the same period of time. We chose to focus on DAS28 because it is the main outcome parameter used in daily clinical practice. Moreover, it is the outcome variable most frequently reported in the selected studies. Upon identification of papers fulfilling the inclusion and exclusion criteria, complementary information was requested from the corresponding author if needed. This was the case if the number of patients that experienced an improvement of the DAS28 equal or superior to 1.2 was either not used as outcome measure or not reported in the paper. Data collection and data extraction were performed by one reviewer (SP) using a predefined form.

Quality assessment

The influence of individual study quality on the results of meta-analysis has been well described (250). Although the use of assessment scales are recommended in systemic reviews and meta-analysis to undergo some type of quality review, it has been demonstrated that use of such scoring methods may not accurately assess the quality measures of interest (251). Furthermore, those scales assess the quality of the data reporting, but not that of the study, especially in the case of brief reports or letters. According to the recommendations of the Cochrane collaboration (252), we used study design as quality variable (prospective clinical trials versus cohort designs) and performed subgroup analysis to evaluate its impact on effect measures.

Statistical analysis

The odds ratio (OR) regarding the dichotomous outcome measure of efficacy (number of patients achieving an improvement of the DAS↗1.2) and the standardized means difference (SMD) regarding the continuous outcome measure of efficacy (mean improvement of the DAS28) were the effect measures of interest to assess the association between the *TNFA*-308G>A gene promoter polymorphism and the response to anti-TNF treatment. For the meta-analysis, summary ORs and summary SMDs were computed using either fixed effects models (for data that did not demonstrate significant heterogeneity, $I^2<50\%$) or random effects models (for data demonstrating significant heterogeneity, $I^2\geq50\%$) (253). To assess potential heterogeneity across studies, we used the I^2 statistic based on Cochran's heterogeneity statistic (Q) (254). Meta-analyses were computed using RevMan analyses software (Review Manager (RevMan), In. Version 5.0 ed. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration; 2008).

Sensitivity analysis: Assessment of the effect of TNF agents, of the potential excessive influence of varying studies and of potential publication bias

This sensitivity analysis was performed on the data from the current meta-analysis including twelve studies. The OR and the SMD of the different classes of anti-TNF agents (TNF receptor fusion protein and antibodies against TNF) were calculated apart in order to determine whether the results were affected by the agent used. Furthermore, to assess whether a specific study could have exerted excessive influence on the final results, we repeated analyses after excluding each study and after excluding studies of varying designs.

Potential publication bias (ie, the association of publication probability with the statistical significance of study results) was investigated using visual assessment of the funnel plot calculated by RevMan analyses software. Funnel plots are plots of effect estimates on the horizontal axis against sample size on the vertical axis and publication bias may lead to asymmetrical funnel plots.

Sensitivity analysis: Assessment of the effect of covariates related to the characteristics of the disease and co-medication

As part of the sensitivity analysis, we performed a separate statistical analysis on individual patients data from two large studies. This analysis aimed to investigate whether potentially important covariates could influence the association between the -308G>A promoter polymorphism and the response to anti-TNF treatment. These covariates consisted of gender, age, disease duration, classes of anti-TNF medication, co-medication with disease modifying antirheumatic drugs and health assessment questionnaire (HAQ) at baseline. This analysis was performed in a subset of 814 well-characterized patients (426 Dutch patients from the DREAM registry and 388 French patients from the large international ReAct clinical trial) (21, 34). In this subset of patients, the *TNFA* –308G>A polymorphism was genotyped by allelic discriminating TaqMan PCR by use of the PreDeveloped TaqMan assay kit C_7514879 (PE Applied Biosystems), according to the manufacturer's protocol. The primary clinical outcome for this analysis was the absolute change in DAS28 at 12 weeks (+/- 2 weeks in cases of infliximab treatment). Bivariate and linear regression analyses were performed to assess association between DAS28 and *TNFA* -308 genotype using R software (R-Developement-Core-Team. R: A Language and Environment for Statistical Computing. In. Vienna, Austria: R Foundation for Statistical Computing; 2008).

> RESULTS

Selected studies

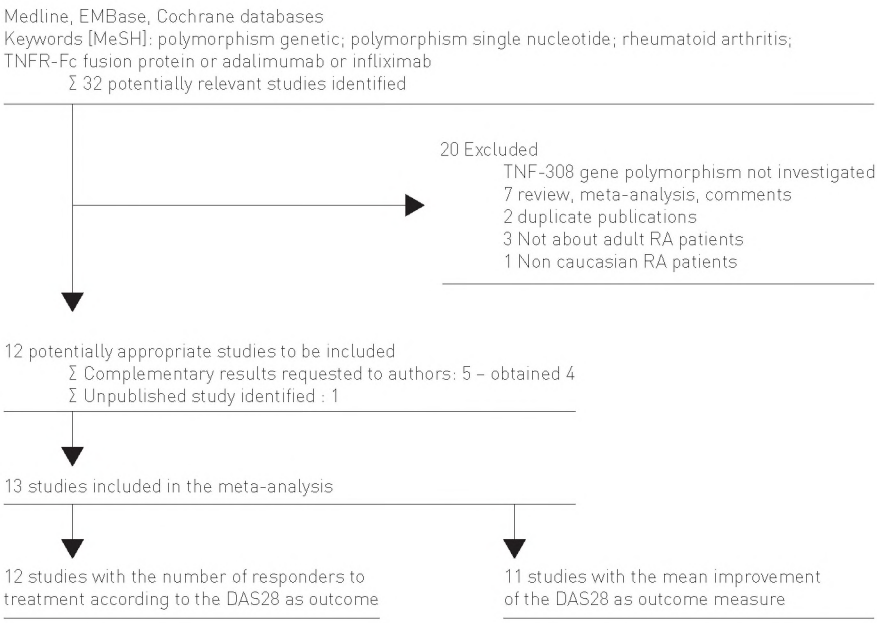
The literature search yielded 32 citations (Figure 1). Twelve published studies met our inclusion criteria and could be included in the meta-analysis after requesting and receiving complementary results from five groups who did not use or report the DAS28 in detail in the original publications. The 20 remaining, non-selected papers encompassed studies in which the *TNFA* -308 gene polymorphism was not investigated, duplicate publications, review and comment articles, and one study on non-Caucasian patients from Korea. A small study with only nine patients with RA was also excluded (56). In addition to the 12 selected studies, data from a total of 426 Dutch patients were included in this meta-analysis. Two studies (43;52) were included in only one of the two analysis (regarding the dichotomous or the continuous variable used as outcome measure) due to the lack of accessible data. Detailed information of the included studies is presented in Table 1.

Table 1 / Characteristics of studies incuded in the meta-analysis of the association of TNFA-308 gene polymorphism with

Author, year (ref.)	Patient selection requirements	study design
Chatzikyriadou *et al*, 2007 (43)	Refractory disease to at least two DMARDs	Retrospective cohort
Criswell *et al*, 2004 (44)	Active disease, early RA	Prospective, subgroup of a RCT
Cuchacovich *et al*, 2006 (59)	Active disease despite DMARDs	Prospective clinical trial
Fonseca *et al*, 2005 (74)	DAS 28≥ 3.2 despite MTX	Prospective cohort
Guis *et al*, 2007 (245)	DAS 28≥ 3.2, refractory to previous treatment by infliximab	Prospective cohort
Marotte *et al*, 2006 (58)	Refractory disease to MTX	Prospective cohort
Maxwell *et al*, 2008 (52)	na	National Cohort
Miceli-Richard *et al*, 2008 (62)	DAS 28≥ 3.2 despite DMARDs	Prospective clinical trial
Mugnier *et al*, 2003 (54)	DAS 28≥ 3.2 despite MTX	Prospective cohort
Padyukov *et al*, 2003(53)	Refractory disease to at least one DMARDs	Retroversive cohort
Pinto *et al*, 2008 (63)	DAS 28≥ 3.2 despite DMARDs	Prospective cohort
Seitz *et al*, 2007 (60)	na	Prospective cohort
Toonen *et al*, reported in the present article	Refractory disease to at least one DMARDs	Prospective cohort
Total Mean [range]		

DMARD: disease-modifying anti-rheumatic drugs, MTX: methotrexate, -: not reported.

Figure 1 / Study selection



response to anti-TNF treatment in RA

No. of patients	Countries	Age, mean, years	Disease duration, mean, years	% female	% RF+	Anti-TNF medication	Date of endpoint, weeks
58	Greece	58	15.4	79	67	infliximab	22
151	USA	50	1.1	74	78	etanercept	52
81	Chile	49	11.5	-	84	adalimumab	24
22	Portugal	-	8.8	77	-	infliximab	25
86	France	56	11.5	69	67	etanercept	26
198	France	50	10.4	79	79	infliximab	30
1050	UK	57	13.9	77		infliximab(=455) adalimumab(n=142) etanercept(n=453)	26
388	France	54	2.7	78	71	adalimumab	12
59	France	56	13.2	78	-	infliximab	22
123	Sweden	52	14.0	81	94	etanercept	12
113	Spain	52	11.6	78	74	infliximab	30
54	Switzerland	57	14.4	68	100	infliximab(n=33) adalimumab(n=9) etanercept(n=12)	24
426	The Netherlands	67	9.4	67	81	infliximab(n=231) adalimumab(n=159) etanercept(n=36)	12
2820							
202		56.7 [50-67]	10.5 [1.1-15.4]	75.4 [67-81]	81.4[67-100]		

Meta-analysis

As the *TNFA*-308 AA genotype is rare, we compared the *TNFA* -308 GG genotype group to the combined AG and AA genotype groups. Considering all anti-TNF agents together, we did not observe any significant association between the *TNFA*-308 G>A poly-

Figure 2a / Meta-analysis of the *TNFA* -308 genotype with response to anti-TNF treatment in rheumatoid arthritis for the outcome:

Study or Subgroup	TNF-308 genotype GG Events	Total	TNF-308 genotype GA/AA Events	Total	Weight
Mugnier	33	41	5	12	1.2%
Padyukov	65	77	34	46	5.3%
Criswell	63	89	43	62	11.8%
Fonseca	11	15	3	7	0.9%
Marotte	93	147	33	51	14.3%
Cuchacovich	45	51	13	19	1.8%
Chatzikariakidou	32	43	11	15	3.3%
Guis	56	68	10	18	2.2%
Seitz	33	37	14	17	1.7%
Toonen	225	297	98	129	26.4%
Pinto	50	86	16	27	8.1%
Micelli	194	261	79	106	23.0%
Subtotal (95% CI)		**1212**		**509**	**100.0%**
Total Events	900		359		
Heterogeneity: Chi² = 16.06, df = 11 (P = 0.14); I² = 31%					
Test of overall effect: Z = 1.80 (P = 0.07)					

M-H= Mantel-Haenszel statistical method; CI = Confidence Interval

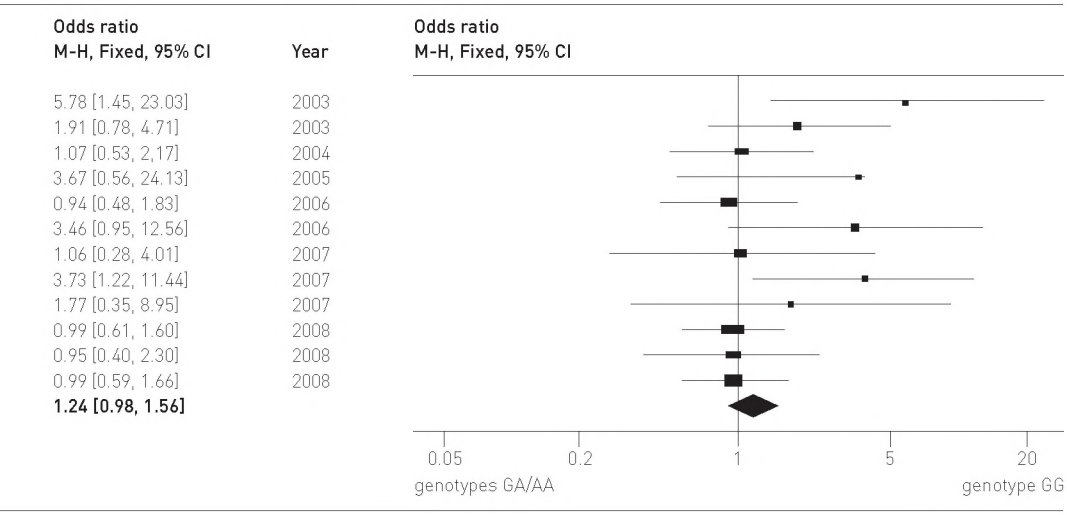
Figure 2b / Meta-analysis of the *TNFA* -308 genotype with response to anti-TNF treatment in rheumatoid arthritis for the outcome:

Study or Subgroup	Weight
Mugnier	5.3%
Criswell	10.6%
Fonseca	3.0%
Cuchacovich	6.9%
Marotte	10.7%
Guis	7.0%
Micelli	5.9%
Seitz	13.2%
Toonen	13.6%
Pinto	8.6%
Maxwell	15.2%
Total (95% CI)	**100.0%**
Heterogeneity: Tau² = 0.05, Chi² = 31.83, df = 10 (P = 0.0004); I² = 69%	
Test of overall effect: Z = 1.87 (P = 0.06)	

M-H= Mantel-Haenszel statistical method; CI = Confidence Interval

morphism and number of patients responders to TNF inhibitors (decrease of DAS28≥1.2): OR=1.24 (95% confidence interval: 0.98 – 1.56) (Figure 2a). The test for heterogeneity was I^2=31% with a p-value=0.07. Similar results were observed when comparing the mean improvement of the DAS28 between genotypes with a non significant effect size of -0.18 point in favour of TNFA-308 GG genotype (95% CI: -0.36 - 0.01) (Figure 2b).

number of responders according to the DAS28 (≥ 1.2).



mean improvement of the DAS28

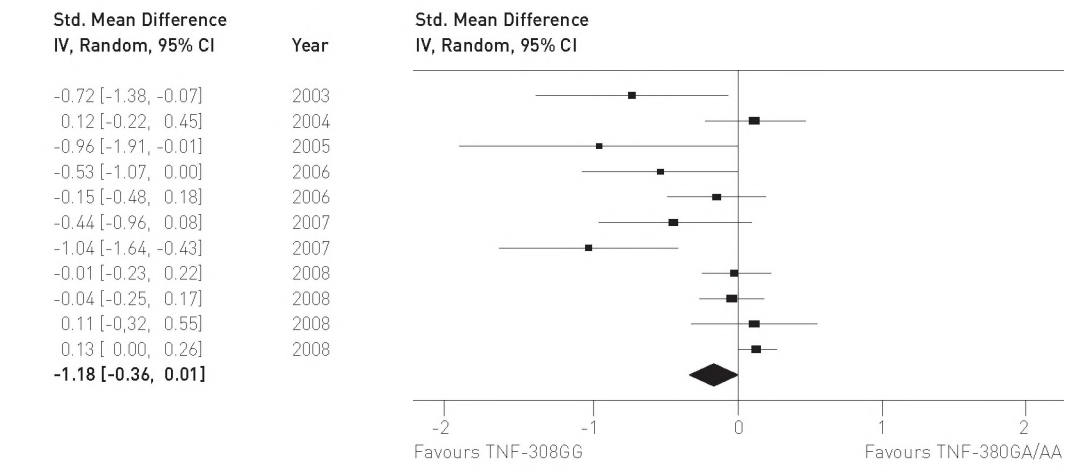


Figure 3a / Meta-analysis of the *TNFA* -308 genotype with response to TNF antibodies medications in rheumatoid arthritis

Study or Subgroup	TNF-308 genotype GG Events	Total	TNF-308 genotype GA/AA Events	Total	Weight
1.2.1 Infliximab					
Mugnier	33	41	5	12	1.4%
Fonseca	11	26	3	10	2.4%
Marotte	93	147	33	51	17.2%
Chatzikariakidou	32	43	11	15	4.0%
Toonen	84	166	35	65	23.8%
Pinto	50	86	16	27	9.8%
Subtotal (95% CI)		**509**		**180**	**58.7%**
Total Events	303		103		
Heterogeneity: Chi² = 6,72, df = 5 (P = 0.24); I² = 26%					
Test of overall effect: Z = 0.41 (P = 0.68)					
1.2.2 Adalimumab					
Cuchacovich	45	51	13	19	2.1%
Toonen	76	106	32	53	11.6%
Miceli	194	261	79	106	27,6%
Subtotal (95% CI)		**418**		**178**	**41.3%**
Total Events	315		124		
Heterogeneity: Chi² = 3,76, df = 2 (P = 0.15); I² = 47%					
Test of overall effect: Z = 1.34 (P = 0.18)					
Total (95% CI)		**927**		**358**	**100.0%**
Total Events	618		227		
Heterogeneity: Chi² = 11,06, df = 8 (P = 0.20); I² = 28%					
Test of overall effect: Z = 1.19 (P = 0.23)					
Test for subgroup differences: Not applicable					

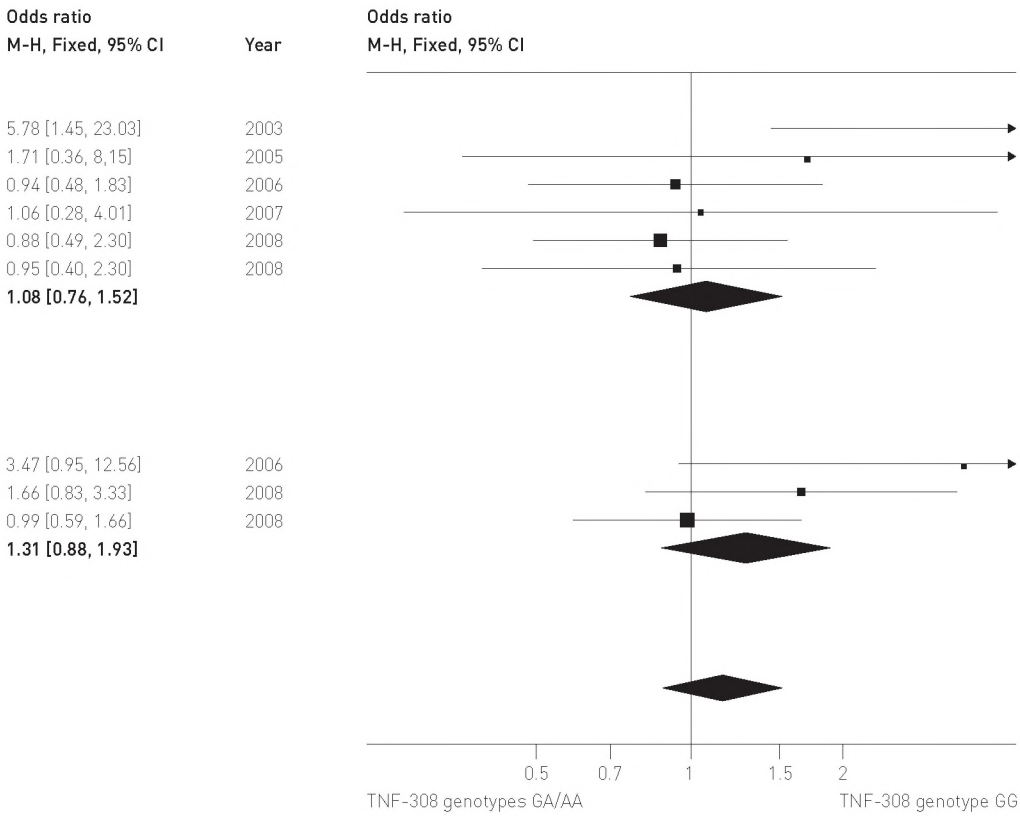
M-H= Mantel-Haenszel statistical method; CI = Confidence Interval

Figure 3b / Meta-analysis of the *TNFA* -308 genotype with response to TNF receptor agonist in rheumatoid arthritis for the

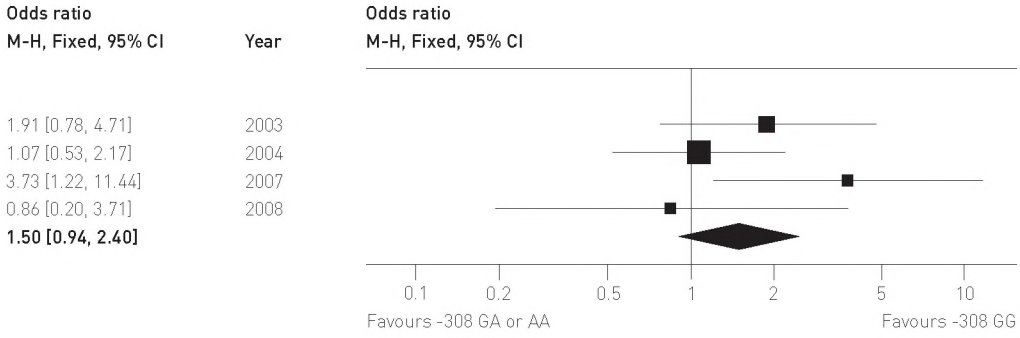
Study or Subgroup	TNF-308 genotype GG Events	Total	TNF-308 genotype GA/AA Events	Total	Weight
1.1.2 Recombinant TNF receptor					
Mugnier	65	77	34	46	23.6%
Criswell	63	89	43	62	52.7%
Guis	56	68	10	18	9.9%
Toonen	15	25	7	11	13.8%
Subtotal (95% CI)		**259**		**137**	**100.0%**
Total Events	199		94		
Heterogeneity: Chi² = 4,26, df = 3 (P = 0.23); I² = 30%					
Test of overall effect: Z = 1.71 (P = 0.09)					

M-H= Mantel-Haenszel statistical method; CI = Confidence Interval

for the outcome: number of responders according to the DAS28 (≥ 1.2).



outcome: number of responders according to the DAS28 (≥ 1.2).



Sensitivity analysis

Analysis of a potential effect of the class of anti-TNF agent anti-TNFalpha antibodies (infliximab and adalimumab) versus the soluble TNF receptor (etanercept) – did not show any association between the *TNFA*-308G>A promoter polymorphism and response to treatment (Figures 3a and 3b). To assess the potential influence of study quality, we repeated the analysis excluding each single study at a time. Only exclusion of the largest studies (52;58;62) would have modified the statistical significance of the mains results. To assess the influence of study quality, we repeated the analysis excluding the studies that were part of randomized clinical trials (RCT). This approach was considered because inclusion criteria in RCT could have selected for a more severe RA phenotype with a higher chance to response to anti-TNF treatment. Nonetheless, the estimate generated was similar to the estimate found in the primary analysis (OR=1.30, 95%CI [0.97-1.73]). The analysis of the funnel plot to check for potential publication bias suggested a random distribution around an OR of 1, consistent with the lack of association between TNFA -308G→A polymorphism and response to treatment (Figure 4).

The effect of potentially important covariates, on the association between the *TNFA* -308G>A polymorphism and response to treatment, was analysed using individual patient data from subset database including 814 patients from The Netherlands and France. The patients' characteristics are presented in Table 2. The covariates studied encompassed class of anti-TNF medication, concomitant use of disease modifying antirheumatic drugs, disease duration, HAQ at baseline, rheumatoid factor positivity, age and gender.

Bivariate and linear regression analysis did not demonstrate association between the different *TNFA* -308 genotypes and response to treatment. The mean absolute variations of the DAS28 after 12 weeks of treatment were -1.38, -1.76 and -1.76 for the *TNFA* -308 genotypes AA (n=28 (3.5%)), AG (n=207 (26.1%)) and GG (n=559 (70.4%)) respectively (p-value=0.33). None of the covariates studied was statistically associated with the *TNFA* -308 genotypes or the response to treatment.

Figure 4 / Funnel plot: assessment of potential bias of publication according to the ORs of association between TNFA -308 gene polymorphism and response to anti-TNF treatment in rheumatoid arthritis

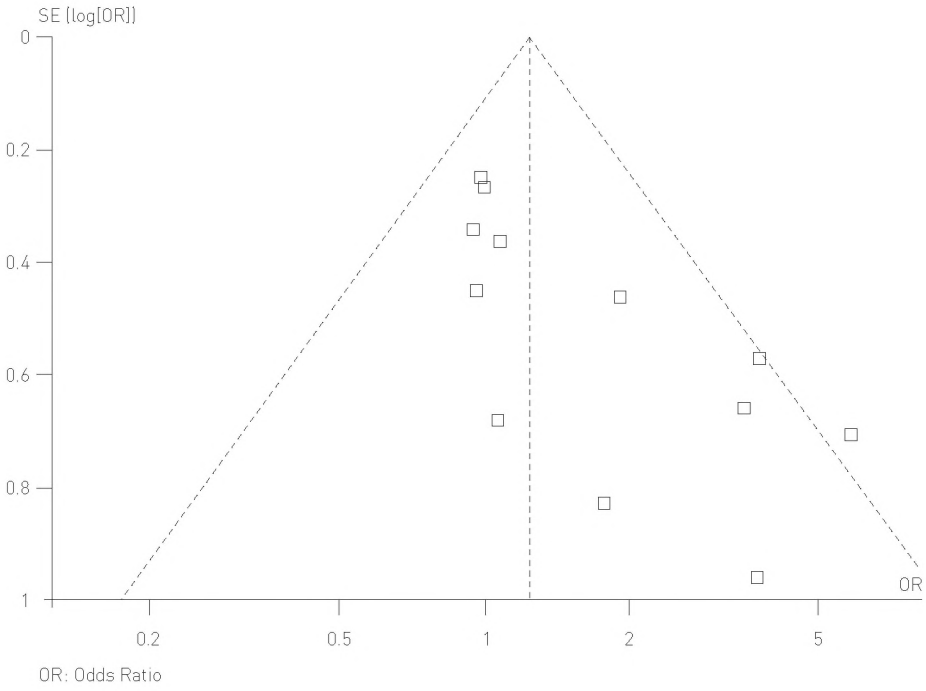


Table 2 / Characteristics of the cohorts from The Netherlands and from France

	Dutch DREAM registry	React French Database	All
Number of patient	426	388	814
Percentage of female	67,4	78,4*	72,6
Age (mean)	65	54*	59,7
Disease duration (mean)	9,4	2,68*	6,17
RF positive (percentage)	81,06	70,54*	76
HAQ baseline	1,48	1,64*	1,57
ESR baseline	28,92	31,59*	30,37
CRP baseline	24,9	27*	26,1
DAS28 baseline	5,47	5,87*	5,66
MTX (percentage)	60,8	46,9*	53,9
Other DMARD (percentage)	45,6	72,1*	58,6
TNF treatment	adalimumab: 159 etanercept: 36 infliximab: 231	adalimumab: 388	adalimumab: 547 etanercept: 36 infliximab: 231

*p<0,001

According to the inclusion criteria required for the clinical trial, RA patients from the React database had a more active disease at baseline than the patients of the DREAM registry. RF: rheumatoid factor, HAQ: health assessment questionnaire, ESR: erythrocytes sendimentation rate, CRP: C-reactive protein, DAS28: disease activity score 28, DMARD: disease-modifying anti-rheumatic drugs.

> DISCUSSION

This meta-analysis did not show any association between the -308G>A *TNFA* polymorphism and response to anti-TNF in the largest cohort of RA patients analyzed up till now. This was still the case after analysis for important covariates such as anti-TNF agents, disease duration, DMARDs use. Our results are not in line with the results presented in two previous meta-analysis including less patients. The study from Lee *et al*. and the study from O'Reilly *et al*. included 311 and 692 RA patients from 6 and 9 different studies, respectively. They suggested that the *TNFA* -308 A allele predisposes to a poorer response to TNF-blocking therapy compared to the -308 G allele (61;248). Several reasons may explain the observed difference between these previous reports and our study. First, one of the obvious differences in favour of our study is that sample sizes were very different. Our meta-analysis involved between 2.5 to 4 times more patients (depending on the end-point) than the previous ones. Moreover, our study of the funnel plot revealed that the largest cohorts did not show any association between *TNFA* -308 GG versus GA/AA genotypes whereas the smallest studies did. Those large studies, published in 2008, were not included in the previous meta-analysis (52;62;63). We identified these studies and we requested to authors details on their negative results. Secondly, the previous meta-analysis disregarded other studies that showed no significant effect of this polymorphism on anti-TNF response which may introduce publication bias. Finally, our and previous studies differ in the use of response criteria. We strictly used response measurements based on the DAS28 whereas Lee *et al*. (61) and O'Reilly *et al*. (248) combined two distinct response measurements, the American College of Rheumatology (ACR) response criteria (158) and the DAS28 (249) in their meta-analysis.

Since the last study included in the meta-analysis of O'Reilly and colleagues, 3 studies investigating the *TNFA* -308G>A polymorphism and anti-TNF treatment outcome in large cohorts of RA patients appeared. Altogether 6 (53;54;57;59;60;245) out of the 13 studies reported an association between the polymorphism and treatment outcome, whereas 7 did not (43;44;49;52;58;62;63). These inconsistent results are largely explained by too low statistical power and small sample sizes. The problem of low power is illustrated even by the largest study published up to date (n=1041 RA patients treated with anti-TNF agents) (52). The authors reported an association only between the very rare homozygous -308AA genotype (n = 7) and response to etanercept (n = 444) but not other TNF neutralizing agents. Comparison of the GG genotype versus the combined AA and AG groups in this and all other studies including our meta-analysis (Figure 3b) showed no difference in the response to any anti-TNF therapy including etanercept. Combining the AA and AG group is in line with the overall hypothesis that the A allele could be associated with anti-TNF non-response (62).

Besides the overall meta-analysis, we also investigated whether potentially important covariates could influence the association of the *TNFA* polymorphism with treatment outcome but this was not the case. Such type of analysis is crucial to assess possible interactions between genes and environmental factors.

Our meta-analysis does not support an association between the *TNFA* -308G>A promoter polymorphism and response to anti TNF therapy. This does not fully rule out that the gene encoding for TNF might influence anti-TNF treatment outcome as we focused on only one genetic variant in the gene. In fact, linkage disequilibrium (LD) is strong in this area and it is difficult to study the role of a SNP in isolation. Therefore, other SNPs of the *TNFA* gene, not in complete linkage disequilibrium with the -308G>A polymorphism deserve further study (87;255). Haplotype analysis of the gene might be an alternative approach to investigating anti-TNF treatment outcome. An example of such an analysis has been shown in the recently published study by our group (62). The latter showed that neither the number of HLA-DRB1 shared epitope copies nor the presence of three TNF polymorphisms (-238A>G, -308G>A and -857C>T) tested separately was significantly associated with response to adalimumab therapy. However, haplotype reconstruction of the TNF locus revealed that the GGC haplotype (-238G, -308G, -857C) in a homozygous form was significantly associated with lower ACR50 response to adalimumab at week 12 (62). Unfortunately the number of studies on the effect of the extended TNFA haplotypes is too small to perform a meta-analysis.

In conclusion, we observed no effect of the *TNFA* –308G>A promoter polymorphism on response to anti-TNF treatment in the largest cohort of RA patients analyzed so far. We conclude that this polymorphism is not a suitable predictor for response to anti-TNF in the clinical practice. Nonetheless, the gene encoding for TNFÐ may still be an interesting candidate gene and other polymorphisms, haplotypes and interactions with environmental factors need to be investigated for their role in anti-TNF response.

> ACKNOWLEDGEMENTS

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> CHAPTER 7

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GENE EXPRESSION PROFILING IN RHEUMATOID ARTHRITIS; CURRENT CONCEPTS AND FUTURE DIRECTIONS

> ABSTRACT

Over the last years microarray technologies have generated new perspectives for high-throughput analysis of biological systems. Nowadays, it is possible to monitor thousands of genes in a single experiment. This molecular profiling technology combined with standardized and validated clinical measurements can allow a more precise characterization of a patient's phenotype, and may lead to the design of therapeutic protocols and procedures better tailored to individual patient's needs.

In this report we provide an overview of expression profiling studies in rheumatoid arthritis (RA). RA is a chronic inflammatory disease in which both genetic and environmental factors are involved. The precise molecular mechanisms underlying RA are not fully understood. A systematic literature search revealed eight array-based expression profiling studies in RA patients. Findings from these studies were compared to those of linkage and genome-wide association (GWA) studies. Although we observed many differences in study design, analysis and interpretation of results between the different studies, we extracted two sets of genes: 1) those differentially expressed in more than one study and 2) genes differentially expressed in at least one of the reviewed studies and present in RA linkage or GWA loci. We suggest that both sets of genes include interesting candidate genes for further study in RA.

> INTRODUCTION

Large-scale analysis of gene expression patterns using (micro-)arrays is spreading over many fields, including that of rheumatology. Nowadays, array-based approaches allow the analysis of thousands of genes in a single experiment (256). There has been special interest in these technologies to elucidate the genetics of heterogeneous autoimmune diseases like rheumatoid arthritis (RA).

Array-based expression analysis is based on the hybridization of an ordered set of probes attached to a surface with a target consisting of cell-/tissue-isolated mRNAs. In general these are mRNAs isolated under different biologic situations, e.g. health and disease, or before and after treatment. The hybridization pattern reflects the relative abundance of each mRNA and leads to the identification of genes up- or downregulated in the test condition compared to the reference.(256) By grouping sets of differentially expressed genes according to function, information can be obtained about key pathways related to disease or treatment (257).

Expression profiling studies in RA can be classified roughly into two categories: 1) those focused on finding (new) candidate genes for disease etiology and understanding its pathogenesis, and 2) those focused on identifying expression patterns typical for a state of RA (e.g. mild versus severe disease or drug-responsive versus non-responsive patients). In the first category expression analysis is often the first step in elucidating gene function, in the second it is aimed at reducing phenotypic heterogeneity or at identifying expression profiles that can serve as diagnostic tools predicting e.g. disease outcome or response to disease modifying anti-rheumatic drugs (e.g. tumor necrosis factor (TNF) blocking agents).

The current study compared the findings of published reports dealing with expression profiling in RA with the aim of identifying genes frequently up- or downregulated. Furthermore, localization of genes was compared to known genetic linkage and association regions for RA. Two sets of genes were derived: 1) those differentially expressed in more than one of the reviewed studies and 2) genes differentially expressed in at least one of the reviewed studies and located within a genetic linkage or association region for RA. Since the large heterogeneity in study design precluded a meta-analysis, we extracted these gene-sets by simple comparison of the studies and report a power estimate of the reviewed genes. We argue that the selected genes are excellent candidates for further research.

> METHODS

Studies were selected by systematically searching MEDLINE (OVID 1966 to January 2007) using the following search terms: rheumatoid arthritis, expression profiling, expression pattern, treatment responsiveness, anti-TNF responsiveness and autoimmune disease. Studies had to deal with expression profiling in RA or other autoimmune diseases, if these also *included* RA samples. Animal studies and expression profiling performed using techniques other than microarray technology were excluded. The criteria were checked for every article independently by two reviewers (ET and MC). In case of disagreement, articles were re-examined and discussed until consensus was achieved. From each selected article, detailed information was extracted on study design, number and characteristics of participants, interventions, outcome measurements and time of follow up.

> RESULTS

Table 1 / Overview of studies, concerning patient characteristics, used tissues and experimental setup

Number of patients (% female)	Tissue	Array
RA: n=29 (75%). Average disease duration: 12 years). No information about ACR criteria was given.	PBMCs	U95Av2 (12626 cDNAs) (Affymetrix Inc., Santa Clara, CA, USA)
RA: n=8 (63%). Mean age 38.25 year. Mean disease duration: 1.7 years. All patients were DMARD-naïve at enrolment, stable dose of NSAIDs and/or prednisolone was allowed.	B-cells	Genome-scale oligonucleotide library containing 21,329 human genes. (Corning UltraGAPS Big Flats, NY, USA)
RA: n=15 (87%). No information on disease duration provided. 14 patients received DMARD medication, 4 received prednisone.	RA synovial tissue	cDNA microarray (24,000 cDNAs) representing a random set of genes (custom made; not further specified)
Early RA: n=11 (82%). Average disease duration 1.1 year. Established RA: n=8 (100%). Average disease duration of 10.5 years.	PBMCs	Gene Filters release 1 (4329 cDNAs) (Research Genetics / Invitrogen)
RA: n=4 (unrelated individuals). Families: 1 parent with RA, 1 unaffected daughter (NS)	PBMCs	Gene Filters GF-211 (4329 cDNAs), (Research Genetics, Huntsville, AL, USA)
Early RA: n=17, Average disease duration 1,1 year. Established RA: n=9. Average disease duration of 10,5 years No information about gender was provided	PBMCs	Gene Filters GF-211 (4133 cDNAs) (Genetics/Invitrogen Carlsbad, CA USA)
RA :n=33 (76%) DAS28 ≥5.1. Average disease duration: 11 to 12 years. Patients were resistance to at least one DMARD (MTX included). Infliximab administration: baseline, weeks 2 and 6, and every 8th week thereafter. Change of DAS28 = 1.2 after 3 months was considered as responder (EULAR criteria (16)).	PBMCs (collected at baseline and after 3 months of treatment)	cDNA microarray with 12,000 cDNA probes for 10,000 non-redundant genes (custom made)
RA: n=10 (80%), median age: 54 years. All patients were treated with MTX, 5 patients received prednisolone. Infliximab administration: baseline, 2 and 6 weeks. Disease activity assessment (DAS28): at baseline and after 12 weeks of treatment. EULAR criteria were used to determine response (16).	RA synovial tissue (taken before and after a median of 9 weeks of treatment. Biopsies were taken from the site of inflammation)	HUM 30k cDNA array containing 30,000 spots (custom made)

All patients met the American College of Rheumatology (ACR) criteria unless indicated otherwise (9). RA: rheumatoid arthritis, NS: not specified, LPS: Lipopolysaccharide, PMA: Phorbol mystrate acetate, IL-1: Interleukin 1, DMARD: disease-modifying antirheumatic drugs, ACR: American College of Rheumatology, SLE: systemic lupus erythematosus, PBMC: peripheral blood mononuclear cells, IDDM: insulin dependent diabetes mellitus, MS multiple sclerosis, MTX: methotrexate SAM: Significance Analysis of Microarrays, SD: standard deviation, DAS28: Disease Activity Score 28, NSAID: non-steroidal anti-inflammatory drugs.

Forty-six studies were identified by the literature search, eight met the selection criteria and were included in this study. Two studies (258;259) dealt with autoimmune diseases in general. Six studies discussed expression profiles of RA patients, specifically (189;190;260-263). Of these, four (260-263) focused on expression patterns typical of a specific disease state, and two studies (189;190) examined expression patterns predictive of anti-TNF respondership. Table 1 summarizes information about patient characteristics, tissues analyzed and experimental parameters of the studies.

Specifics of analysis	Power estimation	Reference
3 criteria for definition of differentially expressed genes in comparison of RA patients and controls: (1) P <0.001 by an unpaired Student's t-test, (2) difference in expression of 100 signal units or greater when comparing the means of two groups, (3) greater than 1.4-fold change in mean gene expression between the two groups.	264 patients and 264 controls are needed to detect differences (1.4 fold change) with a power of 90%	Batliwalla *et al.*, (262)
Genes signal intensities >10 SD above mean of the distribution of random negative controls, and induced or repressed 3.0-fold or greater in RA vs. controls were catalogued and used for further analysis.	12 patients and 12 controls are needed to detect differences (3.0 fold change) with a power of 90%	Szodoray *et al.*, (263)
Transcripts varied in abundance at least two-fold from their median level in at least four tissue samples. Comparison of statistical differences in gene expression were generated by SAM. q-values <5 were considered significant	44 patients and 44 controls are needed to detect differences (2.0 fold change) with a power of 90%	Van der Pouw Kraan *et al.*, (260)
Gene expression data were filtered to include only genes that showed significant variability (3 standard deviations) in the clustering analyses.	Number of analyzed patients is sufficient to detect differences with 90% power	Olsen *et al.*, (261)
Difference in expression intensity between two groups determined by Chen test (264): statistical analysis on the basis of a specified confidence intervals (99% for all tests).	Using the number of samples that were analyzed, the minimal fold change in expression that could be detected with a power of 90% is 4.2	Maas *et al.*, (258)
Microarray data were statistically analyzed using the SAM algorithm (265). SAM uses the SD of repeated gene expression measurements to assign a score to each gene.	Using the number of samples that were analyzed, the minimal fold change in expression that could be detected with a power of 90% is 2.0	Liu *et al.*, (259)
A transcript was considered to be expressed if at least two hybridizations provided a positive signal. Normalized values with abundance differences in two or more comparisons between two samples, using a funnel-shaped confidence interval ($p < 0.05$).	Using the number of samples that were analyzed, the differences in expression as defined in the study could be detected with a power of 90%	Lequerre *et al.*, (189)
Moderate t-test was used to identify differentially expressed genes. Probability of falsely identifying differentially expressed genes was controlled using a false discovery rate approach with q-value (analogous to a p-value) assigned to each gene. Cutoff was set at q=0.025: expected proportion of false positives genes. should be less than 0.025% among differentially expressed	Using the number of samples that were analyzed, the differences in expression as defined in the study could be detected with a power of 90%	Lindberg *et al.*, (190)

In Table 2 we compiled genes found to be regulated in more than one study, irrespective of the above defined categories, but including at least one study comparing expression profiles from RA samples with those of healthy controls.

Table 2 / Genes that show a differential expression in more than one study.

Gene symbol	Gene name	Chromosomal location	Up- or downregulated in RA compared to controls (or otherwise specified)	Reference
CSTF2	Cleavage stimulation factor, 3' pre-RNA, subunit 2	Xq22.1	Upregulated	[258], [261]
SLC genes (SLC7A7, SLC25A4)	Solute carrier family	7A7: 14q11.2 25A4: 4q35	Upregulated	[260], [266]
CSF3R	Colony stimulating factor 3 receptor	1p35-p34.3	Upregulated	[258], [261]
Troponin genes (TNNI1,TNNI2, TNNT2, TNNT3)	Troponin 1, Troponin 2, Troponin T2, Troponin T3	I1: 1q31.3 I2: 11p15.5 T2: 1q32-q42.3 T3: 11p15.5	Upregulated	[261], [260]
ASL	Argininosuccinate lyase	7cen-q11.2	Downregulated	[258], [259]
TP53	Tumor protein p53	17p13.1	Downregulated	[258], [259]
TXK	TXK tyrosine kinase	4p12	Downregulated	[258], [259]
LAMR1	Ribosomal protein SA	3p21.3	Downregulated	[261], [189]
BMP8	Bone morphogenetic protein 8	1p35-p32	Downregulated	[258], [259]
CYP3A4	cytochrome P450, family 3, subfamily A	7q21.1	Ref .[261]: upregulated Ref. [189]: downregulated in infliximab responders vs non-responders	[261], [189]
KNG1	Kininogen 1	3q27	Ref. [258] and ref. [259]: downregulated . Ref. [189]: upregulated in infliximab responders vs non-responders	[258], [259], [189]
S100 genes (S100A10, S100A12)	S100 calcium binding protein	1q21	Ref. [261]: downregulated Ref. [262]: upregulated	[261], [262]
PPP2R3	Protein phosphatase 2 (formerly 2A), regulatory subunit B'', alpha	3q22.1	Ref. [258] and ref. [259]: downregulated. Ref. [260]: Higher expressed in RA-II group compared to RA-I group	[258], [259], [260]
MMP-3	Matrix metalloproteinase 3	11q22.3	Ref. [260]: Higher expressed in RA-I group compared to RA-II group. Ref. [190]: Upregulated in infliximab responders	[260], [190]

Studies focused on finding (new) candidate genes for disease and understanding its pathogenesis

Batliwalla and coworkers (262) performed gene expression profiling using peripheral blood mononuclear cells (PBMCs) from 29 patients with RA and 21 healthy controls (Table 1). Out of the 4500 investigated genes, they identified 81 genes with significantly different expression levels between RA and control; 29 genes were downregulated and 52 genes were upregulated in the RA group. Glutaminyl cyclase (glutaminyl-peptide cyclotransferase; QPCT), IL1 receptor antagonist (IL1RA), S100 calcium binding protein A12 (S100A12) and GRB2-associated binding protein 2 (GAB2) were among the top of overexpressed genes; whereas CD72 and CD79b were most significantly downregulated. Strikingly, many of the overexpressed genes were monocyte-specific. This may have been related to the fact that the RA patients had an active disease and were included before therapy initiation (262).

Szodoray *et al*. (263) used a genome-scale microarray representing 21,329 genes to identify differentially regulated genes in peripheral blood B-cells from eight patients with early RA compared to eight healthy controls (Table 1). Three hundred and five genes were overexpressed in RA-derived B-cells and 231 genes were repressed when compared to controls. Clusters of functionally associated networks of the differentially expressed genes were constructed into which 51 of the 536 genes fitted. Five functional classes were defined: cell activation, proliferation and apoptosis (31 genes); autoimmunity (five genes); cytokines, cytokine-receptors and cytokine-mediated processes (eight genes); neuro-immune regulation (two genes); and angiogenesis (five genes). Quantitative-PCR (q-PCR) confirmed the upregulation of 13 randomly chosen genes in B-cells from RA patients (Supplementary Table 1) (263).

Studies on gene expression patterns typical of a specific state of disease

Studies in this category were aimed at identifying profiles predictive of a disease state, predominantly, but often also included a case-control comparison.Van der Pouw Kraan *et al*. (260) used a custom microarray containing approximately 24,000 cDNA probes to subclassify RA patients (n=15) (Table 1). Hierarchical clustering of gene expression in synovial tissue identified two main patients groups. These groups were indicated as RA-I (n=10) and RA-II (n=5). Gene expression in the RA-I group was indicative of an adaptive immune response, whereas genes identified in the RA-II group were involved in fibroblast dedifferentiation.(260) The RA-I group overexpressed 121 genes compared to RA-II; 39 genes were overexpressed in the RA-II group compared to RA-I Supplementary Table 2 lists the top five discriminators in each group.

Olsen *et al.* (261) aimed at identifying a gene expression pattern specific for early RA which could be used as a diagnostic marker and lead to early therapy and better prognosis. PBMCs from RA patients were analyzed using microarrays containing 4329 cDNAs (Table 1). Eleven patients who had RA for less then two years were compared with eight patients with longstanding RA (≥10 years). The cluster analysis indeed allowed them to discern patients with early RA from those with longstanding disease (261). No clear ranking was provided for genes most significantly over- or underexpressed, but genes that were upregulated more than threefold in early RA included troponin I (TNNI2), troponin T2 (TNNT2), cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4), cleavage stimulation factor (CSTF2) and CSF 3 receptor (CSF3R). Genes downregulated in early RA included S100A10 and ribosomal protein SA (RPSA; LAMR1).

In another study, the same group hypothesized that patients with autoimmune disorders (including RA) exhibit highly reproducible PBMC gene expression profiles resulting from chronic inflammation, other disease manifestations, or from family resemblance.(258) To test the latter hypothesis, PBMC gene expression profiles of individuals with RA (n=4) and SLE (n=4) and their unaffected first-degree relatives were compared (see Table 1). Genes were classified into three major categories: overexpressed genes (n=94), underexpressed genes (n=111) and non-autoimmunity genes (n=3924). Expression profiles in unaffected first-degree relatives resembled those of individuals with autoimmune diseases. Interestingly, this was also true for many of the autoimmune genes. Supplementary Table 3 lists the top 5 under- and overexpressed autoimmunity genes displaying the highest correlation coefficients in relative-pairs. Though the main goal of this study was to identify gene expression signatures across autoimmune disorders and not the identification of RA candidate genes, we added all genes that replicate results from other studies to Table 2.

Another study of this group was aimed at identifying the proportion of a gene expression profile that was *independent* of familial resemblance and determining whether this was a product of disease duration, disease onset or other disease-related factors (259). The study included patients with SLE (n=19) longstanding and early RA (n=9 and 17, respectively), insulin-dependent diabetes mellitus (IDDM; n=5), multiple sclerosis (MS; n=4), healthy controls (n=8) and first-degree unaffected family members of individuals with SLE and RA, (n=8) (Table 1). One hundred genes with shared expression levels between individuals with autoimmune diseases but not unaffected family members or controls were identified (259).

A recent pharmacogenetic study by Lequerré *et al*. (189) set out to identify genes predictive of responsiveness to infliximab (Remicade®), a TNF blocking agent, in PBMCs. Thirty-three patients with highly active disease refractory to

methotrexate (MTX) treatment were included (see Table 1); 16 patients were classified as responders to infliximab, 17 as non-responders. Unsupervised hierarchical clustering of 41 mRNAs differentially expressed in PBMCs prior to treatment perfectly discriminated responders from non-responders. These transcripts included CYP3A4, LAMR1 and KNG1, genes that were also differentially expressed in several other studies and related to disease severity (Table 2). Twenty of the 41 transcripts were assessed by q-PCR in a second set of 10 responders and 10 non-responders to validate their predictive value. This set of transcripts provided 90% sensitivity and 70% specificity for the classification of responders and non-responders (Supplementary Table 4) (189).

Also Lindberg and coworkers (190) examined gene expression profiles in infliximab-treated RA patients (n=10). They included three responders to therapy, five moderate responders and two non-responders (Table 1). Two hundred seventy-nine significant differences in gene expression of synovial (inflamed) tissue were observed between the three good responders and the two non-responders (190). Several of the differentially expressed genes were also observed in patient-control comparisons (Table 2).

Power estimation of expression profiling studies

Several parameters are needed for power analysis of expression profiling studies, such as sample size and standard deviation (SD). SD was not mentioned in most of the studies. Therefore this parameter was estimated using a freely accessible RA expression dataset (Gene Expression Omnibus, GSE 1911; http://www.ncbi.nlm.nih.gov/geo/). Using this dataset, we calculated the SD for the RA group (0,936) and for the control group (0,670) and used these to determine the minimal fold change in gene expression a study was able to detect with a statistical power of 90%. All studies were included (Table 1). In the study of Batliwalla *et al*. (262) a 1.4 fold change in gene expression was considered as threshold for differential expression of genes. We calculated that 264 RA samples and 264 controls would be needed for 90% power to detect a 1.4-fold change; 29 were analyzed (Table 1). Szodoray and coworkers (263) used a 3-fold change as cut-off for differential expression. We calculated that 12 samples in both groups would be needed; eight were analyzed. Van der Pouw Kraan and coworkers (260) used a 2.0 fold change cut-off and therefore 44 samples in each group would have been needed to reach 90% statistical power; 15 were analyzed. The study of Olsen *et al*. (261) considered a difference of 3 SD as threshold for differential expression. The total group of patients analyzed in this study (n=19) was sufficient to detect this difference.

For the studies of Maas *et al*. (258) and Liu *et al*. (259) no information about fold change, effect size or False Discovery Rate (FDR) was provided. Assuming a FDR of 5% for both studies and taking into account the number of samples in the studies, we estimated that Maas and co-workers would have been able to reliably detect a fold change of 4.2 and higher, whereas Liu *et al*. could detect a fold change of Ð2.0. Also Lequerre *et al*. (189) and Lindberg *et al*. (190) did not report the fold change cut-off used. Using their published effect sizes (1.9 for Lequerre *et al*., 2.9 for Lindberg *et al*.) we calculated the minimal fold change. Based on the number of samples analyzed, indeed, both studies appeared sufficiently powered to detect these effects.

Linkage and genome-wide association studies

Additional input for the identification of candidate genes for RA disease susceptibility can be obtained by combining results of different experimental approaches, like expression studies and genetic linkage or association studies (267). Genes that are found differentially expressed in patient-control comparisons and that are located under linkage peaks for RA can be viewed as strong candidates for RA. Several whole-genome scans for RA have been performed (268-273). Recently, also two genome-wide association studies (GWAS) have been published.(187;274) In this study we compared data from linkage meta-analyses (275;276) and the GWAS (187;274) with the results of the expression profiling studies described above. Several differentially expressed genes were located in RA linkage regions (Table 3). As for overlap with the GWAS, tumor necrosis factor, alpha-induced protein 2 (TNFAIP2), which was differentially expressed in the study of Maas and coworkers (258), was also associated with RA in one of the GWAS (187).

Table 3 / Overview of candidate genes located in RA susceptibility loci and differentially expressed in one or more RA expression studies with highest priority given to those genes found in properly powered studies [189;258;259;261]

Gene ID	Gene location	Gene name	Locus identified by RA linkage meta-analysis	References
KNG1	3q27	Kininogen 1	3q27.3-qter	[189;258;259]
CSF3R	1p35-p34.3	Colony stimulating factor 3 receptor	1p35.3-p32.2	[258;259;261]
TNNT2	1q32	Troponin T type 2	1q32-q42.3	[261]
PSMB9	6p21.3	Proteasome subunit β type 9 (LPM2)	6p21.3	[189]
EPS15	1p32	EGF receptor pathway substrate 15	1p35.3-p32.2	[189]
MCP	1q32	Membrane cofactor protein (CD46)	1q32-q42.3	[189]
EPB72	9q34.1	Stomatin	9q33.3-qter	[258]
IRF-4	6p25-p23	Interferon regulatory factor 4	6pter-p22.3	[258]
NCF4	22q13.1	neutrophil cytosolic factor 4	22q13	[260]
IL-8	4q13-q21	Interleukin 8	4q13.3-q24	[260]
GM-CSF (CSF2)	22q13.1	colony stimulating factor 2	22q13	[260]
STMN1	1p36.1	stathmin 1/oncoprotein 18	1p36	[260]
PTPRK	6q22.2-23.1	protein tyrosine phosphatase, receptor type, K	6q23	[260]
AA598840	1p34.3	-	1p35-p32.2	[260]
AA487590	13q12-q13	-	13q12	[260]
HBACH (ACOT7)	1p36.31-p36.11	acyl-CoA thioesterase 7	1p36	[260]

> DISCUSSION AND CONCLUSION

The advent of expression profiling in RA research has served several important purposes: (1) long assumed concepts of RA got additional support on the molecular level. For example the study of van der Pouw Kraan and co-workers confirmed the heterogeneous nature of RA and gave insight in the distinct pathogenic mechanisms contributing to the disease (260). (2) Clinical biomarkers to aid disease diagnosis, prognosis and treatment outcome can be extracted from the genes differentially expressed between RA patients and controls (277;278). The prognostic potential of gene expression profiles has been elegantly shown in cancer (279-283) and is already used in the clinic. Studies dealing with various types of cancer show how gene expression profiling can help to predict treatment outcome in individual patients (284-286). For RA two recent papers suggest feasibility of predicting treatment response by pharmacogenomics, potentially leading to more individualized treatment strategies (189;190). Especially Lequerré and co-workers (189) -by measuring transcript levels at baseline in a well accessible tissue (blood) suitable for implementation into clinical practice- showed that a small subset of discriminative transcripts can provide a tool to predict infliximab efficacy in RA. The genes identified in the treatment response studies were also found differentially expressed in studies related to disease severity, strengthening the view that the same genes and genetic mechanisms may underlie disease severity and response to anti-TNF treatment (53).

In this study we performed a systematic literature search to identify array-based expression profiling studies in RA. Our hypothesis was that genes identified in several of the studies are better candidates for further research into RA susceptibility and progression than those identified only once. Combination of data from several sources may also help in target identification (267), therefore we also included information from RA linkage meta-analysis and the two first GWAS. We describe the similarities and differences of the reviewed studies based on the data presented in the studies, and extract several genes that seem strong candidates for further study (Table 2 and 3). The overlap between the GWAS and the expression profiling studies was somewhat disappointing, but might be related to the low power of the studies performed so far which will certainly improve. A better way to select interesting genes from all studies would be a meta-analysis. However, due to the large heterogeneity between the studies this is impossible. The gene expression data are obtained from studies with different designs (case-control, RA only) using very diverse platforms and probe sets and different sources of patient material or partly overlapping patient samples (Table 1).

The differences in study design also hamper comparisons of the statistical power between studies. However, using a simulation strategy we were able to estimate the statistical power of individual studies. Many of them appeared underpowered. Nevertheless a number good candidates for further study were defined (Table 3). Based on the power analysis the strongest candidate genes would be KNG1 and CSF3R. Both genes were found differentially expressed in three studies with sufficient power (KNG1: (189;258;259), CSF3R: (258;259;261)) and are located in a known RA linkage region (276).

Based on the above it seems very important to reduce heterogeneity between studies and increase power in future studies. One way to achieve a reduction of heterogeneity is to make use of standardized protocols for the description of microarray experiments. MIAME is such a protocol and describes the Minimum Information About a Microarray Experiment that is needed to enable unambiguous interpretation of the study results and reproduction of the experiment (http://www.mged.org/Workgroups/MIAME/miame.html). Of the described studies, only Lequerré et. al. (189) stated that their clinical and experimental data complies with the MIAME recommendations. Besides the use of standardized protocols, when studying complex diseases like RA, the likelihood of identifying important genetic disease determinants can be increased if patients are well characterized and phenotypes are very narrowly defined (287).

In our study we combined expression profiling, linkage and genome-wide association data to predict candidate genes for further research. One might also consider taking additional evidence into account, like proteomics studies that identify proteins differentially expressed between patients and controls or between responders and non-responders to treatment (198;288).

To permit the integration of data from different sources, like expression profiling (in humans and model organisms), linkage, association and proteomics studies, software tools are currently being developed (289) and some are already in use, like the programs 'Prioritizer' and 'Endeavour' (290;291). These perform searches of existing literature as well as of databases containing e.g. gene expression profiles to identify (new) candidate genes for various diseases.

In conclusion, expression profiling opens up a new era in diagnosis, prognosis and treatment of RA and helps to elucidate many of the pathophysiological processes involved in this disease. Combining information from different studies and different sources can aid to find the right genes to study in the maze of different reports. We have identified genes reproducibly regulated in expression profiling studies of RA and/or present in RA linkage and association regions for RA and suggest that these are excellent candidates for further study.

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> SUPPLEMENARY TABLES

Supplementary Table 1 / Genes that were upregulated in RA B-cells compared to control B-cells and were chosen for re-analysis in the study of Szodoray and co-workers [263].

Gene ID	Gene name	Chromosomal location
CNNM4	Cyclin M4	2p12-p11.2
BARD1	BRCA1 associated RING domain 1	2q34-q35
U5-116KD	U5 snRNP-specific protein 116 kDa	17q21.31
TLR9	Toll-like receptor 9, transcript variant A	3p21.3
IL-5RA	Interleukin 5 receptor, alpha	3p26-p24
IL-10	Interleukin 10	1q31-q32
IL-12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	3p12-q13.2
PTX3	Pentaxin-related gene	3q25
CRLF1	Cytokine receptor-like factor 1	19p12
CHRNB1	Cholinergic receptor, nicotinic, beta polypeptide 1	17p13.1
DRD2	Dopamine receptor D2, transcript variant 1	11q23
MMP28	Matrix metalloproteinase 28	17q11-q21.1
VEGFC	Vascular endothelial growth factor C	4q34.1-q34.3

Supplementary Table 2 / Top 5 of higher expressed genes in RA-I vs RA-II group and RA-II vs RA-I group in the study of van der Pouw Kraan and co-workers (260).

Gene ID	Gene name	Fold change	q (%)	Chromosomal location
	Top 5 of genes expressed at higher level in RA-I group compared to RA-II group			
Immunoglobulins (n=10)	-	20.90	0.91	-
NCF1	Neutrophil cytosolic factor 1	20.25	0.91	7q11.23
MMP3	Matrix metalloproteinase 3	12.67	2.03	11q22.3
AA598840	-	11.49	0.91	1p34.3
CXCL10 (IP-10)	Chemokine (C-X-C motif) ligand 10	7.80	0.91	4q21
	Top 5 of genes expressed at higher levels in RA-II group compared to RA-I group			
TNNI1	Troponin I type 1	9.98	2.03	1q31.3
AA196465	-	8.43	2.90	-
KRT17	Keratin 17	6.91	1.87	17q12-q13.2
COL11A1	Collagen type XI	6.74	0.91	1p21
TNNT3	Troponin T type 3	6.02	2.90	11p15.5

Comparison of statistical differences in gene expression between the RA-I vs RA-II were generated by Significance Analysis of Microarrays (SAM). q-values <5 were considered significant

Supplementary Table 3 / Top-5 of under- and overexpressed autoimmune genes displaying the highest correlation coefficient in parent-offspring pairs in the study of Maas *et al* (258).

	Gene ID	Gene name	R_s*	P-value*	Chromosomal location
Underexpressed genes	EPB72	Stomatin	0,98	1.48E -05	9q34.1
	KNG1	Kininogen 1	0,98	1.48E -05	3q27
	ASL	Argininosuccinate lyase	0,98	1.48E -05	7cen-q11.2
	TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	0,98	1.48E -05	14q32
	MADCAM1	Mucosal addressin cell adhesion molecule-1	0,96	1.18E -04	19p13.3
Overexpressed genes	APEX	APEX nuclease (multi functional DNA repair enzyme)	0,98	1.48E -05	14q11.2-q12
	DGKA	Diacylglycerol kinase, alpha	0.98	1.48E -05	12q13.3
	CHGB	Chromogranin B	0.95	3.89E -04	20pter-p12
	GPR30	G protein-coupled receptor 30	0,95	3.89E -04	7p22.3
	NFIL3	Nuclear factor, interleukin 3 regulated	0.95	3.89E -04	9q22

*The corresponding Spearman correlation (R_s) and significance (*P*-value, Student's *t*-test) for each gene are shown

Supplementary Table 4. Transcript levels as predictors of infliximab responsiveness according the study of Lequerré *et al* (189).

Gene ID	Gene name	SAM*	t test (p-value)*	Chromosomal location
AKAP9	A kinase (PRKA) anchor protein 9	-2.83	0.002	7q21-q22
LAMR1	Ribosomal protein SA (37LRP)	-2.43	0.007	3p21.3
FBXO5	F-box protein 5	-2.42	0.006	6q25-q26
RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	-2.41	0.004	2p25.1-p24.1
PFKB4	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	-2.33	0.003	3p22-p21
HLA-DPB1	Major HLA, class II DP beta 1	-2.32	<10-4	6p21.3
RPL35	Ribosomal protein L35	-2.25	NS	9q34.1
RPS16	Ribosomal protein S16	-2.24	NS	19q13.1
RPS28	Similar to 40S ribosomal protein S28	-2.23	NS	19p13.2
PSMB9	Proteasome subunit Ð type 9 (LPM2)	-2.17	0.006	6p21.3
SCAM-1	Vinexinß (SH3-containing adaptor molecule-1	-2.15	NS	8p21.3
EPS15	EGF receptor pathway substrate 15	-2.12	0.003	1p32
TBL2	Transducin (beta)-like 2	-2.12	NS	7q11.23
PTPN12	Protein tyrosine phosphatase, non-receptor type 12	-2.09	NS	7q11.23
MTCBP-1	Membrane-type 1 matrix metalloprotein cytoplasmatic tail binding protein 1	-2.04	0.005	2p25.2
QIL1	QIL1 protein	NS	0.009	19p13.3
COX7A2L	Cytochrome c oxidase subunit VIIa polypeptide 2 like	NS	0.007	2p21
ELMOD2	ELMO domain containing 2	+1.85	NS	4q31.21
MRPL22	Mitochondrial ribosomal protein L22	+1.99	0.009	5q33.1-q33.3
MCP	Membrane cofactor protein (CD46)	+2.30	0.005	1q32

*Significance Analysis of Microarrays (SAM) value as an indicator of significance of transcript variation in responders versus non-responders. A positive or negative value indicates an over- or underexpression at baseline in responders versus non-responders, respectively. *P*-value of a *t* test as an indicator of significant transcript variation in responders versus non-responders ($p < 0.05$ is considered significant). NS, non-significant.

> CHAPTER 8

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WHOLE GENOME EXPRESSION PROFILING OF RHEUMATOID ARTHRITIS PATIENTS TREATED WITH ANTI-TUMOUR NECROSIS FACTOR VALIDATES EXISTING AND REVEALS NEW EXPRESSION SIGNATURES FOR ANTI-TNF RESPONSE

> ABSTRACT

Objective: So far, there are no means of identifying rheumatoid arthritis (RA) patients who will fail to respond to tumour necrosis factor blocking agents (anti-TNF), prior to treatment. We used genome-wide gene expression profiling to identify gene expression signatures predicting therapy outcome. We first tested the validity of three published expression signatures in our patient cohort. Second, we identified a new predictive gene expression signature. Third, we explored potential mechanisms underlying anti-TNF (non-)response.

Methods: Genome-wide expression profiling using Affymetrix GeneChip Exon 1.0 ST arrays was performed on RNA isolated from white blood cells of 42 RA patients starting treatment with infliximab or adalimumab. Clinical response according to EULAR criteria was determined at week 14 of therapy.

Results: Hypothesis-driven analysis of the dataset using two existing gene sets predictive of anti-TNF treatment outcome revealed a sensitivity of 71% and a specificity of 61 and 28%, respectively, for classifying the patients in the current study. Our exploratory analysis identified a set of 113 genes whose expression predicted therapy response with a sensitivity of 79% and a specificity of 84%. Genes involved in guanylate binding, apoptosis, transcription, immune response, inflammation and cellular proliferation were enriched in the set.

Conclusions: We successfully validated one of three previously reported predictive expression profile and identified a new one. The 113 genes associated with anti-TNF treatment response point out specific pathways for further study into mechanisms of anti-TNF action. Our results confirm that gene expression profiling prior to treatment is a useful tool to predict anti-TNF (non) response.

> INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which predominantly involves synovial joints and affects up to 1% of the world's population (20). Tumour necrosis factor (TNF) neutralization is one of the most effective therapeutic strategies in RA. Nonetheless, this approach is not universally effective and approximately 30% of patients treated with TNF blocking agents fail to achieve or maintain clinical improvement (28). The combination of prolonged high disease activity, high costs and risk for adverse effects in these non-responding patients has driven the search for predictive markers – including genetic markers - that are able to predict treatment outcome. Insight into the genetics of anti-TNF therapy may facilitate the choice for the most suitable therapy for an individual patient regarding efficacy and safety, thus leading to more individualized treatment in daily clinical practice (42).

In recent years, genome-wide gene expression analysis using microarrays has become a key component in unravelling the underlying transcriptional regulation of various complex diseases (292-295). Gene expression profiling studies in patients with RA have not only revealed genes associated with the disease itself but also identified molecularly distinct subgroups of RA patients (67;296-298). Gene expression microarray technology has also shown to be able to assist in identifying genes which are involved in treatment response or adverse events associated with a therapy (299-303). To date, five studies used genome-wide gene expression analysis to identify gene expression signatures predicting the response to anti-TNF treatment in patients with RA (67;73;189;191;192;304). Lequerré and co-workers performed a whole genome microarray analysis in peripheral blood mononuclear cell (PBMC) derived RNA from 13 RA patients treated with infliximab. Expression levels prior to treatment initiation of 41 mRNAs were identified that perfectly separated subsequent responders (n=6) from subsequent non-responders (n=7) to infliximab. Validation in 20 other patients reduced the set to 20 transcripts which classify anti-TNF responders and non-responders with a sensitivity of 90% and a specificity of 70%. Further reduction of the transcript set to only 8 transcripts changed sensitivity to 80% and specificity to 100% (189). Lindberg *et. al* (190) examined gene expression profiles in arthroscopic biopsies from 10 RA patients before and after infliximab treatment. Their data revealed 279 genes significantly differentially expressed in responders and non-responders to infliximab (190). More recently, Koczan and co-workers analyzed RNA extracted from PBMCs of 19 RA patients treated with etanercept. Forty-two differentially expressed genes were examined for their ability to discriminate between anti-TNF responders and non-responders, reaching prediction accuracies of 95% for given combinations of genes at day 3 of treatment, but not prior to treatment (192).

Two other studies by Sekiguchi and co-workers and van der Pouw Kraan *et al.* used expression profiling to get more insight into the mechanisms underlying the action of anti-TNF. They did not report predictive values for expression profiles, but their studies suggest that responders to treatment are characterized by a higher level expression of inflammatory genes in synovial tissue (73) and that the increased expression of inflammatory genes in responders normalizes faster than in non-responders (191).

Despite these promising results, the genes identified in each study show little overlap. This can partly caused by the high false positive rate associated with multiple testing in a limited sample, thus necessitating validation in separate cohorts. In this report we used gene expression profiling on white blood cells (WBC) from 42 RA patients treated with the monoclonal anti-TNF antibodies infliximab and adalimumab to validate previously reported gene expression signatures (189;191) for their predictive value in our independent anti-TNF patient cohort. Only patients treated with anti-TNF antibodies (infliximab and adalimumab) and not with a soluble receptor (etanercept) were included in this study to guarantee homogeneity. The expression levels were subsequently used to identify a new gene expression signature predicting therapy outcome. As a last goal, the genes in this newly identified expression signature were analyzed to unravel the underlying biological pathways and mechanisms that might be involved in anti-TNF therapy outcome.

> MATERIAL AND METHODS

Patients

All patients had RA according to the 1987 revised American College of Rheumatology (ACR) criteria (9) and attended the Departments of Rheumatology of the Radboud University Nijmegen Medical Centre or the St. Maartenskliniek in Nijmegen. The patients selected for the current study all participate in the Dutch Rheumatoid Arthritis Monitoring (DREAM) registry. The latter collects detailed clinical information and treatment outcome of patients who start their first course of a TNF-blocking agent according to the Dutch recommendations (Disease Activity Score 28 (DAS28) > 3.2 and previous failure on at least two disease-modifying antirheumatic drugs (DMARDs), one of which has to be methotrexate (MTX)) (219). Response to TNF neutralization was assessed at week 14 according to the EULAR criteria (16). Consecutive patients enrolled in the DREAM study between 2004 and 2008 were included in this study. Only good responders and non-responders at 14 weeks based on the EULAR response criteria were selected for expression analyses. Patients with a moderate response were excluded. This resulted in forty-two patients (18 good responders and 24 non-responders) that were included in the study (Table 1), representing the extremes of a total of 92 patients. Power calculations showed that this sample of 42 patients had a power of 80% to detect a minimal fold change of two with an alpha of 0.0000027. Responders and non-responders were frequency-matched for gender, age, RF-positivity and use of MTX. Blood was sampled prior to treatment start (infliximab or adalimumab). The regional ethics review board had approved the study and all patients had provided written informed consent.

Molecular analyses

All molecular analyses were performed in a CCKL (Coördinatie Commissie ter bevordering van de Kwaliteitsbeheersing van het Laboratoriumonderzoek) -accredited laboratory at the Department of Human Genetics at the Radboud University Nijmegen Medical Centre in Nijmegen. RNA was isolated within 0.5 hours after venapuncture, using the RNeasy midi kit according to the manufacturer's protocol (Qiagen Benelux B.V. Venlo, The Netherlands). To remove residual

Table 1 / Baseline characteristics, disease activity at baseline and DAS28 improvement for responders and non-responders to anti-TNF treatment

	Responders	Non-responders
N (baseline and 14 weeks follow-up)	18 (43%)	24 (57%)
Female gender	16 (89%)	14 (58%)
Age (mean ± SD)	58 ±14.2	57 ±13.6
RF positivity	13 (72%)	19 (79%)
Adalimumab	4 (27%)	11 (52%)
Infliximab	14 (73%)	13 (48%)
MTX-comedication	18 (43%)	24 (57%)
DAS28 baseline (mean ± SD)	5.3 ±1.0	4.8 ±1.5
DAS28 decrease after 14 weeks of anti-TNF therapy (mean ± SD)	2.0 ±0.8	0.1 ±1.0
Percentage DAS28 change 14 weeks of anti-TNF therapy	37 ±10	-1 ±25

Results are number (percentage) or mean (SD). Percentages are expressed in relation to the total number of patients for each response group (except for the total number of patients).

traces of genomic DNA, the RNA was treated with DNase I (Invitrogen, Leek, The Netherlands) while bound to the RNeasy column. Quality and quantity of the purified RNA was controlled using a NanoDrop spectrophotometer (Nanodrop technologies, Montchanin, DE, USA). RNA integrity was investigated by using the 2100 Bioanalyser (Agilent technologies, Philadelphia, PA, USA). RNA was examined for possible degradation using agarose gel electrophoresis. Gene expression profiling was performed using Affymetrix 1.0 Human Exon ST arrays, representing all known genes (17881) (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. The Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay was used to generate amplified and biotinylated sense-strands DNA targets from the entire expressed genome (2.0 µg of total RNA). Arrays were hybridized by rotating them at 60 rpm in the Affymetrix GeneChip hybridization oven at 45 °C for 17 hours. After hybridization, the arrays were washed in the Affymetrix GeneChip Fluidics station FS 450. Arrays were scanned using the Affymetrix GeneChip scanner 3000 7G system.

Data extraction and statistical analyses

For quality control, the Affymetrix CEL-files were first imported into Affymetrix Expression Console version 1.1 where control probes were extracted and normalized using the default RMA algorithm. The Area Under the Curve (AUC) of the Receiver Operator Characteristic was calculated using the positive and negative control probes. All arrays had an AUC score above the empirically defined threshold of 0.85 indicating a good separation of the positive controls from the negative controls. Pearson correlation between arrays showed no outliers. Subsequently, the CEL-files were imported into Partek® (Partek® Genomic Suite software, version 6.4 Copyright ©2008 Partek Inc., St. Louis, MO, USA) where only core probe sets were extracted and normalized using the RMA algorithm with GC background correction. Core transcript summaries were calculated using the mean intensities of the corresponding probe sets, representing the quantitative expression levels of all genes. We performed a mixed model analysis of variance (ANOVA) on the log2 probe intensities (representing the gene expression intensities) based upon the EULAR response criteria after 3 months of treatment. Variance components indicated that the scan date of the samples influenced the expression profiles and this was included in the model as a covariate. A p-value of <0.01 was considered as a statistically significant difference in gene expression levels. No correction for multiple testing was applied.

Validation of previously reported expression signatures

We used the transcript sets from the study of Lequerré and coworkers (189) and the study of Sekiguchi *et al*. (191) for validation in our patient cohort because they matched our experimental set up in the following aspects: 1) both studies present transcript sets that are able to distinguish between responders and non-responders based upon analyses at baseline (before treatment start) and 2) both studies used blood cells as starting material (Lequerré used PBMCs, Sekiguchi used whole blood). The published transcript sets were linked to the corresponding expression values obtained in our analyses. K-means partition clustering was performed using Pearson dissimilarity as a distance measure. The number of partition clusters was set to two (non-responder and responder). The true positive and true negative responses values were calculated. Sensitivity was calculated by the following formula: true positives (true anti-TNF non-responders identified as non-responders) / true positives + false negatives (true non-responders identified as responders). Specificity was calculated by the formula: true negatives (true responders identified as responders) / true negatives + false positives (true responders identified as non-responders).

Identification of a new gene expression signature

Gene expression signatures were identified using K-means partition clustering. Only genes which showed a significant difference ($p<0.01$, no multiple testing correction) after ANOVA were included. The true positive and true negative response values and the sensitivity and specificity were calculated.

Analysis of biological pathways and transcription factor binding sites

A hypothetic gene regulatory network was created from the list of significantly differentially expressed genes using Pathway Studio software version 6.0 (Ariadne Software, Rockville, MD, USA), which contains a large database of literature-mined and curated protein interactions. The Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 web-accessible functional annotation tool was used to identity biological processes enriched in the list of differentially expressed genes (http://david.abcc.ncifcrf.gov/home.jsp; accessed in December 2008) (305). The computational tool rVISTA (http://genome.lbl.gov/vista; accessed in December 2008) was used to search for transcription factor binding sites (TFBS) over-represented (p-value ≤ 0.006) in the 300 base pairs upstream regions of the genes expressed at different levels in responders and non-responders to treatment (306).

> RESULTS

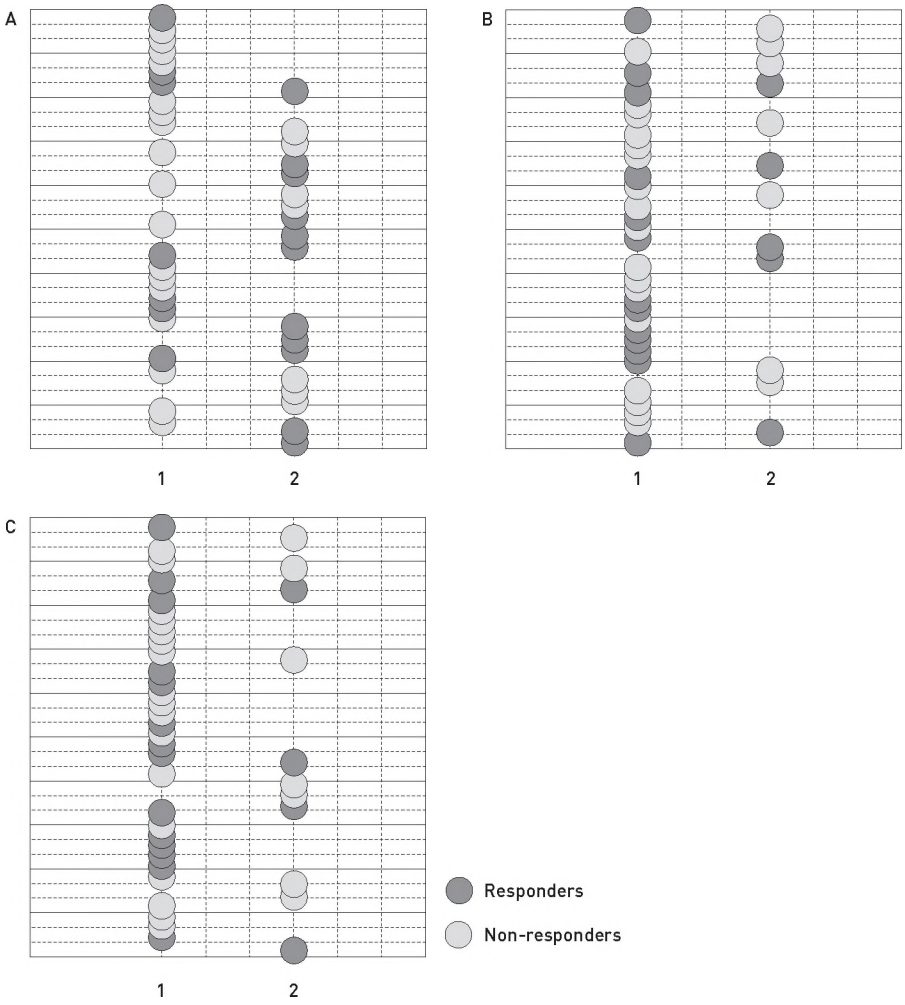
Table 1 shows patients' characteristics, mean disease activity (DAS28) at baseline and DAS28 improvement 14 weeks after treatment start. In total 42 RA patients treated with anti-TNF were included in the study. According to the EULAR definition of response (16), 18 patients in our sample responded well to anti-TNF treatment and 24 patients showed no response to the treatment. Twenty-seven patients were treated with infliximab, 15 were treated with adalimumab. No differences between WBC concentrations (lymphocyts, neutrophils, eosinophils, basophils and monocytes) were observed between the responders and non-responder group.

First, we attempted to validate two previously published expression signatures predicting response to anti-TNF by extracting the expression levels observed for the genes reported by them from our dataset. The transcript sets from the study of Lequerré and coworkers (189) and the transcript set from the study of Sekiguchi *et al.* (191) were linked to the expression values of our 42 RA patients. For the study of Lequerré *et al.*, the transcript set of 20 genes was able to classify our patients as anti-TNF responders and non-responders with a sensitivity of 71% and a specificity of 61%, a transcript set of 8 genes reported by them classified the patients with a sensitivity of 71% and a specificity of 28% (Figure 1A and 1B). The transcript set reported in the study of Sekiguchi and coworkers was able to classify our patient with a sensitivity of 71% and specificity of 28% (Figure 1C).

Subsequently, we performed an exploratory genome-wide analysis of the data and identified 113 genes that, at baseline, were significantly differentially expressed in responders and non-responders to TNF blockade by monoclonal antibodies (Supplementary Table 1). The K-means cluster analysis on these genes divided the patients into two clusters which were identified as a responder and a non-responder cluster (Figure 2), with a sensitivity of 79% and a specificity of 84%.

As shown in Supplementary Table 2, several biological processes were found to be enriched among the 113 differentially expressed genes, including guanylate binding (represented by a number of immune response related genes), apoptosis, transcription and inflammation/immune response. Importantly, most of the genes fitting into these categories were upregulated in responders compared to non-responders to anti-TNF treatment. To get closer to the potential underlying mechanisms regulating response to anti-TNF therapy we performed gene network analysis and searched for transcription factor binding sites enriched in the differentially expressed genes. The latter revealed that 21 genes showed direct interactions with at least one other gene from the list. A

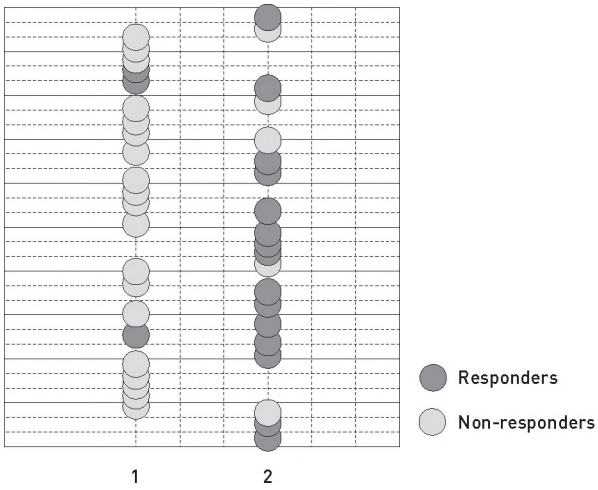
Figure 1 / Cluster analysis for the transcript sets reported in the studies of Lequerré (189) and Sekiguchi (191)



K-means cluster analysis based upon the transcript sets reported by A) Lequerré (20 genes), B) Lequerré (8 genes) and C) Sekiguchi (18 genes). The previously published transcript sets were linked to the expression values of 42 RA patients treated with anti-TNF in our study. The two clusters were identified as the non-responder (1) and responder (2) clusters.

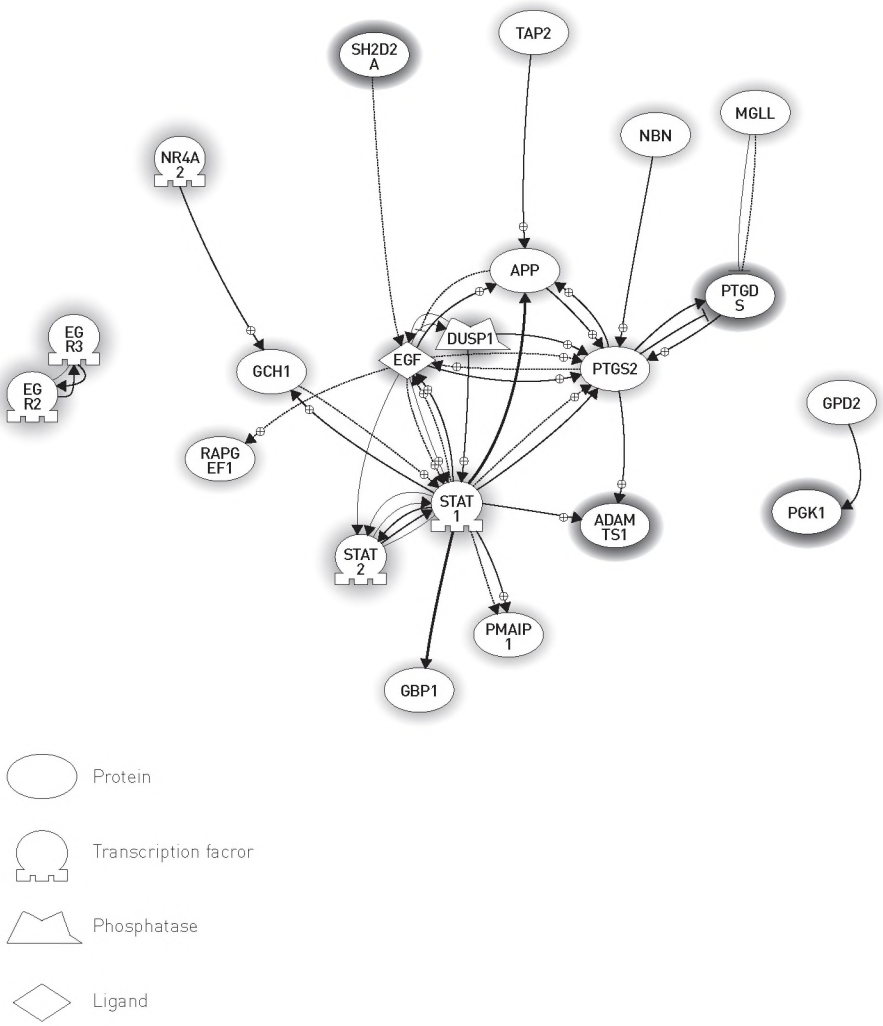
group of 17 genes formed the largest network (Figure 3). The genes EGF (epidermal growth factor), STAT (Signal transducer and activator of transcription) 1 and STAT2 have a central place in this network and are important for cellular proliferation. The search for enriched TFBS was focused on the 61 genes which were upregulated in responders, and the 52 genes which were downregulated in the responders compared to the non responders. The search yielded 9 transcription factors overexpressed in the responders, with ATF1 being most significantly overrepresented (p=1.8·10-12), and only one transcription factor, NANOG downregulated in responders (Supplementary Table 3).

Figure 2 / Cluster analysis



Based upon the expression intensities of these 113 differentially expressed genes the K-means cluster analysis divided the data in two clusters. The two clusters were identified as the non-responder (1) and responder (2) clusters.

Figure 2 / Overview of genes which show an interaction after genetic network modelling



Each gene is represented by a node and gene regulatory interactions are shown by direct lines. Light surrounding indicates that the gene is upregulated in anti-TNF responders compared to non-responders and dark surroundings indicates downregulation when compared to non-responders. Thick arrows indicate promoter binding, thin arrows indicate effect on expression (+ positive, - negative).

> DISCUSSION

Three main objectives were investigated in this study. First we analyzed candidate profiles described in earlier pharmacogenomics studies of anti-TNF treatment in RA. To our knowledge, this is the first study in which previously reported expression signatures for anti-TNF response are re-investigated in an independent patient cohort. Subsequently, we performed a genome-wide analysis to derive a new gene expression signature able to predict outcome at 14 weeks in RA patients treated with TNF blocking agents. Both these analyses were based on whole transcriptome profiling prior to the first anti-TNF administration. As an additional goal of the study we aimed at pinpointing the mechanisms that might explain the mechanisms underlying anti-TNF action and the inter-individual variation in response to treatment.

The expression profiles identified in different studies are often not consistent with each other and different gene sets have been reported to distinguish between responders and non-responders (73;189-192). One reason for the differences between studies might be the limited sample sizes. Other reasons for inconsistent results might be differences in tissues used for analysis (synovial biopsies, PBMCs, whole blood), RNA isolation and analyses at different time points, differences in types and doses of anti-TNF medication, differences in response criteria (ACR, EULAR or DAS28 change), differences in techniques (array platforms, q-PCR methods) and differences in patient ethnicity (Caucasian, Asian). Despite the differences observed between the studies it turns out to be possible to obtain a reasonably good classification of anti-TNF responders and non-responders using one of the three previously described candidate profiles (189;191). One transcript set (20 genes) from the study of Lequerré *et al*. (189) was validated with a sensitivity of 71% and specificity of 61%. The validation of this 20 genes profile results in a relatively good sensitivity and specificity, even in a different type of material (whole blood versus PBMCs). However, we failed to validate two other previously described transcript sets. Possible explanations for this might be caused by the high false positive rate associated with multiple testing in a limited sample, heterogeneous patient cohorts and differences in study design or array platforms between studies.

Our new exploratory expression signature was able to divide the patients in responders and non-responders with a sensitivity of 79% and a specificity of 84%. This confirms that our results for the expression profiling show potential, however, a critical remark is in place. Our investigated cohort of 42 patients consisted of good responders and non-responders according to the EULAR criteria. No moderate responders were included in this cohort. Before such an expression profiling test can be implemented in

the clinic, validation of this expression signature in a larger cohort, consisting of good, moderate and non-responders, is needed. Furthermore, the sensitivity and specificity of the tests might be further enhanced by including into the equation other types of biomarkers, like genetic polymorphisms, and/or clinical characteristics.

The third goal of this study was to gain more insight in the mechanisms underlying therapy outcome. We found that many of the differentially expressed genes are part of biological processes relevant to the disease activity and potentially the etiology of RA, like immune response/inflammation, apoptosis and transcription (Supplementary Table 2). Interestingly, most genes involved in the immune response were upregulated in responders compared to non-responders. These findings in WBC, together with similar results reported by van der Pouw Kraan *et al.* in synovial tissue (73) do suggest that an overall enhanced baseline expression of inflammatory genes is associated with response to TNF neutralization. Interestingly, our study showed that guanylate binding protein (*GBP*) 1, 5 and 7, three genes from the same family, were all upregulated in responders when compared to non-responders. GBPs are induced by interferon gamma (IFN-γ) and are involved in proliferation and regulation of cell growth (307). This family of proteins has an proven role in immunity (307), though it has not been linked to anti-TNF response, before. These results suggest that the underlying cause of RA might be different in patients with increased expression of inflammatory genes, i.e. the anti-TNF responders, than in anti-TNF non-responders. Our study identified an additional immune related gene, *EGF*, which was upregulated in responders. This gene may be an interesting candidate for further study since also Fabre and co-workers showed with protein biochip array technology that high serum levels of EGF, especially if combined with high CRP levels, were associated with a good response to etanercept treatment at 3 months (sensitivity: 87.5% and specificity: 75%) (308).

Also the genes involved in apoptosis are interesting candidates in the context of the efficacy of TNF-blocking therapy. Most genes involved in apoptosis identified in our study were upregulated in responders compared to non-responders. Hlavaty and co-workers investigated the role of genes involved in apoptosis in infliximab treatment in patients suffering from Crohn's disease (CD). They observed that CD patients with a low apoptotic response to infliximab had a higher chance of becoming a non-responders to infliximab treatment compared to patients with a high apoptotic response (309;310). Catrina and co-workers reported a greater increase in apoptosis levels in those RA patients that responded well to treatment with infliximab or etanercept compared to non-responders, but the difference did not reach statistical significance (164). In conclusion, the results of our study and the results reported by Hlavaty *et al.* (309;310) and Catrina *et al.* (164) suggest that apoptotic genes might be useful markers discriminating responders from non-responders to anti-TNF treatment.

The category of genes related to transcriptional biological processes contained a number of immune related genes, as well. Notably, *STAT2* might be a promising candidate gene given its involvement in interferon (IFN) receptor mediated signaling (311). Also Sekiguchi and co-workers reported IFN related genes to be differentially expressed between infliximab responders and non-responders. Their top ten most differentially expressed genes were all IFN related (191). In line with this, as mentioned above, our study showed the *GBP* genes to be differentially expressed in responders and non-responders to anti-TNF. Also these genes are involved in IFN-γ signaling, emphasizing a potentially important role for IFN-γ related pathways in anti-TNF response.

A family member of the *STAT2* gene, *STAT1*, revealed a central role in the reconstructed dynamic network and showed a direct interaction with *PTGS2* (or COX2, the target of selective inhibitors used for pain and inflammation management in arthritis (312)). *STAT1* and *STAT2* are members of the STAT protein family. In response to cytokines and growth factors, STAT family members are capable of inducing a wide range of genes, including the inflammatory genes IFN-gamma, IL10, IL12 and IL13 (313). Polymorphisms in several members of the STAT family of genes have been found to be associated with RA and other auto-immune diseases (314-321). Our own findings now also implicate the STAT genes in anti-TNF response.

The search for common TFBS overrepresented in genes differentially expressed in responders and non-responders identified highly significant overrepresentation of binding sites for several immune-related transcription factors. This analysis might add genes to the list of promising candidates for further study regarding the mechanisms of anti-TNF response.

The current analysis should be viewed in the light of some strengths and limitations. A relative strength of our study is the sample size. Our study investigated a total of 42 well characterized patients. To our knowledge, this is the largest sample investigated for expression profiling to distinguish between anti-TNF responders and non-responders. Another strength of this study is that, for the first time, validations of other expression signatures are incorporated. This leads to a more evidence-based and better argued conclusion in favor of expression profiling as a tool for predicting anti-TNF response then the results from only one single experiment. A limitation of the study is given by the fact that RA is a very heterogeneous disease, which makes it very difficult to select two homogeneous groups (responders and non-responders) for the study. Patients were matched for gender, RF positivity, MTX usage and age and divided into responders and non-responders based upon the EULAR response criteria. Nonetheless, individual patient characteristics like DAS28, CRP, diseased duration, disease onset, age, co-medication, joint erosions, smoking and Health Assessment Questionnaire (HAQ) *within* the two groups were slightly different. This might have

limited our power to detect gene expression differences in the comparison *between* the two groups. In addition, only two genes with a fold change of >2 were detected (*MOP1* and *G0S2*). Therefore the patient population investigated should be increased to have sufficient power (80%) to detect fold changes <2. Another possible limitation of our study arises from the fact that we used RNA isolated from peripheral blood. Synovial biopsy samples are likely to reflect the consequences of inflammation at a specific joint and the therapeutic response to anti-TNF more directly (190;260), however, obtaining such samples is difficult. Especially, this would limit their use for diagnostic testing, for which easy accessibility of material is an important aspect. In addition, we analyzed the two anti-TNF agents together, and it is possible that genetic markers associated with response differ between these agents. The last limitation is that we did not correct for multiple testing, and this increases the chances of false positive findings. However this study should be viewed as a hypothesis generating study, in order to investigate if our identified expression profile is really able to separate the responders from the non-responders, we suggest to validate this expression profile in an independent and lager patient cohort.

To conclude, this study successfully validated an earlier reported gene expression profile predictive of anti-TNF treatment outcome and indentified a new pharmacogenomic expression profile of 113 genes suitable for prediction of response to anti-TNF therapy. Our findings confirm earlier findings suggesting that responders to treatment show a higher level of inflammation compared to anti-TNF non-responders, indicating potentially different mechanisms contributing to disease in the latter group.

> ACKNOWLEDGEMENTS

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> SUPPLEMENARY TABLES

Supplementary Table 1 / Genes that are differentially expressed between anti-TNF responders and non-responders (113 genes)

Gene symbol	Gene name	Chromosomal location
MOP-1	MOP-1	4q21.22
G0S2	G0/G1switch 2	1q32.2-q41
PTGS2	Prostaglandin-endoperoxide synthase 2	1q25.2-q25.3
GBP1	Guanylate binding protein 1, interferon-inducible	1p22.2
EGR3	Early growth response 3	8p23-p21
NR4A2	Nuclear receptor subfamily 4, group A, member 2	2q22-q2
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	18q2
EGR2	Early growth response 2	10q21.1
GBP5	Guanylate binding protein 5 (GBP5), mRNA	1p22.2
MGLL	Monoglyceride lipase	3q21.3
PDE3A	Phosphodiesterase 3A, cGMP-inhibited	12p12
EGF	Epidermal growth factor	4q25
AXUD1	AXIN1 up-regulated 1	3p22
RAPGEF1	Rap guanine nucleotide exchange factor (GEF) 1	9q34.3
HSPC159	Galectin-related protein	2p14
GPD2	Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	2q24.1
STAT2	Signal transducer and activator of transcription 2	12q13.2
PCSK6	Proprotein convertase subtilisin/kexin type 6	15q26.3
PSTPIP2	Proline-serine-threonine phosphatase interacting protein	18q12
C7orf41	Chromosome 7 open reading frame 41	7p15.1
PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 1	19q13.2
PCGF5	Polycomb group ring finger 5	10q23.32
GMPR	Guanosine monophosphate reductase	6p23
TPM1	Tropomyosin 1 (alpha)	15q22.1
SESTD1	SEC14 and spectrin domains 1	2q31.2
TMOD2	Tropomodulin 2 (neuronal)	15q21.1-q21.2
NBN	Nibrin	8q21
PKHD1L1	Polycystic kidney and hepatic disease 1	8q23.1-q23.2
NEXN	Nexilin (F actin binding protein)	1p31.1
DUSP1	Dual specificity phosphatase 1	5q34
GRAMD1B	GRAM domain containing 1B	11q24.1
STAT1	Signal transducer and activator of transcription 1	2q32.2
MPP7	Membrane protein, palmitoylated 7	10p11.23
YPEL5	Yippee-like 5 (Drosophila)	2p23.1

Biological process	P-value	Fold change
Unknown	0.00065481	2.901743504
Regulation of progression through cell cycle	0.0006411	2.41228439
Fatty acid biosynthetic process, prostaglandin biosynthesis, inflammation	0.00050209	1.99835099
Immune response	0.00669153	1.700536533
Transcription, apoptosis	0.00259073	1.64545891
Transcription	0.00753406	1.627096706
Cytochrome c from mitochondria	0.00194964	1.616888282
Regulation of transcription, apoptosis	0.00234606	1.521790752
Immune response	0.00669932	1.497960934
Lipid metabolic process	0.00601595	1.452521985
Lipid metabolic process	0.00293713	1.442938689
Activation of MAPKK activity, immune response	0.00617846	1.429251694
Apoptosis, regulation of transcription	0.00210008	1.427301383
Signal transduction, apoptosis	0.0020499	1.406305467
Unknown	0.00703397	1.368684572
Glucose catabolic process	0.00118695	1.364989639
Transcription, response to cytokines, immune response	0.00981475	1.345730947
Proteolysis	0.009852	1.344434994
Unknown	0.00302269	1.343913236
Unknown	0.00252184	1.337946103
Apoptosis, cell cycle arrest, regulation of transcription	0.00188278	1.328639766
Transcription	0.00112379	1.327811176
Metabolic process	0.0087634	1.310484237
Cell motility	0.00040307	1.30658411
Unknown	0.00115557	1.300755674
Nervous system development	0.00228299	1.296839555
DNA damage checkpoint, cell proliferation	0.00494905	1.292836648
Unknown	0.00369549	1.291278329
Regulation of cell migration	0.00865698	1.284182283
Protein amino acid dephosphorylation	0.0024407	1.279694998
Unknown	0.00183788	1.278746242
Regulation of progression through cell cycle, immune response	0.00518544	1.270503192
Unknown	0.00858529	1.265361846
Unknown	0.00511528	1.26523029

Supplementary Table 1 / Genes that are differentially expressed between anti-TNF responders and non-responders (113 genes)

Gene symbol	Gene name	Chromosomal location
C9orf91	Chromosome 9 open reading frame 91	9q32
GCH1	GTP cyclohydrolase 1 (dopa-responsive dystonia)	14q22.1-q22.2
NT5C3	5'-Nucleotidase, cytosolic III	7p14.3
GBP7	Guanylate binding protein 7	1p22.2
RNF11	Ring finger protein 11	1pter-p22.1
XRN1	5'-3' Exoribonuclease 1	3q23
TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	6p21.3
VEPH1	Ventricular zone expressed PH domain homolog 1 (zebrafish)	3q24-q25
GCNT2	Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	6p24.2
FRMD3	FERM domain containing 3	9q21.32
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	6q23
GTPBP2	GTP binding protein 2	6p21-p12
GTF2F2	General transcription factor IIF, polypeptide 2	13q14
PPCDC	Phosphopantothenoylcysteine decarboxylase	15q24.2
APP	Amyloid beta (A4) precursor protein	21q21.3
PDE4B	Phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4B)	1p31
SLK	STE20-like kinase (yeast)	10q25.1
TAS2R50	Taste receptor, type 2, member 50	12p13.2
UVRAG	UV radiation resistance associated gene	11q13.5
CMIP	C-Maf-inducing protein	16q23
MED12L	Mediator complex subunit 12-like	3q25.1
CD97	CD97 molecule	19p13
MAP4K2	Mitogen-activated protein kinase kinase kinase kinase 2	11q13
HERPUD2	HERPUD family member 2	7p14.2
SQSTM1	Sequestosome 1	5q35
C5	Complement component 5	9q33-q34
RANBP17	RAN binding protein 17	5q34
PIH1D2	PIH1 domain containing 2	11q23.1
LRRC44	Leucine rich repeat containing 44	1p31.1
KCNJ13	Potassium inwardly-rectifying channel, subfamily J, member 13	2q37
BAT1	HLA-B associated transcript 1	6p21.3
PXT1	Peroxisomal, testis specific 1	6p21.31
ARL6	ADP-ribosylation factor-like 6	3q11.2
ZNF425	Zinc finger protein 425	7q36.1
C7orf46	Chromosome 7 open reading frame 46	7p15.3
ELOVL4	Elongation of very long chain fatty acids	6q14
FLJ30672	Hypothetical protein FLJ30672	Xq26.3
HSCB	HscB iron-sulfur cluster co-chaperone homolog (E. coli)	22q12.1
OGG1	8-Oxoguanine DNA glycosylase	3p26.2
LOC652968	Unknown	22q12

(continued)

Biological process	P-value	Fold change
Unknown	0.00989524	1.255524342
L-phenylalanine catabolic process, immune response	0.00939347	1.245159019
Pyrimidine nucleoside metabolic process	0.00637296	1.243804723
Immune response	0.00297358	1.241479122
Protein ubiquitination	0.00651408	1.235923973
Cell cycle	0.00686137	1.234562607
Protein complex assembly, immune response, antigen presentation	0.00666743	1.226570365
Unknown	0.0029713	1.22616234
Glycosaminoglycan biosynthetic process	0.00610597	1.218156929
Cytoskeletal protein binding	0.00814001	1.216056281
Ubiquitin cycle, apoptosis, immune response	0.00567462	1.214910469
Small GTPase mediated signal transduction	0.00176723	1.212235502
Transcription	0.00962998	1.200678584
Coenzyme A biosynthetic process	0.00998291	1.199871576
Cellular copper ion homeostasis	0.00853138	1.198657926
Signal transduction	0.00716894	1.190716533
Nucleotide-excision repair	0.00655314	1.181099084
Signal transduction	0.00698444	1.166818366
DNA repair	0.0088686	1.161871082
Unknown	0.00458458	1.161806656
Transcription, proliferation	0.00341711	1.13723468
Cell motility, immune response, inflammatory response	0.00791707	1.133512264
Protein amino acid phosphorylation, immune response	0.00937475	1.11359142
Protein modification process	0.00129877	1.107402743
Ubiquitin-dependent protein catabolic process, apoptosis	0.00269138	1.10629796
Activation of MAPK activity, inflammation	0.00930742	1.102867902
Protein import into nucleus, docking	0.00101526	1.063382036
Unknown	0.00548372	-1.02431539
Unknown	0.00825136	-1.03227689
Ion transport	0.00460367	-1.05643939
Nuclear mRNA splicing, via spliceosome	0.00418843	-1.05720855
Unknown	0.00854238	-1.06394236
Small GTPase mediated signal transduction	0.00712878	-1.07250918
Transcription	0.0094925	-1.07424272
Unknown	0.00315607	-1.07509936
Fatty acid biosynthetic process	0.00474756	-1.07516643
Unknown	0.0012008	-1.07788256
Protein folding	0.0096215	-1.08232217
Base-excision repair	0.00764588	-1.093634
Unknown	0.00609432	-1.09471855

Supplementary Table 1 / Genes that are differentially expressed between anti-TNF responders and non-responders (113 genes)

Gene symbol	Gene name	Chromosomal location
SLC7A6OS	Solute carrier family 7, member 6 opposite strand	16q22.1
ATPBD1B	ATP binding domain 1 family, member B	1p36.11
HIRIP3	HIRA interacting protein 3	16p11.2
GLT25D2	Glycosyltransferase 25 domain containing 2	1q25
FAIM	Fas apoptotic inhibitory molecule	3q22.3
OR6C74	Olfactory receptor, family 6, subfamily C, member 74	12q13.13
CATSPER3	Cation channel, sperm associated 3	5q31.1
BEX2	Brain expressed X-linked 2	Xq22
ZNF2	Zinc finger protein 2	2q11.2
FSCN1	Fascin homolog 1, actin-bundling protein	7p22
EDC3	Enhancer of mRNA decapping 3 homolog (S. cerevisiae)	15q24.1
ATP6V0E2	ATPase, H+ transporting V0 subunit e2	7q36.1
GPR175	G protein-coupled receptor 175	3q21.2
AK123815	Unknown	13q11
C15orf40	Chromosome 15 open reading frame 40	15q25.2
PIGV	Phosphatidylinositol glycan anchor biosynthesis, class V	1p36.11
GSX1	GS homeobox 1	13q12.2
EIF4E2	Eukaryotic translation initiation factor 4E family member	2q37.1
TUSC4	Tumor suppressor candidate 4	3p21.3
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	21q21.2
PGK1	Phosphoglycerate kinase 1	Xq13
RAD23A	RAD23 homolog A (S. cerevisiae)	19p13.2
HOP	Homeodomain-only protein	4q11-q12
AY358807	Unknown	6p25.1
RAMP3	Receptor (G protein-coupled) activity modifying protein 3	7p13-p12
AY358772		3q13.2
ZBTB6	Zinc finger and BTB domain containing 6	9q33.2
FBXO10	F-box protein 10	9p13.2
OR2L3	Olfactory receptor, family 2, subfamily L, member 3	1q44
SH2D2A	SH2 domain protein 2A	1q21
TMEM186	Transmembrane protein 186	16p13
PTGDS	Prostaglandin D2 synthase 21kDa (brain)	9q34.2-q34.3
CLIC3	Chloride intracellular channel 3	9q34.3
ATP6V0A2	ATPase, H+ transporting, lysosomal V0 subunit a2	12q24.31
KIAA1274	KIAA1274	10q22.1
UQCRFS1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	19q12-q13.1
DND1	Dead end homolog 1 (zebrafish)	5q31.3
OSM	Oncostatin M	22q12.2
IL8	Interleukin 8	4q13-q21

(continued)

Biological process	P-value	Fold change
Unknown	0.00465625	-1.09644998
Unknown	0.00417439	-1.09816132
Chromatin assembly or disassembly	5.9045E-05	-1.09920464
Lipopolysaccharide biosynthetic process	0.00620079	-1.10307432
Apoptosis	0.00514745	-1.1050181
Signal transduction	0.00391734	-1.10651269
Ion transport, cell differentiation	0.0090418	-1.10705738
Unknown	0.00499863	-1.10845484
Transcription	0.00332552	-1.10925419
Cell proliferation	0.0058707	-1.11044659
Unknown	0.00565166	-1.11127047
Ion transport	0.00942957	-1.11570839
Lipid metabolic process	0.0018316	-1.11755048
Unknown	0.0094493	-1.11955857
Unknown	0.00441213	-1.12104174
GPI anchor biosynthetic process	0.00064269	-1.1346049
Regulation of transcription, DNA-dependent	0.00999428	-1.13553329
Translational initiation	0.0015043	-1.13572221
Cell cycle	0.00045971	-1.13899388
Proteolysis, regulation of cell proliferation	0.00890078	-1.13929393
Glycolysis	0.00975208	-1.14329682
Nucleotide-excision repair	0.00851234	-1.16870434
Regulation of transcription	0.00635068	-1.16910135
Unknown	7.9314E-06	-1.18634202
Intracellular protein transport, immune response	0.0028468	-1.1917817
Unknown	0.00424962	-1.1987327
Transcription	0.00086149	-1.19886566
Protein ubiquitination	0.00948124	-1.22914064
Signal transduction	0.00749111	-1.24228828
Angiogenesis, cell differentiation, signal transduction	0.00148433	-1.25690011
Unknown	0.0034506	-1.28575878
Prostaglandin biosynthetic process	0.00686992	-1.31895011
Ion transport	0.00047798	-1.33213473
Ion transport, immune response	0.00892385	-1.33726001
Unknown	0.00754343	-1.3458802
Electron transport	0.00493438	-1.39646557
Multicellular organismal development	0.00147899	-1.42935077
Regulation of cell growth, immune response	0.00344552	-1.87191769
Angiogenesis, immune response	0.00538486	-1.92406295

Supplementary Table 2 / Enriched biological processes among the identified differentially expressed genes

Biological process	p-value	Gene symbol	Gene name	Up-/downregulated*
Guanylate binding	$5.9 \cdot 10^{-8}$	*GBP7*	Guanylate binding protein 7	Upregulated
		GBP1	Guanylate binding protein 1, interferon-inducible	Upregulated
		GBP5	Guanylate binding protein 5 (GBP5), mRNA	Upregulated
Apoptosis	$5.3 \cdot 10^{-3}$	*PPP1R15A*	Protein phosphatase 1, regulatory (inhibitor) subunit 1	Upregulated
		RAPGEF1	Rap guanine nucleotide exchange factor (GEF) 1	Upregulated
		AXUD1	AXIN1 up-regulated 1	Upregulated
		EGR2	Early growth response 2	Upregulated
		EGR3	Early growth response 3	Upregulated
		SQSTM1	Sequestosome 1	Upregulated
		OSM	Oncostatin M	Downregulated
		FAIM	Fas apoptotic inhibitory molecule	Downregulated
Transcription	$2.0 \cdot 10^{-2}$	*MED12L*	Mediator complex subunit 12-like	Upregulated
		PCGF5	Polycomb group ring finger 5	Upregulated
		PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 1	Upregulated
		AXUD1	AXIN1 up-regulated 1	Upregulated
		GR2	Early growth response 2	Upregulated
		EGR3	Early growth response 3	Upregulated
		GTF2F2	General transcription factor IIF, polypeptide 2	Upregulated
		STAT1	Signal transducer and activator of transcription 1	Upregulated
		NR4A2	Nuclear receptor subfamily 4, group A, member 2	Upregulated
		STAT2	Signal transducer and activator of transcription 2	Upregulated
		ZNF2	Zinc finger protein 2	Downregulated
		HOP	Homeodomain-only protein	Downregulated
		ZNF425	Zinc finger protein 425	Downregulated
		GSX1	GS homeobox 1	Downregulated
		ZBTB6	Zinc finger and BTB domain containing 6	Downregulated
Immune response and inflammation	$4.4 \cdot 10^{-2}$	*PTGS2*	Prostaglandin-endoperoxide synthase 2	Upregulated
		TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	Upregulated
		MGLL	Monoglyceride lipase	Upregulated
		STAT1	Signal transducer and activator of transcription 1	Upregulated
		EGF	Epidermal growth factor	Upregulated
		TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	Upregulated
		CD97	CD97 molecule	Upregulated
		C5	Complement 5	Upregulated
		MAP4K2	Mitogen-activated protein kinase kinase kinase kinase 2	Upregulated
		GCH1	GTP cyclohydrolase 1 (dopa-responsive dystonia)	Upregulated
		STAT2	Signal transducer and activator of transcription 2	Upregulated
		IL8	Interleukin 8	Downregulated
		ATP6V0A2	ATPase, H+ transporting, lysosomal V0 subunit a2	Downregulated
		RAMP3	Receptor (G protein-coupled) activity modifying protein 3	Downregulated
		OSM	Oncostatin M	Downregulated

* Up- or downregulated in anti-TNF responders compared to non-responders

Supplementary Table 3 / Transcription factor binding sites (TFBS) overrepresented in genes differentially expressed in responders and non-responders

Transcription factor	p-value	Overexpressed genes in which TFBS are found
TFBS enriched in genes upregulated in treatment responders		
ATF1	$1.8 \cdot 10^{-12}$	*EGR2, EGR3, NR4A2, GTF2F2, PPP1R15A, DUSP1, C7orf41, RNF1,1 AXUD1*
CREBP1	$2.4 \cdot 10^{-7}$	*EGR2, EGR3, NR4A2, GTF2F2, PPP1R15A, DUSP1, C7orf41*
CREBATF	$5.6 \cdot 10^{-7}$	*EGR2, EGR3, NR4A2, GTF2F2, PPP1R15A, DUSP1, C7orf41, RNF11, AXUD1, TPM1, FRMD3, YPEL5, SESTD1, GTPBP2, NT5C3, HERPUD2, C9orf91*
E4F1	$6.9 \cdot 10^{-5}$	*EGR2, EGR3, NR4A2, PPP1R15A, DUSP1, C7orf41*
TAXCREB	$1.4 \cdot 10^{-4}$	*NR4A2, PPP1R15A, DUSP1, C7orf41, RNF11, AXUD1, HERPUD2*
ATF4	$1.2 \cdot 10^{-3}$	*EGR2, EGR3, NR4A2, GTF2F2, PPP1R15A, DUSP1, C7orf41, RNF11, AXUD1, TPM1, FRMD3, YPEL5, SESTD1, GTPBP2, NT5C3, HERPUD2, XRN1, TNFAIP3*
MINI20	$2.8 \cdot 10^{-3}$	*EGR3, NR4A2, GTF2F2, PPP1R15A, DUSP1, C7orf41, RNF11, AXUD1, TPM1, FRMD3, YPEL5, GTPBP2, TNFAIP3, MED12L, TMOD2, CMIP, STAT1, APP, MGLL, VEPH1, UVRAG, GCNT2,*
BARBIE	$3.7 \cdot 10^{-3}$	*EGR2, EGR3, NR4A2, PPP1R15A, GTPBP2, MED12L, PTGS2, PCGF5, SLK, MAP4K2, GCH1, EGF, GCNT2,* TAP2, , *NEXN, RAPGEF1*
ZNF219	$4.3 \cdot 10^{-3}$	*EGR3, DUSP1, FRMD3, YPEL5, SESTD1, CMIP, MGLL, VEPH1,*
TFBS enriched in genes downregulated in treatment responders		
NANOG	$1.4 \cdot 10^{-5}$	*OSM, DND1*

> CHAPTER 9

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SIRPB1 AND ITS RELATION TO ANTI-TNF RESPONSE IN PATIENTS WITH RHEUMATOID ARTHRITIS

> ABSTRACT

Objective. The purpose of this study was to investigate whether anti-TNF treatment outcome was associated with differences in RNA expression at the exon level using a gene expression microarray covering the human genome.

Methods. RNA expression analysis in 42 rheumatoid arthritis (RA) patients before the start of anti-TNF treatment was used to identify alternatively spliced genes. Quantitative PCR was used to validate (n=42) and replicate (n=34) the results obtained by microarray analysis. In order to investigate two partly overlapping deletions in the *SIRPB1* gene we performed multiplex ligation probe analysis (MLPA) in a cohort of 523 RA patients treated with anti-TNF.

Results. Expression analysis of 42 RA patients revealed reduced expression of two adjacent exons in the *SIRPB1* gene in anti-TNF non-responders when compared to responders (p=0.033). This gene encodes signal-regulatory protein B1, a receptor-type transmembrane glycoprotein known to be involved in the positive regulation of receptor tyrosine kinase-coupled signaling processes. After validation, combination with an additional, independent sample showed an association between the RNA expression levels of exon 5 and 6 of *SIRPB1* and anti-TNF treatment response in 76 patients (p=0.011), with good responders showing higher expression. The decrease in expression of the two exons is a direct result of two partly overlapping deletions in the *SIRPB1* gene. We observed no association of the deletions at the DNA level with response to treatment in a cohort of 523 RA patients treated with anti-TNF medication.

Conclusion. The RNA expression levels of exon 5 and 6 in the *SIRPB1* gene showed an association with anti-TNF treatment response in RA patients in a sample of 76 patients. However, at the DNA level no relation between the deletions in the gene and anti-TNF response was found in a larger sample of 523 patients. Further study is needed if the *SIRPB1* gene is involved in response to anti-TNF treatment.

> INTRODUCTION

Rheumatoid arthritis (RA) is a common multifactorial autoimmune disease, characterized by a chronic inflammation of the synovial joints with a prevalence of approximately 1 percent worldwide (1).

Tumor necrosis factor alpha (TNFα) is a key inflammatory mediator in RA (20). This cytokine binds to two receptors, the type 1 TNF receptor (p55) and the type 2 TNF receptor (p75) (49). Evidence for the essential role of TNFÐ in the pathogenesis of RA has resulted in the development of therapeutic interventions targeting this cytokine (25;322). Currently, three TNF antagonists, Infliximab (Remicade®), Adalumimab (Humira®) and Etanercept (Enbrel®) are commercially available and two new anti-TNF agents, golimumab (CNTO 148) and certolizumab pegol (Cimzia®), will most likely be approved in 2009. The molecular mechanisms for these TNF inhibitors are similar: they block the binding of TNFα to its cell-surface receptors and thereby limiting the signaling of TNFα induced pathways. Infliximab is a chimeric mouse-human antibody, while Adalimumab is a fully humanized antibody. Etanercept is a dimeric TNF receptor-IgG fusion protein and mimics the inhibition effects of soluble TNF receptor by binding to TNFα (66). The new anti-TNF agents golimumab and certolizumab pegol are a fully human monoclonal anti-TNF antibody and a humanized monoclonal anti-TNF antibody, respectively (26). Although the therapeutic effect of TNF neutralization is well established, patients show substantial heterogeneity in their response. Approximately 30 percent of the patients treated with TNF blocking agents do not show clinical improvement (28). Non-response, but also adverse events and the high costs of anti-TNF blocking agents, have motivated the search for genetic markers to predict treatment outcome. Insight into the genetics of anti-TNF therapy may facilitate the choice for the most appropriate therapy for an individual patient (42;213).

In the last few years, genome-wide gene expression analysis using microarrays has become a useful tool to help unravel disease etiology of various complex diseases and traits (292-295;323). Gene expression profiling of patients with RA has yielded specific sets of genes (genetic markers) associated with disease and has demonstrated molecularly distinct subgroups of RA patients (67;296-298). In addition, gene expression microarray technology can help to identify genes involved in treatment response or adverse events associated with a certain therapy (299-303;324;325).

Gene expression analysis of RA patients treated with anti-TNF has led to the identification of gene expression signatures which may discern responders from non-responders to treatment (67;73;189-192), although these studies were limited by small

sample sizes, so far. Nowadays it is also possible to monitor expression of individual exons in genome-wide expression studies. Differential expression of certain exons or exon clusters points to the presence of alternative spliced variants. Dysregulation of splice variants of specific genes might form a marker for therapy outcome (326).

In this study we used the GeneChip Human Exon 1.0 ST array to analyze expression levels of individual exons in white blood cell (WBC) derived RNA from 42 RA patients treated with the monoclonal anti-TNF antibodies infliximab or adalimumab, selected for either a good treatment response (n=18) or non-response (n=24) at 14 weeks of therapy. This analysis led to the identification of a differentially expressed exon cluster in the signal-regulatory protein B1 (*SIRPB1*) gene associated with anti-TNF treatment response. Quantitative PCR at the RNA level and multiplex ligation-dependent probe amplification (MLPA) analysis at the DNA level were used to further investigate this exon cluster and its potential role in the response to anti-TNF treatment.

> MATERIAL AND METHODS

Patients

Patients included in the study are all part of the Dutch Rheumatoid Arthritis Monitoring (DREAM) registry (www.dream-registry.nl) and attended the Department of Rheumatology of the Radboud University Nijmegen Medical Centre or the St. Maartenshospital in Nijmegen. The regional ethics review board had approved the study and all patients had given written informed consent. The DREAM registry collects detailed clinical information and regular measurements of treatment outcome in patients who start their first course of a TNF-blocking agent according to the indications in The Netherlands. These encompass a Disease Activity Score 28 (DAS28) > 3.2 at baseline and failure on at least two disease-modifying antirheumatic drugs (DMARDs), one of which has to be methotrexate (MTX)) (219). All patients in this study fulfilled the American College of Rheumatology (ACR) criteria (9). In the current RNA expression study, we included 18 good responders and 24 non-responders after 14 weeks of anti-TNF therapy (Infliximab or Adalimumab) according to the EULAR criteria (16). The patients were matched for age, sex, and MTX. These had been selected from a total sample of 100 patients for whom RNA had been collected just before anti-TNF treatment start and after 14 weeks of treatment. The replication sample consisted of 34 RA patients from the Autoimmune Biomarkers Collaborative Network (ABCoN) cohort which is previously described (66).

After finding an association between *SIRPB1* deletions and anti-TNF response at the RNA level, a total of 523 patients were genotyped using MLPA analysis at the DNA level.

Molecular analyses

All molecular analyses were performed in a CCKL (Coördinatie Commissie ter bevordering van de Kwaliteitsbeheersing van het Laboratoriumonderzoek) -accredited laboratory at the Department of Human Genetics at the Radboud University Nijmegen Medical Centre. RNA was isolated within 0.5 hours after blood extraction using the RNeasy midi kit according to the manufacturer's protocol (Qiagen Benelux B.V. Venlo, The Netherlands). To remove residual traces of genomic DNA, the RNA was treated with DNase I (Invitrogen, Leek, The Netherlands) while bound to the RNeasy column. Quality and quantity of the purified RNA was controlled using a NanoDrop spectrophotometer (Nanodrop Technologies, Montchanin, DE, USA). RNA integrity was investigated by using the 2100 Bioanalyser (Agilent technolo-

gies, Philadelphia, PA, USA). RNA was examined for possible degradation using agarose gel electrophoresis. DNA isolation was performed as described by Miller *et al.* (206).

(A) ***Gene expression profiling***

Gene expression profiling was performed using Affymetrix 1.0 Human Exon ST arrays, representing all known exon clusters. RNA isolated from blood taken before anti-TNF treatment start was analyzed. Performance was according to the manufacturer's instructions (Affymetrix Inc., Santa Clara, CA, USA). Briefly, the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay was used to generate amplified and biotinylated sense-strand DNA targets from the expressed genome (2.0 µg of total RNA). Arrays were hybridized by rotating them at 60 rpm in the Affymetrix GeneChip hybridization oven at 45 ºC for 17 hours. After hybridization and washing in the Affymetrix GeneChip Fluidics station FS 450, arrays were scanned using the Affymetrix GeneChip scanner 3000 7G system.

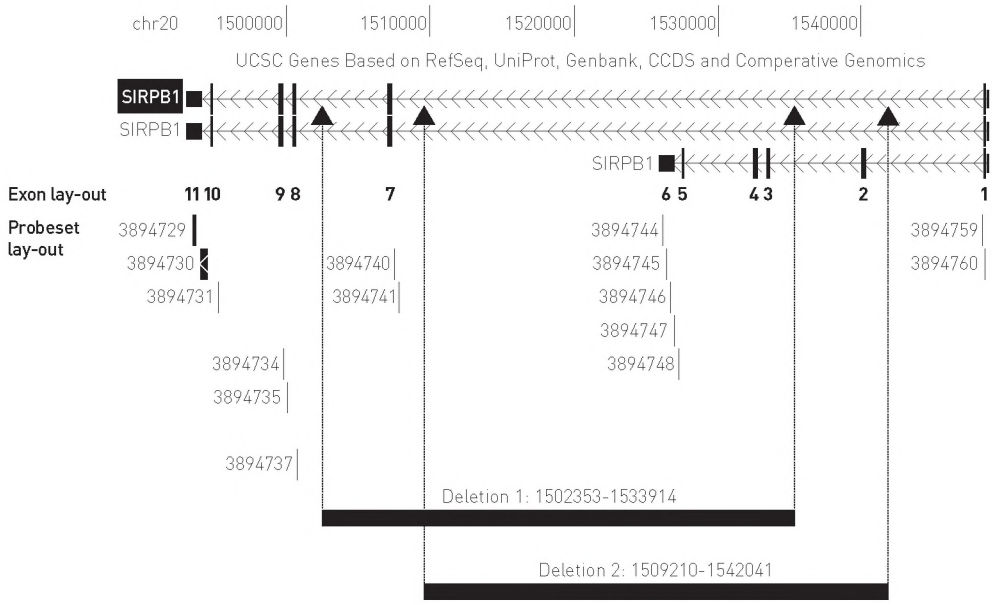
(B) ***Synthesis of cDNA and quantitative PCR***

Total RNA (1 µg) was reverse transcribed using a commercially available cDNA synthesis kit (iScript, BioRad Laboratories, Hercules, CA, USA). Primers were developed for *SIRPB1* exon 1-2/1-7, exon 5-6 and exon 10-11 using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Table 1, Figure 1). All primer pairs spanned an exon-intron boundary, to ensure amplification of RNA exclusively. The first primer pair, annealing to either exon 1 and 2 or exon 1 and 7 (Figure 1), was able to detect all three *SIRPB1* splice variants. Primers for *SIRPB1* exon 1-2/1-7 and exon 10-11 were used as positive controls to detect expression of the specific splice variants (Figure 1) (327). The general housekeeping gene *GUSB* (glucuronidase beta) showing a stable expression in leukocytes was used as endogenous control (328). All PCR products were selected to be between 80 and 120 bp long. Quantitative PCR (q-PCR) was performed by SYBR Green-based quantification according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The expression levels of specific *SIRPB1* exons were analyzed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster city, Ca, USA) according to the following protocol: 3 min. denaturation at 95°C, and 40 cycles of 30 sec. denaturation at 95°C, 30 sec. annealing and extension at 60°C. All primer pairs were validated in triplicate using serial cDNA dilutions that resulted in a final concentration of the equivalent of 800, 400, 200, 100 and 50 pg/µl input of total RNA in the first strand synthesis. Primer pairs that were 100Ð10% efficient (which implies a doubling of PCR product in each cycle) were used to quantify mRNA levels.

Table 1 / Primer sequences *SIRPB1* used for qPCR

Location	Size (bp)	Forward sequence	Reverse sequence
Exon 1-2/1-7	114	TCCTGCTGATGACGCTACTG	GAGTGGCCGACTCTCCAG
Exon 5-6	104	TGTCATCTATGTCTACTGGAAGCAG	ATTTCCTGGGAGGCTGTTG
Exon 10-11	103	CTCTGCCATCTACATCTGCTG	TCGCCAGCTCCTTCTCTC

Figure 1 / Schematic overview of *SIRPB1* as indicated in the UCSC Genome Browser (http://genome.ucsc.edu/)



Exon organization is as well as the GeneChip Human Exon 1.0 ST array probesets layout in *SIRPB1* are depicted in the figure. Three transcripts of the gene are known, all with different exon organizations. Also two overlapping deletions as described in literature are depicted

Table 2 / Primer sequences *SIRPB1* used for MLPA

Location	Size (bp)	5' probe (5'→3')	3' probe (5'→3')
Exon 1	100	CAGCACAGACGTTTGGACAGAGCAGGCTC	CTAAGGTCTCCAGAATGCCCGTGCCAGCC
Exon 2 and 7	112	GAGCAGAGCTGCATCATCTCAGAATGTCCCTCACCTCAGGA	AAGAGGTCCCACAAGTGGGTGACTTAGGG
Exon 4	96	GACCACGCCCCCAGGTGACCATGAGTG	GTCAAAGCAAGGGCCTCCTTTGGGTCC
Exon 5	108	GCAGAAGGCCTGACTGTAAGTGTGGGGGA	TGGATGCCTGACCAAGGGCTGCTCCAAAAGGGCAGGG
Exon 6	88	ATGGCCCCTTCCTCCCACAGCAA	CTTCCCTCCTGGGACATCATGGC
Exon 7a	92	CCCTGATCCCTGTGGGGCCCATCAT	GTGGTTTAGAGGAGCTGGAGCAGGC
Exon 7b	92	GGAGAGTCGGCCACTCTGCGCTGTG	CTATGACGTCCCTGATCCCTGTGGG
Exon 9	104	GGATGCCTTCTGTGACGTGCTGCATCAGTGG	CATGGGAGCCTAGTGAGAGACTGTGCCCTAG
Exon 10 and 11	124	CCCAGGGAGTGTGCAGGAGTTGGGATGGTCAAGGGTATTAC	AGGAGGTGGGATGCTTCAAGGGGGTTCAAAGTTGTCCCCAG

(C) ***Multiplex ligation probe analysis (MLPA)***

Probes were designed within or in close proximity of the exons of the SIRPB1 gene (Table 2). Quality control and reference probes were selected outside the region. To ensure specific hybridization, the BLAT program from the UCSC website (http://genome.ucsc.edu) was used to exclude the presence of repetitive sequences (329). Due to high sequence similarity of exons 2 and 7 as well as exons 10 and 11 of the *SIRPB1* gene it was not possible to develop specific probes for these exons (Table 2). The hybridizing regions of the probes had a Tm of at least 67.5oC and GC content between 40 and 60 percent. Both aspects were defined by the use of MELTingeny 1.0 software (Ingeny International, Goes, The Netherlands). Each end of the designed probes contained universal primer sequences, which have been described before (330), allowing simultaneous probe amplification with one pair of primers. Probes within each set were designed to produce PCR products ranging in size from 88 bp to 136 bp with a size separation of at least 4 bp. Probes were designed using the MELTingeny 1.0 software and ordered from Biolegio (Nijmegen, The Netherlands). MLPA reaction reagents were ordered from MRC-Holland (Amsterdam, The Netherlands). For each reaction, 200 ng of genomic DNA was heated to 98°C for 5 minutes. The DNA was cooled down to room temperature and subsequently 1.5 µl probe mix (4 fmol/µl for each probe set) and SALSA hybridization buffer were added to each sample. The mix was denaturated at 97°C for 1 minute, followed by hybridization for at least 16 hours at 60°C. Next, ligation was performed at 54°C for 15 minutes, followed by heat inactivation at 98°C for 3 minutes.

PCR amplification was carried out for 30 cycles and 1 µl of product from each PCR reaction was mixed with 8.5 µl formamide and 0.5 µl LIZ500 as a size standard (Applied Biosystems). Product separation was performed using capillary electrophoresis on an ABI 3730 sequencer (Applied Biosystems). For analysis, data were retrieved using Genemapper software following the manufacturer's protocol (Applied Biosystems). These data were exported to an Excel worksheet for further analysis (331).

Statistical analysis

(A) *Data extraction and statistical analysis of expression arrays*

For quality control, the Affymetrix CEL-files produced by the Affymetrix station were first imported into Affymetrix Expression Console version 1.1 where control probes were extracted and normalized using the default Robust Multichip Average (RMA). The Area Under the Curve (AUC) of the Receiver Operator Characteristic (ROC) was calculated using the positive and negative control probes present on the Affymetrix expression array. All arrays had an AUC score above the empirically defined threshold of 0.85, indicating a good separation of the positive controls from the negative controls. Furthermore spike-in control probes and intensity histograms for all arrays were checked. Pearson correlation between arrays showed no outliers. Subsequently, the CEL-files were imported into Partek® (Partek® Genomic Suite software, version 6.4 Copyright © 2008 Partek Inc., St. Louis, MO, USA), where only core probesets were extracted and normalized using the RMA algorithm with GC background correction. GC RMA is an extension of the RMA algorithm that incorporates some mismatched probe information by using physical models of nonspecific hybridization based on the GC-content of the probes. Core transcript summaries were calculated using the mean intensities of the corresponding probesets, representing the quantitative expression levels of all genes. We performed a mixed model analysis of variance (ANOVA) on the log2 probe intensities (representing the exon expression intensities) using the EULAR response criteria after 3 months of treatment. Variance components indicated that the scan date of the samples influenced the expression profiles and this was included in the model as a covariate. A p-value of <0.01 was considered as a statistically significant difference in probe expression level. Alternative splicing (AS) analysis was performed using the RMA normalized intensity values of the core probesets. For each transcript we used the mean of the corresponding probeset intensities. Transcripts with mean expression across the different groups lower than 70 were removed. To decrease the number of false positives, probesets with Detection Above Background (DABG) p-value above 0.01 were removed as were probesets of which the expression showed a five-fold difference compared to the expression of the corresponding

transcript. After filtering we calculated for each probeset a normalized value by dividing its intensity through the intensity of the transcript. For both sample groups (anti-TNF responders and anti-TNF non-responders) we compared the mean normalized probeset intensities using an ANOVA and corrected the resulting p-values using Benjamini-Hochberg correction for multiple testing (332). In addition we calculated for each combination of groups the splicing index (SI) of the probeset which is defined as the log2 of the ratio of normalized probeset intensities. The maximum splicing index was used as selection criterion for the sample group that contains alternatively spliced probesets. Only probesets with a maximum SI above 1.0 and a corrected ANOVA p-value below 0.0001 were considered as candidate genes showing alternative splicing. Finally, candidate probesets were grouped by their corresponding transcript and for each transcript we calculated the number of candidate probesets showing alternative splicing, the average maximum SI, the average corrected ANOVA p–value, and the most occurring group with the maximum SI.

(B) ***Analysis of quantitative PCR***

Threshold cycle numbers (referred to as Ct) were obtained using the 7500 System SDS software version 1.4 (Applied Biosystems). All samples were measured twice and duplicate samples with a standard deviation (SD) larger than 0.5 were excluded from the analysis. The relative quantity (RQ) of the gene-specific mRNA was calculated from the average value of the ΔCt (target gene Ct – endogenous control gene Ct) for each of the analyzed samples. Differences in expression between two samples were calculated by the $2^{\Delta\Delta Ct}$ method (241;333) and tested for association with anti-TNF treatment outcome using the independent Student's t-test. A p-value of <0.05 was considered statistically significant. Analyses were performed using SPSS for Windows, version 14.0 (SPSS, Chicago, IL, USA).

(C) ***Analysis of MLPA analysis***

To assess the effect of the deletions on anti-TNF response, a two-tailed Fisher's exact test was performed (SPSS for Windows, version 14.0). The patients were subdivided into two groups based on their genotype. The groups consisted of (1) patients showing deletions of exon 5 and 6 on both alleles (n=309) and (2) patients either heterozygous or homozygous for the absence of the deletion (n=214).

> RESULTS

RNA taken before treatment start (=baseline) of a total of 42 RA patients treated with anti-TNF (infliximab or adalimumab) (18 good responders and 24 non-responders at 14 weeks of treatment) were analyzed on GeneChip Human Exon 1.0 ST arrays to obtain genome-wide exon expression profiles. Table 3 shows patient characteristics at baseline and after 14 weeks of treatment.

Table 3 / Baseline characteristics, disease activity at baseline and DAS28 improvement for responders and non-responders (based on the EULAR criteria) to anti-TNF treatment

	Responders	**Non-responders**
N (baseline and 14 weeks follow-up)	18 (43%)	24 (57%)
Female gender	16 (89%)	14 (58%)
Age	58 ±14.2	57 ±13.6
RF positivity	13 (72%)	19 (79%)
Adalimumab	4 (27%)	11 (52%)
Infliximab	14 (73%)	13 (48%)
MTX as co-medication	18 (43%)	24 (57%)
DAS28 baseline	5.3 ±1.0	4.8 ±1.5
DAS28 decrease after 14 weeks of anti-TNF therapy	2.0 ±0.8	0.1 ±1.0
Percentage DAS28 change 14 weeks of anti-TNF therapy	37 ±10	-1 ±25

Results are number (percentage) or mean ± standard deviation (SD). Percentages are expressed in relation to the total number of patients for each response group (except for the total number of patients (N baseline and 14 weeks follow-up)).

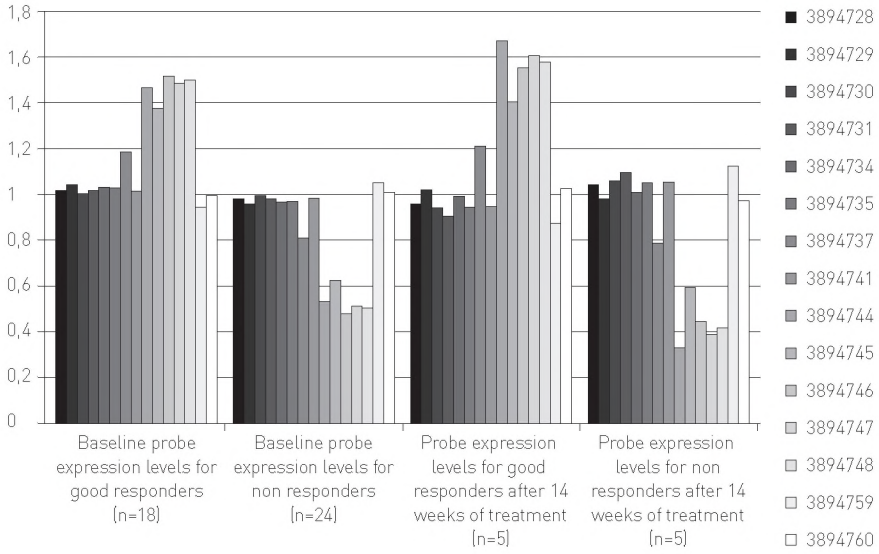
Expression array analysis

Individual probeset analysis revealed several genes that showed indications of alternative splicing based on the splicing index and the ANOVA. The gene with the highest splicing index (SI= 6.53) and most significant p-value (p= 0.000483371) was the *SIRPB1* gene (signal-regulatory protein beta 1; located on chromosome 20p13). *SIRPB1* is a member

of the signal-regulatory protein (SIRP) family and encompasses 11 exons (http://genome.ucsc.edu/). Figure 1 shows a schematic outline of the gene. Exon organization is illustrated as well as the GeneChip Human Exon 1.0 ST array probesets layout in *SIRPB1*. Three transcripts of the gene are known, all with different exon organizations. Transcript 1 consists of exon 1, 7, 8, 9, 10 and 11; transcript 2 consists of exon 1, 7, 10 and 11; transcript 3 consists of exon 1, 2, 3, 4, 5 and 6 (Figure 1). Five consecutive probesets located in exons 5 and 6 of *SIRPB1* (3894744, 3894745, 3894746, 3894747, 3894748) showed a decrease in mean expression in the array analysis in non-responders compared to responders (Figure 2).

Closer examination showed that other findings with high splicing indices and low p-values were either due to an exceptionally high splicing index in only one or two patients, or to highly variable expression levels between patients. These findings were not selected for follow-up.

Figure 2 / Normalized mean expression of probesets located in *SIRPB1*



SIRPB1 is covered by 15 probesets of the GeneChip Human Exon 1.0 ST array (for localization see Figure 1). Each probeset is indicated by specific color bar which corresponds to its unique probenumber outlined in the legend. Normalized mean expression values of each probeset is shown for 4 groups (anti-TNF responders and non-responders at baseline and after 14 weeks of treatment). Five consecutive probesets showed a decrease in mean expression in the non-responder groups when compared to the responders groups.

Quantitative PCR analysis of exon 5 and 6 of the SIRPB1 gene

Quantitative PCR (qPCR) was used to validate the lower expression of exon 5 and 6 of the *SIRPB1* gene observed on the array in anti-TNF non-responders. The qPCR analysis was first performed in the same 42 patients used for the initial array analysis, confirming the association found on the array (p=0.033, Table 4) and second in an independent sample of 34 patients. A combined analysis in all 76 patients also demonstrated an association between anti-TNF treatment response and the expression levels of exon 5 and 6 of *SIRPB1* (p= 0.011; Table 5), with those patients expressing the exons more likely to be good responders to anti-TNF therapy.

Table 4 / *SIRPB1* exon 5 and 6 expression distribution according to the EULAR response criteria; validation of the array results in 42 patients.

	Response to anti-TNF treatment		
	Good response	**Non-response**	**Total**
Expression of exon 5 and 6	10 (63%)	6 (37%)	16 (100%)
No expression of exon 5 and 6	8 (31%)	18 (69%)	26 (100%)
Total	18 (43%)	24 (57%)	42 (100%)

In the table the number of patients and percentage are presented. The Fisher exact test showed an association between the expression of exon 5 and 6 and anti-TNF treatment response (p=0.033).

Investigating the deletion in SIRPB1 at the DNA level using Multiplex ligation probe analysis (MLPA)

The complete absence of exon 5 and 6 expression detected by qPCR in 58% of the patients (Table 5) suggested a possible deletion at the DNA level. A recent publication (334) and information on copy number variation from the UCSC Genome Browser (http://genome.ucsc.edu/) showed the existence of two partially overlapping deletions in SIRPB1 (Figure 2). Hence, we investigated by MLPA if the reported DNA deletions were responsible for the absence of expression of *SIRPB1* exons 5 and 6 in our patients. MLPA analysis in the initial cohort of 42 patients showed that all 26 patients without expression of exon 5 and 6 at the RNA level indeed had a

homozygous deletion including exon 5 and 6 at the DNA level whereas all patients showing expression of exons 5 and 6 of the gene had at least one non-deleted allele. In the latter group the number of non-deleted alleles did not seem to influence the extent to which the exons were expressed. The MLPA results also confirmed the existence of two partly overlapping deletions located in the *SIRPB1* region as reported by Kidd and co-workers (334) (Figure 3).

In a sample of 523 RA patients we subsequently investigated by MLPA whether the deletion of exon 5 and 6 of *SIRPB1* was indeed associated with non-response to TNF neutralization (Table 6). The genotype distribution in this cohort is outlined in Table 7.

For this association analysis we categorized the patients into two genotype groups (Table 8); one including patients with exons 5 and 6 present (genotypes: homozygous for no deletions or heterozygous for either no deletion/deletion 1 or no deletion/deletion 2) and one with patients carrying homozygous deletions of exon 5 and 6 (genotypes: homozygous for deletion 1 or deletion 2 or compound-heterozygous for deletion 1/deletion 2). When evaluating the course of DAS28 change compared to baseline over the first year of anti-TNF treatment in the patient groups, we found that patients homozygous for the deletions showed a smaller change in DAS28 (expressed as percentage) 12 months after treatment initiation compared to patients that were either heterozygous did not show deletions (Figure 3). However, the observed difference was not statistically significant (p=0.329).

Table 5 / *SIRPB1* exon 5 and 6 expression distribution according to the EULAR response criteria in the combined cohort of 76 patients.

	Response to anti-TNF treatment		
	Good response	**Non-response**	**Total**
Expression of exon 5 and 6	21 (66%)	11 (34%)	32 (100%)
No expression of exon 5 and 6	16 (36%)	28 (64%)	44 (100%)
Total	37 (43%)	39 (57%)	76 (100%)

Fisher exact test, p=0.011.

Table 6 / Baseline characteristics, disease activity at baseline and DAS28 improvement for responders and non-responders to anti-TNF treatment (based on the EULAR criteria) included in the MLPA analysis.

	Responders	Non-responders
N (baseline and 14 weeks follow-up)	371 (71%)	152 (29%)
Female gender	257 (69%)	92 (61%)
Age	56 ±12.2	58 ±14.1
RF positivity	304 (82%)	123 (81%)
Adalimumab	167 (45%)	44 (29%)
Infliximab	145 (39%)	90 (59%)
Etanercept	59 (16%)	18 (12%)
MTX as co-medication	258 (70%)	97 (64%)
DAS28 baseline	5.6 ±1.13	5.1 ±1.54
DAS28 decrease after 14 weeks of anti-TNF therapy	2.0 ±0.88	-0.1 ±0.8
Percentage DAS28 change 14 weeks of anti-TNF therapy	37 ±14.5	-4.6% ±22

Results are number (percentage) or mean ± standard deviation (SD). Percentages are expressed in relation to the total number of patients for each response group (except for the total number of patients (N baseline and 14 weeks follow-up)).

Table 7 / Genotype percentages of the two deletions in *SIRPB1*

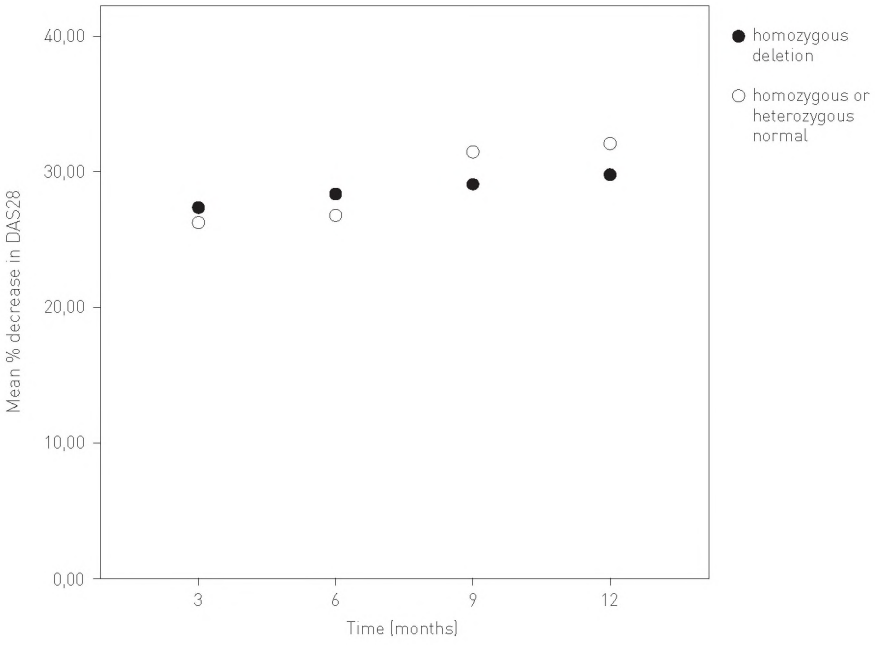
Genotype *SIRPB1*	Percentage
Normal/Normal	8%
Normal/Del 1	7%
Normal/Del 2	26%
Del 1/Del 1	2%
Del 2/Del 1	17%
Del 2/Del 2	40%
Total	100%

Table 8 / Association analysis of the *SIRPB1* gene and anti-TNF treatment outcome.

	Response to anti-TNF treatment		
Genotype *SIRPB1*	**Response**	**Non-response**	**Total**
Normal/Normal	22 (56%)	18 (44%)	40 (100%)
Normal/Del 1	24 (68%)	11 (32%)	35 (100%)
Normal/Del 2	98 (71%)	40 (29%)	138 (100%)
Del 1/Del 1	8 (78%)	2 (22%)	10 (100%)
Del 2/Del 1	62 (70%)	27 (30%)	89 (100%)
Del 2/Del 2	156 (74%)	55 (26%)	211 (100%)
Total	371 (71%)	152 (29%)	523 (100%)

A Fisher exact test demonstrated no association between the *SIRPB1* genotype and anti-TNF treatment response (P=0.395). In the table the number of patients and percentage for each genotype group are depicted for the responders and non-responders to anti-TNF treatment.

Figure 3 / Course of percentage DAS change compared to baseline in all patients (n=523)



Percentage decrease in DAS28 at three, six, nine, and twelve months after treatment start for the patients homozygous for a deletion of exon 5 and 6 of *SIRPB1* and patients heterozygous or homozygous no deletion.

> DISCUSSION

In this study we aimed at identifying alternatively spliced genes with an effect on the response to TNF neutralization therapy in rheumatoid arthritis. In a genome-wide analysis, one gene showed particularly strong effects, with two exons being frequently repressed in a sample of 42 patients. Association testing revealed an association of the downregulation of these exons with non-response to anti-TNF. Combined analysis with an additional 34 patients from an independent cohort confirmed this association. Downregulation was subsequently identified as being due to two overlapping deletions located in the *SIRPB1* gene, there was a perfect correlation between the presence of (homozygous or compound heterozygous) deletions and absence of transcription of the exons. MLPA analysis in much larger cohort of 523 anti-TNF treated patients for whom DNA and treatment response data were available revealed no significant association between (either of) the two deletions and anti-TNF response, though a trend for reduced response at prolonged treatment was observed in deletion carriers.

In addition to *SIRPB1*, two other members, *SIRPA* and *SIRPG*, have been characterized in humans (335;336). Similar to *SIRPB1*, *SIRPA* and *SIRPG* have three Ig-like domains in their extracellular regions. The structures of their cytoplasmic regions however, are quite different. *SIRPB1* has a very short cytoplasmic region and lacks signaling motifs. The protein associates with DAP12 (which is a demeric adaptor protein containing an immunoreceptor tyrosine-based activation motif (ITAM)), through a basic amino acid residue in the transmembrane region. *SIRPA* has a longer cytoplasmic region which consists of several putative tyrosine phosphorylation sites. Also *SIRPG* has a short cytoplasmic region, but it lacks a charged amino acid residue in its transmembrane region and therefore does not associated with DAP12 (336). The *SIRP* family might be an interesting candidate family for further study in RA and other autoimmune related disorders. Both *SIRPA* and *SIRPG* are expressed by T-cells and binds CD47 molecules. CD47 molecules are known to be involved with several autoimmune associated cell functions such as integration, migration, phagocytosis, T cell activation, anergy and appotosis (337). Olsson and co-workers suggested that the CD47-SIRPA interaction is important in the induction of autoimmune disorders such as autoimmune hemolytic anemia (AIHA) (338). Furthermore, in the study of Kawasaki *et al*. it is stated that CD47-SIRPG induced T-cell signaling is increased during the active stages of systemic lupus erythematosus (SLE), indicating that this signaling pathway is involved in SLE (339). However, the ligand for *SIRPB1* is (still) unknown, making it difficult to unravel its precise functions.

Based upon our initial results that *SIRPB1* is involved in anti-TNF response and on literature concerning the *SIRP* family, *SIRPB1* is an interesting candidate gene for further study. Unfortunately, we were not able to confirm our initial results with MLPA in a cohort of 523 RA patients. Possible explanations for not confirming SIRPB1 as a marker for anti-TNF response might be heterogeneity in both the disease itself and its response to therapy. Therefore, *SIRPB1* might be responsible for anti-TNF non-response in a subset of patients. For example, the patients that were investigated at the level of RNA was only a small subgroup of the patient cohort that was investigated at the DNA level. Subtle differences in disease characteristics (CRP, anti-CCP, DAS28, joint damage) or co-medication between the small RNA cohort and the larger DNA cohort might be responsible for not confirming *SIRPB1* as a marker. Investigation of the deletions located in the *SIRPB1* gene in larger, well characterized RA patient cohorts with longer follow-up is needed to unravel the precise role of *SIRPB1* in anti-TNF response. Since we were able to show a trend towards lower anti-TNF response rates in patients with a deletion after a longer time of treatment (12 months), it is possible that *SIRPB1* is a marker for response after a longer period of treatment.

Given the equivocal findings of our association studies and the potential functional implications of the deletion for *SIRPB1* protein function, larger association studies should be performed before the *SIRPB1* deletions are discarded as candidate biomarkers for anti-TNF response.

> CHAPTER 10

GENERAL DISCUSSION

The main goal of the studies presented in this thesis was the identification of genetic markers which are able to predict response to anti-tumour necrosis factor (anti-TNF) treatment in patients with rheumatoid arthritis (RA). To do so, we used both a hypothesis-driven and a non-hypothesis driven approach. The hypothesis-driven approach is presented in the first part of this thesis. Here, we investigated whether several polymorphisms located in candidate genes are involved either in the response to anti-TNF therapy (*TNFRSF1B* and *TNFA*) and/or in RA disease severity (*HMOX1*, *TNFRSF1B*, and *TNFA*) We investigated both treatment outcome and disease severity as the anti-TNF therapy target TNF plays a pivotal role in the pathogenesis of RA and it is expected that the same genes might be involved in both processes (42;53). The results of the non-hypothesis-driven approach are shown in the second part of this thesis. Whole genome gene expression profiling of RA patients was performed prior to their first anti-TNF administration in order to identify new genes/expression profiles involved in anti-TNF response. Both the hypothesis and non-hypothesis approach will be discussed in this chapter.

Our aims directed to identify genetic markers predictive for anti-TNF response are hampered by several difficulties. RA disease severity and response to anti-TNF treatment might, like RA itself, be multifactorial events. In such case, the genetic dissection of a complex phenotype is as difficult as defining the genetic background of complex diseases. Studies directed to the identification of genetic factors associated with a complex trait often have a low power as the trait is influenced by several genetic and non-genetic (environmental) factors, with each of those having probably small individual effects. In addition, disease severity and treatment response can be defined at several time-points using diverse outcome measures making comparisons between studies difficult. Therefore, comparison between studies is often hampered by differences in ethnicity (differences between genetic backgrounds) between the investigated patient cohorts. These difficulties may lead to contradictory results between studies resulting in failure to validate previous results in independent patient cohorts. These difficulties will be addressed in this chapter.

> CANDIDATE GENE STUDIES (HYPOTHESIS-DRIVEN APPROACH)

The focus of previous anti-TNF pharmacogenetic studies has been mainly on (functional) polymorphisms located in candidate genes (43-45;49;50;52-54;56;57;59;60;114;156;159;160;234;245). The latter encompass genes involved in the pathogenesis of RA, implicated in the mechanisms of action of TNFα or involved in the breakdown and clearance of anti-TNF agents. Such studies have not resulted in the identification of genetic markers that can predict anti-TNF treatment outcome with high sensitivity and specificity. As shown in chapter 2, the literature on these candidate gene studies is plagued by contradictory results (42). This can be related to the use of different ethnic groups or different outcome definitions, but most often seems due to power problems since the patient cohorts studied are generally very small (44;50;53-57;59-63;234;245;247;340). One way to draw more definite conclusions concerning the association of a genetic variant with anti-TNF response is combining studies in a meta-analysis, as shown in chapter 6 of this thesis. The polymorphisms investigated in this thesis, have a (possible) effect on receptor shedding (*TNFRSF1b*) and the degree of gene expression (*TNFA*, *HMOX1*). In case that a (supposed) functional polymorphism is associated with outcome, in our case the *TNFA* and *HMOX1* genes in relation to disease severity, it is still possible that the effect is not caused by the polymorphism itself but by *another* polymorphism which is in linkage disequilibrium (LD) with the former. LD describes a situation in which some combinations of alleles or genetic markers occur more or less frequently in a population than would be expected from a random formation of haplotypes from alleles based on their frequencies. The causal polymorphism co-segregates with the genotyped polymorphism located in the same haplotype. LD allows the identification of genetic variation without genotyping every single SNP in a chromosomal region (341). These representative SNPs in a region with high LD are often referred to as 'tag SNPs' (342;343). Even though LD based analysis in a certain candidate region has long served as a tool for human genetic research it became more generally applied in the analysis of complex traits after the elucidation of the LD structure of the human genome by the HapMap consortium (341). The last few years LD based analysis is also increasingly applied in pharmacogenetic research (344).

Although the hypothesis-based candidate gene approach is appealing, it does not account for the potential role of genes other than the obvious candidates, includ-

ing the genes of unknown function. Therefore, a candidate driven approach is limited by the existing knowledge about disease aetiology and modes of action of the therapeutic agent under study. An approach to (partially) solve this problem is the so called 'candidate pathway' approach. This method takes into account complete downstream and interacting signalling pathways of a candidate gene. Pathways of genes harbouring allelic variants may have more impact on drug response than one polymorphism in an individual gene, as several polymorphisms in networks of genes may interact. One study investigated genes located in the within the TNF receptor superfamily member 1B signalling pathway. They identified two SNPs located in the genes MAP3K1 and MAP3K14 to be associated with anti-TNF treatment response (345).

> GENOME WIDE ASSOCIATION STUDIES (NON-HYPOTHESIS DRIVEN APPROACH)

Since the present knowledge on the aetiology and course of RA and on the modes of action of new therapeutics like anti-TNF is still limited, more non-hypothesis-driven approaches ('fishing expeditions') seem also warranted. The best example of the latter are studies aimed to search for genes involved in complex traits in a genome-wide fashion, like a genome-wide association study (GWAS) (187). This technique makes it feasible to analyse hundreds of thousands of SNPs in one single experiment (39;64;65). Association is detected via a surrogate marker (tag SNP) in linkage disequilibrium with the true causal variant (which can be a SNP, copy number variant (CNV) or repeat-type polymorphism). A GWAS has the advantage of investigating many genes at the same time including those genes whose function in relation to the studied trait is not yet fully understood or recognized (346). The results of our expression profiling study in chapter 9 of this thesis suggest that such genes are important in anti-TNF response since the most significant finding is for an unknown gene. The fact that a GWAS can identify genes with an indirect or unknown link to a trait or disease is also illustrated by the recent study of The Wellcome Trust Case Control Consortium (187). These authors identified common genetic variants located in or nearby the genes MMEL1, ANAPC4, CRYL1 and the unknown gene AK_094492 to be associated with RA susceptibility (187). Limitations of the GWAS are the (still) relatively high costs and the large samples required to account for multiple testing in combination with small effect sizes of individual variants (347;348). A less expensive way to increase power is a meta-analyis in which relevant studies are combined as shown in the study of Raychaudhuri *et al*. (349). So far, one GWAS for identifying genetic markers for anti-TNF response was performed and a SNP located in interleukin 10 (*IL10*) was identified as a (weak) marker for anti-TNF response (66). However, the number of patients analyzed is small.

As already mentioned, many genetic variants are likely to have a role in therapy response and the contribution of each single polymorphism is probably modest and therefore difficult to identify. For these reasons, it is of utmost importance for candidate gene based as well as GWAS to investigate very large sample sizes and that those patient cohorts are well-characterized for demographics, disease characteristics and therapy response at baseline and during follow up. Large samples increase the study power and a thorough patient characterization allows better patient stratification, increasing the chance of identifying true associations (350).

Nonetheless, the candidate gene as well as the GWAS are only the first steps in identifying genetic markers/genes for anti-TNF response. Especially results from GWAS, studies need not only to be replicated in additional, independent patient samples to guarantee their validity and generalizability (351) but also followed up by more focused studies searching for the causal variants in the region of interest. The identification of causal variants is of particular interest to gain more insight in the mechanisms underlying anti-TNF treatment response.

> GENE EXPRESSION STUDIES AND COMBINATION WITH OTHER 'OMICS' STRATEGIES (NON-HYPOTHESIS DRIVEN APPROACH)

Another non-hypothesis driven approach to search for genes involved in anti-TNF treatment outcome is the use of genome-wide gene expression studies. During the last decade, gene expression profiling has emerged as a key tool in the study of various complex diseases and traits (292-295;323). Several studies focusing on RA identified gene expression signatures predicting the response to anti-TNF treatment in patients with RA (73;189-192). Quite a few of the identified genes did not only have a (potential) role in anti-TNF response but are also candidate genes for RA severity, backing up the hypothesis that (a subset of) the same genes are involved in both disease severity and anti-TNF treatment. Unfortunately there is little overlap between the expression signatures reported in different studies, as yet, probably for the same reasons discussed above for the gene polymorphism analyses.

Especially in microarray experiments there is an increased chance for false positives because of the multiplicity problem: thousands of hypotheses (in this case genes) are tested simultaneously for their association with anti-TNF response. Therefore there is a need to correct for multiple testing when assessing the statistical significance of findings. Due to the high costs and the time consuming procedure to collect sufficient samples in a standardized manner, microarray experiments are often underpowered. When multiple testing correction is applied in these underpowered studies, not a single gene or polymorphism will be identified as true positive. Yet, this will not automatically mean that the results obtained in these studies are not informative and there is reason for debate. The biological functions and mechanisms underlying the identified genes and polymorphisms are often involved in the mechanisms underlying the investigated complex trait (67;192;256). Therefore, the last few years a shift is observed in the interpretation of microarray experiment results. The focus is no longer on multiple testing correction but on validation of the exploratory results in independent patient cohorts. In chapter 8 we show that some of the previously reported expression signatures are able to provide a reasonable classification of anti-TNF responders and non-responders in an independent patient cohort. This clearly shows that validation of exploratory expression signatures is possible and that these signatures are useful to predict anti-TNF treatment outcome.

In addition to gene expression profiling, studies on protein profiles –so-called proteomics- are also of interest (193-198;352;353). For example, a sustained increase of soluble CD30 levels has been recently reported to be associated with non-response to anti-TNF therapy (353). Integration of the results of the above described techniques (candidate gene analysis, GWAS, expression profiling) with those of an analysis at the level of the proteome (two dimensional gel electrophoresis, surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF), clinical markers (rheumatoid factor positivity, C-reactive protein, anti-CCP) and important environmental factors is probably the most powerful way to identify markers for anti-TNF therapy outcome.

> STRENGTHS AND LIMITATIONS OF THE STUDIES PRESENTED IN THIS THESIS

In light of our results, we have to conclude that since the start of our pharmacogenomic research five years ago, we were not able to identify a genetic marker for anti-TNF response with high sensitivity and specificity so far. To identify such markers, both a hypothesis as non-hypothesis driven strategy was used. One could argue whether the right patient cohorts and strategies were used in these studies and if the right hypotheses were made. To draw sensible conclusions from an association study, it is of utmost importance that the investigated patient cohort is well characterized regarding disease activity/severity, response anti-TNF medication, response to co-medication and clinical markers such as rheumatoid factor positivity, anti-CCP and C-reactive protein. A well-characterized cohort makes it possible to correct for non genetic confounders in the analysis. The patient cohort used for our pharmacogenomic studies was derived from the DREAM registry (www.dreamregistry.nl), in which many disease characteristics are monitored. In our analysis also these clinical parameters were integrated, however they did not have any effect on the results.

One of the two strategies we used to identify genetic variants associated with anti-TNF outcome was a hypothesis driven candidate gene strategy. Based upon the pharmacogenomic knowledge from five years ago, this strategy was a good and affordable method to investigated (possible) associations between genetic variants and response to medication. For example, one of the earliest clinically accepted pharmacogenetic test available is used to determine different alleles of the gene coding for the enzyme thiopurine S-methyltransferase (*TPMT*), which has a important role in metabolizing thiopurine drugs. Also the cytochrome P-450 drug metabolizing enzyme cytochrome P-450 2DS (*CYP2D6*) represents an example of a successful pharmacogenomic candidate gene study. Polymorphisms in this gene showed a direct association with the ability of patients to oxidize or metabolize certain drugs. Both the *TPMT* and the *CYP2D6* genes were obvious candidate genes, seen their direct interactions with the investigated drugs. Both pharmacogenomics tests are still used in a daily clinical setting.

Seen these results in pharmacogenomic research in which the hypothesis-driven candidate gene strategy was applied, it was common sense to select this approach as one of the strategies to investigate the pharmacogenomics of anti-TNF treatment.

Unfortunately we have not been able to identify genetic variants in the genes investigated. It might be possible that other variants in the investigated genes explain the differences in response to anti-TNF medication or we simply investigated the wrong candidate genes. A genome-wide association study allowing the investigation of the complete genome in a non-biased manner would be the best approach to circumvent the candidate gene selection.

In our non-hypothesis (genome-wide) approach directed to the identification of differences in gene expression levels between responders and non-responders to anti-TNF treatment we only identified genes with small differences in expression between the two groups (fold changes <2). Replication studies including sufficient patients are needed to be able to draw definite conclusions concerning the association of these genes with anti-TNF outcome. However we have been able to validate existing gene expression profiles with a relatively good negative and positive predictive value suggesting that the method we used is suitable to identify biomarkers predicting anti-TNF treatment outcome. In the study of Acharya and co-workers it is demonstrated that the genome-wide approach is able to successful identifying genetic markers which can be used for optimizing individual therapeutic strategies (354). This study uses genome-wide expression profiling to demonstrate the value of integrating genomic information with clinical and pathological risk factors, to refine prognosis, and to improve therapeutic strategies for early stage breast cancer. They conclude that incorporation of gene expression signatures into clinical risk stratification can refine prognosis and therapeutic strategies (354).

All data generated so far suggests that anti-TNF (non-)response is a more complex phenomenon as was anticipated five years ago. It is expected that the genetic variants involved in non-response to anti-TNF therapy have small effect sizes instead of the effect sizes seen in the pharmacogenetic tests that are used in daily practice nowadays. Drugs can be metabolized by a different sets of enzymes, transported by different types of protein carriers and interact with one or multiple targets. Most drug effects are polygenic, meaning that the drug processing pathway (metabolism, transportation, target interaction) displays genetic variation between patients. Clear-cut frequency distributions as observed for tests used in daily practice would be replaced by multiple overlapping distributions, thereby obscuring the relationship between drug and target. To identify these genetic variants it is of utmost importance to investigate large preferably homogeneous patient cohorts. Our patient population might have been underpowered to detect these small effects in the genes we investigated. As a result, it is far more difficult to identify genes which are associated with anti-TNF therapy response and consortiums with access to large well characterized populations are needed to tackle the problem of anti-TNF non-response.

To conclude, the investigated patient population is very well characterized and therefore it is (probably) not the reason for not identifying genetic marker for anti-TNF (non-)response. It is more likely that the effect sizes for the investigated SNPs are very small and that the patient cohort has insufficient power to detect them. It is also possible that the wrong candidate genes are investigated. Ways to improve power are: 1) including more patients and 2) improve follow-up which will generate possibilities to do extensive longitudinal analyses.

> TOWARDS PERSONALIZED MEDICINE?

As mentioned above, it is unlikely that a single genetic variant will predict anti-TNF treatment outcome with sufficient sensitivity and specificity. It seems more likely that a set of genes and genetic variants will together predict anti-TNF treatment response. In this respect it is important to take additive effects as well as interactions between genetic polymorphisms into consideration in pharmacogenetic studies. Besides these gene-gene interactions, individual disease course and treatment response is also influenced by gene-environment interactions. For example, a prominent gene-environment interaction was identified between smoking and the HLA-DRB1 shared epitope (SE) alleles (355-357). Källberg and co-workers investigated in a recent study the interaction between the *HLA-DRB1* alleles and another environmental factor: alcohol consumption. They reported that alcohol consumption was dose-dependently associated with reduced risk of RA in the investigated patient cohorts. The absolute risk reduction associated with alcohol consumption was more pronounced among smokers carrying one or two of the *HLA-DRB1* SE alleles (358). Since gene-gene and gene-environment interactions are also involved in therapy response, the main goal in pharmacogenomic research is to incorporate genomic information with demographic and environmental covariates to obtain a genotype-to-phenotype map which can be used to personalize a treatment regimen for a disease. Such a multivariate analysis for therapy response in RA is elegantly shown in the study of Wessels and colleagues (359). In this study, a clinical pharmacogenetic model was developed which can be used to predict methotrexate (MTX) response in patients with RA. This model included four polymorphism located in MTX response candidate genes (*AMPD1*, *ATIC*, *ITPA* and *MTHFD1*) together with sex, rheumatoid factor, smoking status and the disease activity score (DAS). Using this model, 60% percent of the patients were categorized as either a MTX responder or non-responder, whereas only 32% of the patients were categorized when a non-genetic model was applied. They conclude from this comparison that pharmacogenetic is of great value for predicting response to MTX (359). I suggest that such a pharmacogenetic model is also developed for anti-TNF treatment. At this moment it is too early to point at the specific DNA variants that should be included in this model. However, in the next few years these will be identified by large international GWAS studies. Potentially important phenotypic and environmental factors that need to be taken into account regarding anti-TNF response are the formation of antibodies directed against anti-TNF agents and smoking. Some studies suggest that both may be associated with non-response to anti-TNF agents (199;200). The process of antibody

formation against biologicals is increasingly suggested to be one of the mechanisms of drug failure (360). Dose and frequency adjustments of anti-TNF therapy due to insufficient response are often applied in RA patients (29;361). In addition, many patients who fail to respond to the first anti-TNF agent, benefit from a switch to a second anti-TNF agent. This suggests a mechanism of neutralizing antibodies directed against the first used anti-TNF agent (362;363). Indeed, several studies showed a correlation between clinical response and the formation of anti-TNF antibodies (201;364-368). In the study of Hyrich and co-workers it is suggested that smoking is associated with anti-TNF non-response. They reported lower response rates among RA patients, particularly in patients receiving infliximab (200).

When such pharmacogenetic models are developed for several RA treatment strategies, it will increase the possibility to identify the best treatment regimen for each individual patient. Thereby, the identification of RA severity genes will make it possible to diagnose patients with rapid disease development in an early stage. This will result in a more aggressive and targeted treatment soon after diagnosis.

However, it is methodologically demanding to investigate the relationship between several (genetic and environmental) factors as these factors may be inter-related in varying degrees between RA patients. Therefore, completeness of information and non-biased recruitment of the study cohorts is crucial for the reliability of results.

> FUTURE RESEARCH PERSPECTIVE

Pharmacogenetic studies reported so far are only beginning to unravel the genetic complexity underlying the mechanisms of anti-TNF response. In the next few years the focus will shift from the hypothesis-driven pharmacogenomics approaches towards the non-hypothesis driven approaches in which the entire genome, transcriptome and proteome is screened for relevant variation associated with response to anti-TNF treatment. In addition, the technique of next generation sequencing (or resequencing) is very promising (369;370). This technique offers high throughput sequencing of the entire genome for relatively low costs, thereby providing information on literally every nucleotide in the genome. Within three to five years this technique will be standard for genomic screening of large patient cohorts. Genomic data from such screens (GWAS or next generation sequencing) will be combined with data obtained from transcriptomic and proteomic screens using sophisticated statistical methods for the identification of markers that can predict treatment outcome in a substantial part of the patients (371). However, these large genome-wide screens on the DNA, RNA and protein levels will only be successful if they are applied on large well-characterized cohorts. Therefore, multicenter pharmacogenomics collaborations should concentrate on collecting these well-defined patient cohorts, which provide sufficient statistical power to detect the supposedly small genetic effects associated with treatment outcome and/or disease course.

Although pharmacogenetic testing is yet in its early infancy in complex diseases, the proposed approaches will most certainly lead to the identification of markers for anti-TNF therapy response resulting in a more personalized treatment strategy. The application of 'tailor-made' personalized medicine in the daily clinic will be of great benefit to the individual patient.

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> SUMMARY

Rheumatoid arthritis (RA) is a common chronic inflammatory disease that may lead to severe joint destruction and disability. The exact cause of RA remains to be elucidated but it is generally accepted that both genetic and environmental factors play a role in initiation and outcome of the disease. Since there is no cure for RA, therapeutic intervention is focused on minimizing joint damage and functional loss. One of the most widely used treatment approaches for RA patients with active disease is tumour necrosis factor (TNF) neutralization. Anti-TNF treatment is highly effective, however, approximately 30% of the RA patients treated with TNF-blocking agents fail to show clinical improvement initially or loose response after short or longlasting therapy. Non-response, together with the occurrence of toxicities and the high costs of TNF-blocking agents have driven the search for genetic markers to predict treatment response, since a better insight into the pharmacogenetics of anti-TNF therapy may facilitate the choice for the most suitable medication and/or dosing regimen for an individual patient in daily clinical practice.

Goal of this thesis is the identification of genetic markers which are able to predict anti-TNF therapy outcome in patients with RA. Since it can be assumed that overlapping (inflammatory) genes have a critical role in both anti-TNF response and the pathogenesis of RA, we also aimed at identifying genetic markers associated with RA disease severity and radiologic joint damage.

This thesis consists of two parts. The first part focuses on the identification of polymorphisms responsible for anti-TNF (non-)response and disease severity in a hypothesis driven manner. Following a general introduction, chapter 2 gives an overview of the current literature concerning anti-TNF pharmacogenetics, as well as technologies currently in use for the identification of genetic markers associated with anti-TNF outcome. In chapter 3 to 6 three polymorphisms in candidate genes are investigated for their potential role in anti-TNF response and/or RA severity using two large patient cohorts. The cohort used to study anti-TNF response included patients from the Dutch Rheumatoid Arthritis Monitoring (DREAM) registry with detailed information on their response to anti-TNF therapy (infliximab and adalimumab). The RA severity cohort consisted of patients from a long-term observational early inception cohort who are regularly monitored for their disease activity and progression.

In chapter 3 it was investigated if a $(GT)_n$ repeat polymorphism in the heme oxygenase-1 (*HMOX1*) promoter is associated with RA susceptibility, disease severity and radiologic joint damage. Recently, it was demonstrated that HMOX-activity protects against the onset of RA and that individuals with short $(GT)_n$-repeats (SS; $n<25$) induce higher levels of HMOX-activity more rapidly than those with long repeats (LL; $n\geq25$). A

total of 325 well-characterized RA patients and 273 controls were investigated. No significant differences in genotype or allele frequency were found between RA patients and controls, but patients with the SS-genotype had a significantly more favorable radiologic outcome over nine years than those carrying the LL-genotype. These results suggest that the *HMOX1* gene is involved in RA disease severity.

In chapter 4 the effect of a functional polymorphism (676T>G, M196R) in the Tumor Necrosis Factor Receptor Super Family 1b (*TNFSF1B*) gene on disease activity, radiologic joint damage and response to infliximab and adalimumab therapy in patients with RA was investigated. For this study, DNA was available for 234 patients from the DREAM registry and for 248 patients from the early inception cohort. Analyses showed that the 676T>G polymorphism was not associated with response to anti-TNF medication after three or six months of therapy or with disease severity. These results suggest that the *TNFRSF1B* 676T>G polymorphism does not have a large impact on either anti-TNF response or disease severity in our population.

Next, the *TNFA* -308G>A promoter polymorphism and its relation to RA disease severity (chapter 5) and anti-TNF treatment (chapter 6) was investigated. For the analyses regarding disease severity and radiologic joint damage, a total of 208 patients from the early inception cohort were genotyped. To learn more about the mechanism behind the effect of the polymorphism, RNA of an additional 66 RA patients was used for *TNFA* gene expression analysis by quantitative PCR. The -308G allele was found associated with more radiologic joint damage progression compared with the A allele over 9 years of follow up. No differences in *TNFA* gene expression between alleles was observed. These data confirm that the *TNFA* -308G>A promoter polymorphism is associated with RA severity, but this does not seem to be mediated by expression differences between alleles. To investigate the *TNFA* -308G>A promoter polymorphism and its influence on anti-TNF response, a meta-analysis was performed combining data from 13 previously published studies and one unpublished dataset from the DREAM registry resulting in a patient cohort of 2829 patients. The two subclasses of anti-TNF treatment, i.e. monoclonal anti-TNF antibodies and TNF receptor antagonists were analyzed separately. No association was observed between the promoter polymorphism and response to treatment in this large patient cohort. Therefore, it was concluded that this polymorphism does not play a role in the response to anti-TNF treatment.

The second part of this thesis concentrates on the non-hypothesis driven gene discovery technique, whole genome expression profiling, to investigate anti-TNF response in RA patients at the transcriptional level. In chapter 7 a systematic literature search was performed and this revealed eight array-based expression profiling studies in RA patients. Two of those investigated the response to anti-TNF treatment, the other six were focused on studying differences in disease etiology. Results from these eight studies were compared

to those of linkage and genome-wide association (GWA) studies and two sets of genes were extracted: 1) those differentially expressed in more than one study, and 2) genes differentially expressed in at least one of the reviewed studies and present in RA linkage or GWA loci. Both sets of genes were identified as interesting candidate genes for further study in RA and potentially in anti-TNF treatment response. One of these genes was also identified in a genome-wide expression profiling studies focusing on RA susceptibility.

Subsequently, in chapter 8, a genome-wide expression profiling study was performed to identify gene expression signatures predicting anti-TNF therapy outcome in a patient cohort of 42 RA patients. First, the validity of two previously published gene sets predictive of anti-TNF treatment outcome was tested in our patient cohort. Analyses revealed a sensitivity of 71% and a specificity of 61% and 28%, respectively, for the two previously published gene sets. Secondly, whole-genome expression profiling identified a new predictive gene expression signature of 113 genes whose expression predicted therapy response with a sensitivity of 79% and a specificity of 84%. These 113 genes associated with anti-TNF treatment response point out to specific pathways for further study into the mechanisms of anti-TNF action. These results confirm that gene expression profiling prior to treatment is a useful tool to predict anti-TNF (non-)response.

Expression analysis at the level of exons also revealed that two adjacent exons in the *SIRPB1* gene were decreased in expression in the anti-TNF non-responders when compared to the responders. In chapter 9 the decrease in expression of these two exons in the *SIRPB1* gene was further investigated for its involvement in anti-TNF response. The decrease in expression was validated and replicated by Q-PCR in a combined cohort of 76 RA patients from the DREAM registry and the Autoimmune Bio-markers Collaborative Network (ABCoN) cohort. Further analysis revealed that the decrease in expression was a direct result of two partly overlapping deletions in the *SIRPB1* gene. However, no association was observed between the two DNA deletions and anti-TNF response in a larger cohort of 466 RA patients treated with anti-TNF medication. Despite the initial results, it was concluded that the observed association at the RNA level could not be confirmed at the DNA level and that the *SIRPB1* deletions are probably not associated with the response to anti-TNF treatment.

In the general discussion (chapter 10) the results of the research presented in this thesis are briefly summarized and placed in a broader perspective concerning pharmacogenetics and its role in the daily treatment of RA in a clinical setting. Several different approaches for pharmacogenetic research are discussed. Future studies should improve and integrate different approaches on multiple levels (DNA, RNA and protein expression) to identify those (genetic) markers responsible for therapy outcome in RA. Once identified and validated for their clinical use, these pharmacogenetic markers may be of great clinical benefit for personalized treatment in RA patients.

> NEDERLANDSE SAMENVATTING

Reumatoïde artritis is een veel voorkomende ontstekingsziekte die kan leiden tot ernstige gewrichtsschade en tot verlies van functie van de aangedane gewrichten. De oorzaak van RA is grotendeels onbekend maar het wordt algemeen aangenomen dat zowel genetische als omgevingsfactoren een rol spelen in het ontstaan en het verloop van de ziekte. RA kan niet worden genezen en daarom is de behandeling van de ziekte erop gericht om gewrichtsschade en functie verlies van de gewrichten zoveel mogelijk te beperken. Een van de meest toegepaste therapieën bij RA patiënten met een actief ziektebeeld is neutralisatie van tumor necrosis factor (TNF). Anti-TNF therapie is zeer succesvol, echter, ongeveer 30% van de behandelde RA patiënten reageert onvoldoende na de start van de behandeling of na enige tijd van behandelen. Gezien het percentage patienten dat onvoldoende reageert, samen met de bijwerkingen en hoge kosten van anti-TNF therapie, is het waardevol om in staat te zijn de uitkomst van anti-TNF therapie te kunnen voorspellen *voordat* daadwerkelijk met de therapie wordt begonnen. Het onderzoeksveld van de farmacogenetica richt zich op het identificeren van genetische variatie die verantwoordelijk is voor het verschillend reageren van patienten op medicatie. Beter inzicht in de farmacogenetica van anti-TNF therapie maakt het mogelijk om de keuze voor het type anti-TNF medicatie en hoogte van de dosering beter af te stemmen op de individuele patiënt.

Doel van dit proefschrift is het identificeren van genetische variatie die het mogelijk maakt om te voorspellen hoe RA patienten zullen gaan reageren op anti-TNF medicatie. Omdat wordt aangenomen dat de (inflammatoire) genen verantwoordelijk voor de response op anti-TNF medicatie en voor ziekte ernst/activiteit grotendeels overlappen, is dit proefschrift er ook gericht op het identificeren van genetische variatie verantwoordelijk voor ziekte ernst en radiologisch gewrichtsschade.

Dit proefschrift betaat uit twee delen. Het eerste deel richt zich op de identificatie van polymorfismen verantwoordelijk voor anti-TNF (non-)response op een hypothese gedreven manier. Na een algemene introductie wordt in hoofdtuk 2 een overzicht gegeven van de actuele literatuur betreffende anti-TNF farmacogenetica en de huidige technieken die gebruikt worden voor de identificatie van genetische variatie. In hoofdstuk 3 tot en met 6 worden achtereenvolgens drie polymorfismen in kandidaat genen onderzocht op hun (potentiële) rol in anti-TNF response en/of RA ziekte ernst. Voor deze studies is gebruikt gemaakt van twee grote RA patiënten cohorten.Voor het onderzoek naar anti-TNF response is gebruikt gemaakt van het Dutch Rheumatoid Arthritis Monitoring (DREAM) register, een cohort wat gedetailleerde informatie bevat

over de response van patiënten op anti-TNF medicatie (infliximab en adalimumab). Voor onderzoek naar RA ziekte ernst is gebruik gemaakt van een lange termijn observationeel cohort, waarin patiënten meteen naar de diagnose worden geïncludeerd en worden gevolgd met betrekking tot hun ziekte activiteit en ziekte ernst.

In hoofdstuk 3 wordt onderzocht of een $(GT)_n$ repeat polymorfisme in de heme oxygenase-1 (*HMOX1*) promoter is geassocieerd met RA gevoeligheid, ziekte activiteit en radiologische gewrichtsschade. Onlangs is aangetoond dat HMOX activiteit beschermd tegen het ontstaan van RA en dat individuen met korte $(GT)_n$ repeats (SS; $n<25$) sneller hogere niveau's van HMOX1 activiteit induceren dan individuen met lange repeats (LL; $n\geq25$). In totaal zijn 325 goed gekarakteriseerde RA patiënten en 273 controle personen bestudeerd. Er zijn geen significanten verschillen in genotype of allel frequenties gevonden tussen RA patiënten en controle personen, maar patiënten met een SS genotype hadden na negen jaar significant minder radiologische schade ontwikkeld dan patienten met een LL genotype. Deze resultaten suggereren dat het *HMOX1* gen betrokken is bij RA ziekte ernst.

In hoofdstuk 4 wordt het effect onderzocht van een functoneel polymorfisme (676T>G, M196R) in het Tumor Necrosis Factor Receptor Super Family 1b (*TNFRSF1B*) gen op ziekte activiteit, radiologische schade en de response op infliximab en adalimumab therapie in patiënten met RA. Voor deze studie was DNA beschikbaar van 234 patiënten uit het DREAM cohort en 248 patiënten uit het lange termijn observationeel cohort. Analyse laat zien dat het 676T>G polymorfisme niet is geassocieerd met response op anti-TNF medicatie na drie of zes maanden. Daarnaast is in deze studie het polymorfisme ook niet geassocieerd met RA ziekte ernst. Deze resultaten suggereren dat het *TNFRSF1B* 676T>G polymorfisme geen grote invloed heeft op zowel anti-TNF response als RA ziekte ernst in deze populatie.

Vervolgens is onderzocht of het *TNFA* -308G>A promoter polymorfisme is geassocieerd met RA ziekte ernst/radiologische schade (hoofdstuk 5) en response op anti-TNF medicatie (hoofdstuk 6). Voor de analyses met betrekking tot RA ziekte ernst en radiologische schade zijn 208 patiënten van het lange termijn obervationeel cohort gegenotypeerd. Om meer inzicht te krijgen in het mechanisme achter het effect van het polymorfisme, is RNA van 66 additionele patiënten gebruikt voor *TNFA* gen expressie analyse door middel van quantitative PCR. Het -308G allel bleek geassocieerd te zijn met een grotere toename in radiologische gewrichtsschade na negen jaar in vergelijking met het A allel. Geen verschillen in *TNFA* expressie tussen het G en A allel zijn waargenomen. Deze data bevestigen dat het *TNFA* -308G>A polymorfisme is geassocieerd met RA ziekte ernst, maar wordt waarschijnlijk niet gemedieerd door expressie verschillen tussen de allelen. Om het *TNFA* -308G>A polymorfisme te bestuderen in relatie tot anti-TNF response, is een meta-analyse uitgevoerd waarbij 13 eerder

gepubliceerde studies plus een ongepubliceerde dataset uit het DREAM cohort zijn samengevoegd. Dit resulteerde in een dataset van 2829 patiënten. De twee sub-klassen anti-TNF medicatie, monoclonale antilichamen en TNF receptor antagonisten, zijn apart geanalyseerd. Geen associatie is aangetoond tussen het promoter polymorfisme en de response op anti-TNF medicatie. Geconcludeerd is dat het polymorfisme geen rol speelt in de response op anti-TNF medicatie.

Het tweede gedeelte van dit proefschrift richt zich op de techniek van genoom-wijd expressie profilering voor het bestuderen van anti-TNF response in RA patiënten. Deze techniek maakt het mogelijk de response op anti-TNF medicatie te onderzoeken op een niet-hypothese gedreven manier. In hoofdstuk 7 zijn, met behulp van een systematische literatuur analyse, acht expression profiling studies met RA patiënten geïdentificeerd. Twee van deze studies hebben de response op anti-TNF medicatie bestudeerd, de andere zes hebben zich gericht op verschillen in RA ziekte etiologie. De resultaten uit deze acht studies zijn vergeleken met resultaten uit linkage en genoom-wijde associatie (GWA) studies en hieruit zijn twee sets van genen naar voren gekomen: 1) genen die differentieel tot expressie kwamen in meer dan één studie, en 2) genen die differentieel tot expressie kwamen in minstens één van de acht studies en die voorkomen in linkage of (GWA) loci. Beide genensets zijn interessante kandidaat genen voor verdere studie naar RA en de response op anti-TNF medicatie.

In hoofdstuk 8 wordt met behulp van genoom-wijde expressie profilerings in 42 RA patiënten een expressie profiel van 113 genen aangetoond dat in staat is anti-TNF responsers te kunnen onderscheiden van anti-TNF non-responders met een sensitiviteit van 79% en een specificiteit van 84%. Deze 113 genen geasocieerd met anti-TNF response duiden specifieke transductieroutes aan die verder bestudeerd kunnen worden met betrekking tot de achterliggende mechanismen van anti-TNF response. Daarnaast is het patiënten cohort van 42 RA patiënten gebruikt om de validiteit van 2 eerder verschenen expressie profielen te testen. Analyse laat een sensitiviteit van 71% zien voor beide expressie profielen en een specificiteit van 61% en 28%.

Expressie analyse op exon niveau laat twee aangrenzende exonen in het *SIRPB1* gen zien waarvan de expressie is verlaagd in anti-TNF non-responders ten opzichte van responders. In hoofdstuk 9 wordt deze afname in expressie verder bestudeerd. De verlaagde expressie van deze twee exonen in anti-TNF non-responders werd gevalideerd en gerepliceerd met quantitative PCR in een gecombineerd cohort van 76 RA patiënten afkomstig uit het DREAM register en het Autoimmune Bio-markers Collaborative Network (ABCoN) cohort. Verdere analyse laat zien dat de afname in expressie een direct gevolg was van twee gedeeltelijk overlappende deleties in het *SIRPB1* gen. Echter, er bleek geen associatie te zijn tussen de twee DNA deleties en anti-TNF response in een groter cohort van 466 RA patiënten behandels met anti-TNF medicatie. De eerdere

bevindingen op RNA niveau konden niet bevestigd worden op DNA niveau en er moet geconcludeerd worden dat de deleties in het *SIRPB1* gen waarschijnlijk niet geassocieerd zijn met anti-TNF response.

In de algemene discussie (hoofdstuk 10) worden de resultaten van dit proefschrift samen gevat en geplaatst in een breder perspectief betreffende de farmacogenetica en de rol daarvan bij de behandeling van RA in de kliniek. Verschillende benaderingen voor farmacogenetisch onderzoek worden besproken. Toekomstige studies zullen verschillende strategieën op meerdere niveau's (DNA, RNA, eiwit) moeten verbeteren en integreren om zo (genetische) markers te kunnen identificeren die verantwoordelijk zijn voor therapie uitkomsten in RA. Eenmaal geïdentificeerd en gevalideerd voor klinisch gebruik, zullen deze farmacogenetische markers veel bijdragen aan de ontwikkeling van gepersonaliseerde behandel methoden voor RA patiënten.

> LIST OF PUBLICATIONS

Macrophage migration inhibitory factor polymorphisms do not predict therapeutic response to glucocorticoids or to tumour necrosis factor alpha-neutralising treatments in rheumatoid arthritis.

Radstake TR, Fransen J, **Toonen EJ**, Coenen MJ, Eijsbouts AE, Donn R, van den Hoogen FH, van Riel PL. *Ann Rheum Dis*. 2007 Nov; 66(11): 1525-30. Epub 2007 Apr 24.

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Coenen MJ, **Toonen EJ**, Scheffer H, Radstake TR, Barrera P, Franke B. *Pharmacogenomics*. 2007 Jul; 8(7): 761-73

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The functional variant (Asp299gly) of toll-like receptor 4 (TLR4) influences TLR4-mediated cytokine production in rheumatoid arthritis.

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> CURRICULUM VITAE

Erik Toonen werd geboren op 10 mei 1977 te Molenhoek. In 1998 behaalde hij zijn VWO diploma aan de Nijmeegse Scholen Gemeenschap (NSG) Groenewoud. In datzelfde jaar begon hij aan de studie Gezondheidswetenschappen aan de Universiteit Maastricht, met als afstudeerrichting Biologische Gezondheidskunde. Zijn afstudeerstage verrichtte hij aan de afdeling Moleculaire Dierfysiologie binnen het Nijmegen Centre for Molecular Life Sciences (NCMLS). Onder de leiding van ir. Marcel Coolen en prof. Gerard Martens onderzocht hij, door middel van *Xenopus Laevis* transgenese, de rol van Aph-1b, een kandidaat eiwit voor schizofrenie. Na het behalen van zijn doctoraal begon hij in april 2004 als Junior Onderzoeker aan een promotieonderzoek bij de afdeling Antropogenetica van het UMC St Radboud te Nijmegen. Dit promotieonderzoek, een samenwerking tussen de afdelingen Antropogenetica en Reumatische Ziekten, werd begeleid door dr. Marieke Coenen, dr. Barbara Franke, dr. Pilar Barrera, prof. Han Brunner en prof. Piet van Riel. Doel van dit onderzoek was het identificeren van genetische variatie verantwoordelijk voor response op anti-TNF medicatie en voor ziekte ernst bij patiënten met reumatoïde artritis. De resultaten van het onderzoek staan beschreven in dit proefschrift. In het laatste jaar van zijn promotie ontving Erik Toonen tijdends de EULAR (European League Against Rheumatism) annual meeting 2008 de EULAR Scientific Travel Award. Sinds maart 2009 is hij werkzaam als postdoc bij de afdeling Immune Therapeutics binnen MSD (voormalig Organon) te Oss, waar hij onderzoek doet naar de metabole bijwerkingen van glucocorticoïden.

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